

**The Relationship between Insulin Resistance
and
The Atherogenic Lipoprotein Phenotype**

Chee-Eng Tan, M.B., B.S. (S'pore), M Med (Int Med) (S'pore), FAMS (S'pore)

**Institute of Biochemistry
Royal Infirmary
Glasgow**

**Thesis submitted for the degree of Doctor of Philosophy in the Faculty of Medicine,
University of Glasgow, UK.**

Submitted April 1996

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Abstract

The prevalence of the insulin resistance syndrome in a non diabetic population had been estimated to be about 25% whilst the prevalence of the atherogenic lipoprotein phenotype is thought to be about 30%. There are some evidence to suggest that there may be a close relationship between these two syndrome and that relationship was explored in this thesis. Risk factors for cardiovascular disease often show clustering that cannot be accounted for by chance occurrence. This has led to the hypothesis that clustering of risk factors such as insulin resistance and dyslipidaemia, may be an indication of an underlying metabolic disorder which increases the risk for premature atherosclerosis.

Plasma triglyceride was found to be the most important determinant of LDL subfraction distribution in a group of normal individuals. The least dense species, LDL-I, decreased as the level of this plasma lipid rose. LDL-II in both men and women exhibited a positive association with plasma triglyceride in the range 0.5 to 1.3 mmol/L; it increased from about 100 to 200 mg/dL. In contrast, within this triglyceride range the LDL-III concentration was low (about 30 mg/dL) and changed little. As plasma triglyceride rose from 1.3 mmol/L to 3.0 mmol/L there was a significant fall in LDL-II concentration in males but not in females. Conversely, above the triglyceride "threshold" of 1.3 mmol/L there was a steeper rise in LDL-III concentration in males than females; 42% of men had an LDL-III in the range associated with high risk of heart disease (> 100 mg lipoprotein/dL plasma) compared with only 17% of women. In a group of young Singaporean males, the relationship of plasma triglyceride with LDL-II failed to show a biphasic pattern but rather, increasing levels of plasma triglyceride was associated with decreasing levels of LDL-II. The LDL-I showed a similar negative relationship and the LDL-III a positive relationship with plasma triglyceride. Other influences of the LDL subfraction profile were the activities of lipases and parameters indicative of the presence of insulin resistance. Males on the average had twice the hepatic lipase activity of females. This enzyme in males was not strongly associated with variation in the LDL subfraction profile whereas in the females it was correlated with LDL-III and remained a significant predictor in multivariate analysis. Increased WHR, fasting insulin and glucose were shown to be correlated with LDL-I (negatively) and LDL-III (positively), primarily, at least in the case of LDL-III, through raising plasma triglyceride.

Subjects with proven coronary artery disease were also found to have impaired triglyceride metabolic capacity during the administration of an oral fat tolerance test. The triglyceride rose to a higher level when compared to age matched controls. The exaggerated hypertriglyceridaemia may be related to the preheparin lipoprotein lipase activity, which was demonstrated to be significantly lower in CAD patients compared to age matched controls. The CAD patients also demonstrated some features of insulin resistance, such as hyperinsulinaemic and hyperglycaemic response, both after the standardised fat meal as well as after a modified glucose meal carried in almost fat free yoghurt. Lipoprotein lipase activity is believed to be modulated by hormonal influence such as insulin and resistance of LPL to insulin may be responsible for the lower preheparin LPL documented in CAD patients. The metabolic implications of impaired

preheparin LPL during alimentary lipaemia is presently unclear as there is no correlation with adipose tissue and skeletal muscle LPL.

The prevalence of insulin resistance and the atherogenic lipoprotein phenotype in Singapore may be higher than other population because of the high prevalence of diabetes mellitus. A group of normal Singaporean male, aged 30 to 45 years old were studied and found to have a high prevalence of both syndromes. More than half of this sampled group had LDL-III concentration that was above 100 mg lipoprotein/dL plasma and this may be related to the higher mean plasma triglyceride levels (1.8 mmol/L). Ethnic differences were noted and men with predominantly Asian ancestry had higher mean LDL-III concentrations and plasma triglyceride, while those with some European descent (Eurasians) had levels of LDL-III and plasma triglyceride similar to age matched Scottish cohort. There were also ethnic differences in markers of insulin resistance such as fasting insulin and fasting glucose and these difference paralleled that seen in LDL-III concentration. Ethnic differences suggest that genetic factors may determine manifestation of both the atherogenic lipoprotein phenotype and the insulin resistance syndrome, as socio-economic factors are similar between the various ethnic groups.

The data presented in this thesis suggest a strong relationship between the atherogenic lipoprotein phenotype and the insulin resistance syndrome. The clustering of coronary risk factors such as dyslipidaemia and insulin resistance in patients with premature atherosclerosis may be an indication of an underlying metabolic dyslipidaemic syndrome.

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Acknowledgements

I would like to acknowledge my two supervisors and mentors, Professor James Shepherd and Professor Chris Packard for their encouragement and support, without which this thesis would not have come to fruition. They have also been very patient and were willing to set aside valuable time to discuss and guide me throughout the four years from 1992 to 1996, both in Scotland and even while I was back in Singapore. The training received from them goes even beyond the scope of this thesis and space does not allow me to go into detail my gratitude for their "mentorship".

I would like to thank A/Professor William Chew, for his support and encouragement during my formative years in training and for arrangements of my training at the Glasgow Royal Infirmary.

I would also like to express grateful thanks to Ms Nancy Thomson and Sheena Brownlie for the secretarial assistance both in Scotland and at home in Singapore, Mrs Muriel Caslake for the invaluable help in the setting up of the LDL subfraction systems in Singapore, Mr Philip Stewart for assistance in computing, Dr Lorne Squires for assistance in patient recruitment and sampling, Mrs Dorothy Bedford for assistance in laboratory techniques, Dr Chio Lee Foon and Mr Roland Chu for assistance in laboratory work in Singapore and last but not least, all the staff at the Lipid section of the Department of Pathological Biochemistry at the Glasgow Royal Infirmary

Author's Declaration

The work presented in this thesis was performed solely by the author, except where the assistance of others is acknowledged.

Dr Chee-Eng Tan
April 1996

Dedication

This thesis is dedicated to my wife, Janice who had been very supportive and a source of great encouragement during the four years needed to complete this challenging but rewarding task. It is also dedicated to my two sons, Nathanael and Euan, who have been a great joy to me.

Chapter 1 Introduction

The fear of the Lord is the beginning of wisdom, And the knowledge of the Holy One is understanding.
Proverbs 9:10

1.1 Historical Background of Insulin Resistance

The association between excessive concentrations of insulin and development of atherosclerosis first appeared in the mid 1960s and since then, numerous clinical and experimental data have accumulated, most of it supporting the link between insulin resistance and atherosclerosis. In the 1930's, Himsworth and Kerr introduced the first standard approach to measuring insulin sensitivity in vivo.^{1,2,3} Two oral glucose tolerance tests were performed in individual subjects, with and without a concomitant intravenous insulin injection. Insulin sensitivity was expressed as the ratio of the areas under the respective glucose tolerance curves. Himsworth thus showed that young, thin, ketosis prone diabetics were more sensitive to exogenous insulin than older, nonketotic patients. He also reported relative insulin insensitivity to insulin for nondiabetic obese² and elderly individuals⁴ and demonstrated that high carbohydrate, low fat diets increased insulin sensitivity.^{5,6} However, such measure of insulin sensitivity was viewed as inferential because of the absence of a sensitive specific measurement of plasma insulin and a variety of mechanisms could contribute to the differences observed. These include differential effects of insulin on gastrointestinal glucose absorption, differences in inhibition of endogenous insulin secretion during the test, and variability in clearance of exogenously administered insulin.

The development of the radioimmunoassay (RIA) by Yalow and Berson⁷ satisfied the need for a sensitive specific measure of plasma insulin. This was soon followed by development of various methods for assessing in vivo effects of changes in plasma insulin, described in detail in subsequent sections.

1.11 Insulin Resistance in Diabetes Mellitus

For many years, diabetes was thought to be a disease of insulin deficiency and that those with diabetes had low circulating insulin levels. As our knowledge of diabetes advances, we know now that many non-insulin dependent diabetes mellitus (NIDDM) patients have insulin levels which are higher than nondiabetic subjects. The insulin response to an oral glucose tolerance test (OGTT) becomes elevated as glucose tolerance declines from normal.^{8,9} With severe glucose intolerance and fasting hyperglycaemia, insulin levels become normal or subnormal.⁸

In insulin-dependent diabetes mellitus (IDDM), there is a deficiency of insulin which may be absolute or relative. Unlike in NIDDM patients where there is resistance to insulin mediated glucose disposal, IDDM patients do not have much inherent insulin resistance. However, in the treatment of IDDM, insulin is delivered in non-physiological ways with respect to route and control of delivery. Insulin is secreted into the portal circulation, where 50% is cleared on first passage through the liver. Thus insulin concentrations in the portal vein are several times higher than the peripheral circulation.¹⁰ Subcutaneous insulin

on the other hand, circulates in high concentration to achieve its desired effect on its main target organ i.e. the liver. With subcutaneous insulin injections, insulin levels remain high between meals and overnight,^{11,12} unlike in normal physiology where insulin secretion is finely regulated, with increase stimulated by meals and a rapid fall to basal levels after meals. The IDDM patients, like the NIDDM patients are thus exposed to higher levels of insulin as a consequence of therapy.

There is now a large body of evidence showing that the vast majority of patients with impaired glucose tolerance (IGT) or NIDDM are insulin resistant, compared with appropriately matched individuals with normal glucose tolerance.¹³ Different parameters of insulin resistance were used in various studies, but whether it be fasting insulin, insulin-glucose ratio, plasma C-peptide with or without glucagon stimulation, glucose and insulin response to OGTT, almost all reported studies have shown a relationship between such parameters and cardiovascular disease in both NIDDM and IDDM patients.^{14,15,16,17,18,19} These studies show that insulin resistance in NIDDM predisposes to atherosclerosis and thereby to cardiovascular diseases. It is interesting to note that even IDDM patients demonstrate insulin resistance, possibly secondary to prolonged iatrogenic hyperinsulinemia during therapy and consequently IDDM patients also have increased risk of cardiovascular disease.

1.12 Insulin Resistance in Coronary Heart Disease

The knowledge that diabetes is associated with an increased frequency of cardiovascular disease prompted numerous studies looking at insulin resistance in patients with ischaemic heart disease. The findings of these studies are summarised in an excellent review by R W Stout,²⁰ with the majority showing fasting hyperinsulinaemia or postglucose challenge hyperinsulinaemia. One of the largest study of insulin and cardiovascular disease was the Caerphilly, Wales, Heart Disease study, involving 2512 men aged 45-59 years. This study showed an association between fasting plasma insulin levels and prevalent ischaemic heart disease independent of body mass index (BMI), age, systolic blood pressure, and triglyceride levels.²¹ It would appear that insulin resistant individuals may be able to limit the degree of deterioration in glucose tolerance by compensatory hyperinsulinaemia, although this is not without a price. Endogenous hyperinsulinaemia has been shown to be a risk factor for coronary heart disease in prospective studies.^{22,23,24,25}

1.13 Insulin Resistance in Essential Hypertension

Hypertension is an important risk factor for cardiovascular disease, particularly for stroke, myocardial infarction and peripheral vascular disease. One of the earliest study to suggest a relationship between insulin and hypertension found that hypertensive patients had higher insulin levels in the fasting state and after a 50g OGTT when compared with normotensive controls.²⁶ Obesity and age are known to be confounding factors for hyperinsulinaemia in such hypertensive patients. However, we have found that even in the young (<35 years) and nonobese (BMI <26) hypertensive patients, 40% demonstrated insulin resistance after an intravenous glucose challenge (IVGTT).²⁷ Insulin may be causally related to hypertension because of an effect of insulin on renal sodium

reabsorption,²⁸ enhanced sympathetic nervous system activity in hyperinsulinaemic states.^{29,30}

1.14 Insulin Resistance in relatives of Diabetic patients

The high incidence of NIDDM among first degree relatives of type 2 diabetic patients and the high concordance in identical twins provide strong evidence that genetic factors underlie susceptibility to this disease.³¹ Normoglycaemic offspring of diabetic parents have defects in insulin sensitivity and glucose disposal, which are important predictors of their risk of NIDDM.³² This is further supported by other studies which showed that relatives of patients with NIDDM are also at risk of developing diabetes.^{33,34,35} Sarlund et al suggested that a family history of diabetes adds substantially to the risk of atherosclerosis, particularly in subjects with impaired glucose tolerance (IGT).³⁶ Perhaps one of the reasons why relatives of NIDDM are more prone to develop diabetes and atherosclerosis is because they are insulin resistant, as studies have shown that first degree relatives of patients with NIDDM are more insulin resistant than subjects without a family history of diabetes.³⁷

1.15 Insulin Resistance in relatives of Hypertensive patients

Since hypertensive patients are known to be insulin resistant, and this is independent of obesity^{27,38} or treatment,^{39,40} and that hypertension is often clustered with coronary heart disease and diabetes mellitus, all of which have strong genetic elements, one would thus expect relatives of hypertensive patients to also show insulin resistance. The evidences to date are not as clear as those in relatives of NIDDM patients because of the confounding factors like obesity and age of the study population. In a study of 33 obese women⁴¹ who were neither diabetic or hypertensive, a significant positive correlation was seen between fasting serum insulin and diastolic blood pressure. Insulin levels were also slightly higher in those with a positive family history of hypertension. First degree relatives of patients with high blood pressure have been shown to be insulin resistant and hyperinsulinaemic when compared to a control group without a family history of hypertension.⁴²

1.16 Genetic syndromes with extreme Insulin Resistance

The genes that determine an individual's susceptibility to NIDDM and insulin resistance have not been identified. Most of our understanding of insulin resistance stems from studies of patients with genetic syndromes associated with extreme insulin resistance and acanthosis nigricans. Hence this next section will highlight some of these genetic syndromes whilst the subsequent sections will elucidate some possible mechanisms for the insulin resistance.

Type A Insulin Resistance

This is characterised by the triad of insulin resistance(IR), acanthosis nigricans(AN) and hyperandrogenism(HA)⁴³ in the absence of obesity or lipotrophy and is sometimes also known as the HAIR-AN syndrome. The diagnosis of type A insulin resistance was originally reserved for females because of hyperandrogenism as one of the manifestations.

However, it is now clear that males can also be affected by a similar syndrome.⁴⁴ Most patients with type A insulin resistance are not overtly diabetic, although they are all hyperinsulinaemic and many have IGT.

Leprechaunism

This is a congenital syndrome associated with extreme insulin resistance and patients are glucose intolerant, often with peak insulin levels more than a 100-fold above the normal range. They have multiple abnormalities, including intrauterine growth retardation and fasting hypoglycaemia.⁴⁵ Some cases of leprechaunism have been reported to live into childhood and adolescence with short stature but normal intellectual development.⁴⁶

Rabson-Mendenhall syndrome

This syndrome is characterized by multiple features, including extreme insulin resistance, acanthosis nigricans, abnormalities of teeth and nails, and pineal hyperplasia.⁴⁷ Clinically, it may be difficult to distinguish some patients with Rabson-Mendenhall from patients with leprechaunism who have survived beyond the first year of life.

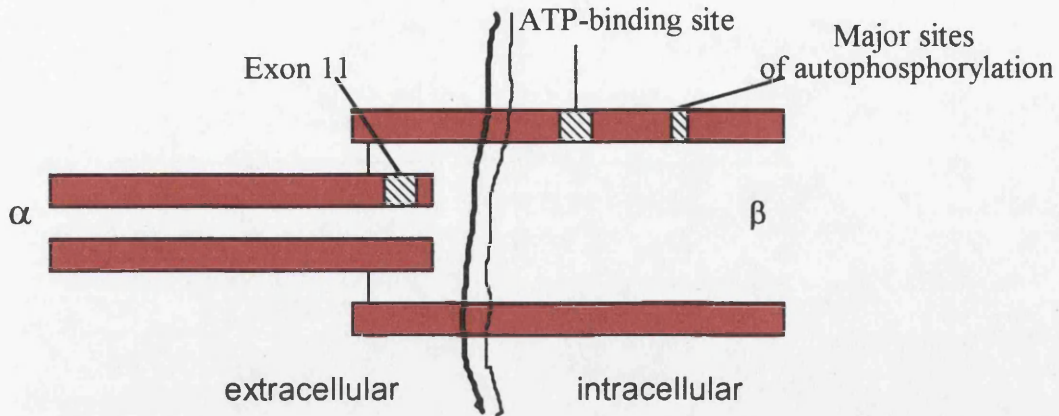
Other syndromes

Several other syndromes associated with insulin resistance have been described that appear to have a genetic aetiology but these need further research before such syndromes can be attributed to mutations in the insulin-receptor gene.

1.17 The Insulin Receptor

The insulin receptor has been identified as a membrane glycoprotein composed of two extracellular α -subunits and two transmembrane β -subunits (Fig 1). Kasuga, Karllson and Kahn demonstrated that the insulin receptor is an insulin activated enzyme, which undergoes autophosphorylation on tyrosine residues after insulin binding, which then becomes competent to phosphorylate cellular substrates on tyrosine residues.^{48,49} This suggested the likely existence of a kinase cascade, through which many or all of the effects of insulin might arise. It is also now known that the insulin receptor is the product of a very large gene on chromosome 19.^{50,51}

Fig 1: The Insulin Receptor

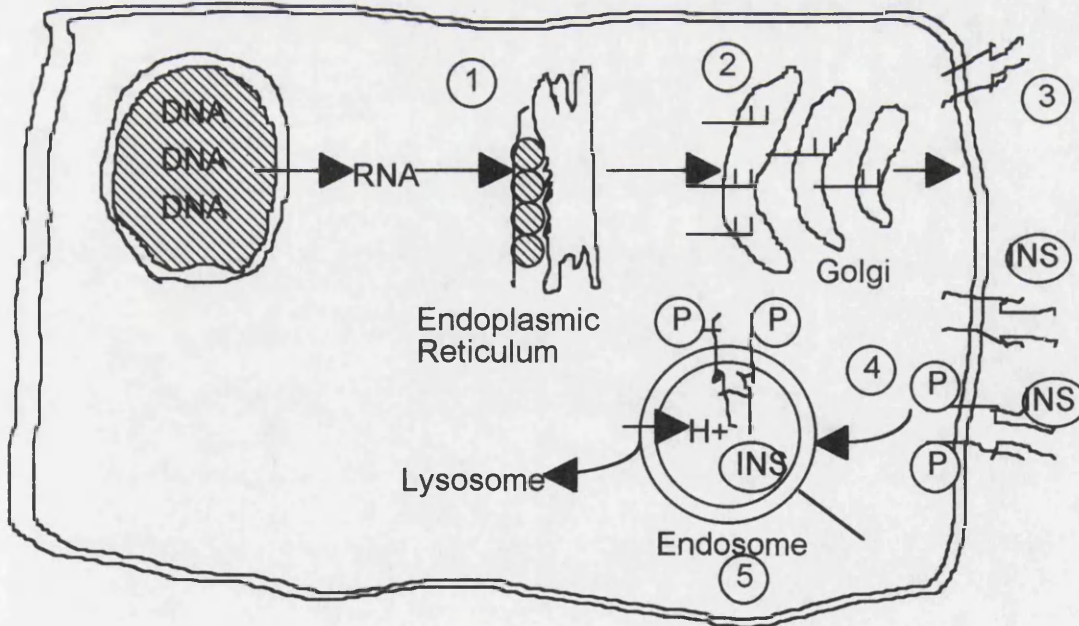


1.18 Mutations in the Insulin-Receptor Gene

Mutations of the Insulin-Receptor Gene can be classified into 5 categories summarised in Fig 2.⁵²

- Class 1: Mutations that impair receptor biosynthesis
- Class 2: Mutations that impair transport of receptors to cell surface
- Class 3: Mutations that decrease the affinity of insulin binding
- Class 4: Mutations that impair receptor Tyrosine kinase activity
- Class 5: Mutations that accelerate receptor degradation

Fig 2: Classifications of mutations in insulin-receptor gene



However, it would appear that the cellular defects of insulin resistance found in NIDDM and cardiovascular disease, cannot be explained by major impairments in the structure or

function of the insulin-receptor gene. Although genetic factors are thought to play a significant role in insulin resistance and NIDDM, no deleterious mutations of candidates have yet been found to explain the disease.

1.19 Fasting insulin as a marker of insulin resistance

The fasting plasma insulin concentration is largely determined by the glucose concentration⁵³ and the degree of basal hyperglycaemia is in turn determined by a combination of β -cell deficiency and insulin resistance. Fasting plasma insulin as a marker of insulin resistance is the simplest measure and large studies often employ this parameter because of ease of obtaining the sample. Hence it is important to consider whether fasting plasma insulin is a good measure of insulin resistance. Evidence suggests that in the absence of marked hyperglycaemia, fasting insulin correlated with other estimates of insulin resistance and the insulin concentration is a simple function of insulin resistance.⁵⁴ Findings from published studies regarding fasting insulin as a risk factor for coronary heart disease have been inconsistent²⁰ although two large prospective studies, the Helsinki Policeman Study²² and the Paris prospective study²⁴ have shown a significant association between coronary heart disease and fasting insulin, with the later study showing fasting insulin to be the only independent risk factor for coronary heart disease in a multivariate analysis. It would appear that a single fasting insulin sample is unlikely to be a reliable guide to a subject's insulin resistance or β -cell function.

1.1.10 Oral Glucose Tolerance Test

Appropriately elevated plasma insulin, in the face of normal or supranormal plasma glucose pattern has been accepted as evidence for diminished overall tissue sensitivity to insulin.^{55,56,57} However, the heterogeneity of OGTT patterns amongst normal, glucose intolerant, and non-ketotic diabetic patients has been demonstrated.⁵⁵

1.1.11 Intravenous Glucose Tolerance Test

The insulin response to intravenous glucose tolerance test (IVGTT) have been inconsistent although hyperinsulinaemia has been demonstrated in patients with coronary heart disease.^{28,58,59,60,61,62} The diverse response to IVGTT led investigators to suggest that a gastrointestinal stimulus mediated the exaggerated response to oral glucose.^{58,63}

1.1.12 Insulin Suppression Test

The principle of the insulin suppression tests involves the pharmacologic inhibition of endogenous secretion of insulin during exogenous insulin and glucose infusion. Inhibition is accomplished with either epinephrine infusion and propranolol-induced β -adrenergic blockade (E/P)⁶⁴ or with somatostatin (SRIF).⁶⁵ In the E/P protocol, steady state glucose is achieved within 90 minutes of combined infusion of propranolol, epinephrine, glucose and insulin (propranolol and epinephrine being replaced by cyclic somatostatin in the SRIF protocol). Steady-state plasma glucose (SSPG) is defined as the average glucose during a steady-state observation period. SSPG is considered a measure of insulin resistance.⁶⁴

1.1.13 Glucose Clamp Studies

The principle of the glucose clamp employs the knowledge that blood glucose concentration is relatively constant in the postabsorptive state and thus the rate at which glucose is produced by the body (R_a) is equal to the rate of glucose utilisation (R_u). When insulin is administered exogenously, R_u normally increases, whilst R_a decreases, so that $R_u > R_a$. The imbalance between glucose utilisation and endogenous production would normally result in a decline in plasma glucose which can be countered by a rate of exogenous glucose infusion calculated to equal the total of decreased glucose production and increased glucose utilisation. The rate of glucose infusion needed to maintain basal glucose level is a measure of the net effect of the insulin infusion to alter the production and utilisation of glucose by the body. After an appropriate period, at which steady-state is presumed to be reached, the rate of glucose infusion is considered an indication of insulin action. In some instances, sequentially increasing rates of insulin infusion were used to establish a dose-response relationship between plasma insulin concentration and insulin action. Variations of the clamp protocol have been employed and glucose levels may be targeted at the fasting level,⁶⁶ hypoglycaemic levels⁶⁷ or in subjects with elevated fasting glucose, at hyperglycaemic levels.⁶⁸

1.1.14 Minimal Model Approach

Glucose achieves an elevated value after a glucose injection and thereafter begins to decline and the rate of decline is dependent on the abrupt insulin secretory response. Therefore the extent to which a given plasma insulin response accelerates the decline of glucose after injection is a measure of the insulin sensitivity. The minimal model is able to infer insulin sensitivity by the use of a computer model which simulates plasma glucose dynamics when plasma insulin dynamics are supplied. The dynamic insulin response to glucose injection is considered as the input whilst the observed plasma glucose pattern is the output. The computer model chosen is the simplest physiologically based representation which can account for the input-output relationship under various conditions.⁶⁹ Although the measure of insulin sensitivity from the minimal model approach is not identical to that obtained from the glucose clamps, the good correlation between the two does suggest they measure closely related physiologic processes.

1.1.15 Homeostasis Model Assessment Method

This model is a computer-solved model of insulin: glucose interactions which had been used to plot an array of fasting plasma insulin and glucose concentration that would be expected for varying degrees of β -cell deficiency and insulin resistance. The β -cell function for any patient can be calculated from the formula: β -cell function (%) = $20 \times \text{insulin} / (\text{glucose} - 3.5)$ and Insulin resistance = $\text{insulin} / (22.5 e^{-\ln \text{glucose}})$.⁵⁴ The accuracy and precision of this model has been found to correlate well with independent measures of insulin resistance obtained by hyperglycaemic and euglycaemic clamps and intravenous glucose tolerance test. However, the model is limited by low precision and a single fasting sample taken in the outpatient clinic is unlikely to be a reliable guide to a

subject's insulin resistance or β -cell function. Rather, 3 separate samples over a 15 minute period at 5 minutes interval is advocated to improve precision.

1.1.16 The Insulin Resistance Syndrome

Reaven first proposed a widespread role of insulin resistance in human disease in the Banting Lecture in 1988. The introduction of the insulin resistance syndrome (IRS) or syndrome X as a plurimetabolic disorder related to increased coronary heart disease risk (CHD) associated with hypertriglyceridaemia, depressed high density lipoprotein (HDL) cholesterol, central obesity, hypertension, impaired glucose metabolism, has since been confirmed by other authors.^{70,71,72} It is now recognised that an insulin-resistant hyperinsulinaemic state is not associated with substantial changes in low density lipoprotein (LDL) cholesterol concentrations but rather with marked increases in LDL apolipoprotein (apo) B levels.⁷³ Recently, insulin resistance has been shown to be associated with an increased proportion of small, dense, LDL particles.^{74,75} Both genetic and environmental factors play determinant roles in its development. Obesity has been associated with insulin resistance⁷⁶ but the syndrome can also develop in nonobese individuals.¹³ Increasing age has also been shown to increase insulin resistance.⁷⁷ Support for a genetic element in the development of insulin resistance can be seen from the Bogalusa Heart study involving 2856 children which showed positive relationships between insulin and other cardiovascular risk factors such as blood pressure, triglyceride and VLDL levels and an inverse relationship with HDL cholesterol, even at a young age.⁷⁸

1.2 Lipids and Lipoproteins in Coronary Heart Disease

The Framingham Study is one of the most often quoted study on lipids, lipoproteins and risk of coronary heart disease. This study showed that amongst the identified host factors associated with increased susceptibility to coronary heart disease, blood lipids are among the strongest.⁷⁹ The data suggested that in men, a moderately elevated cholesterol regardless of the metabolic aberrations responsible or how it is transported or partitioned among the lipoproteins, are associated with increased risk of coronary heart disease. The multiple risk factor intervention trial (MRFIT) then showed that the relationship between serum cholesterol and CHD is not one of threshold, with increased risk confined to the two highest quintiles, but rather a continuously graded relationship that powerfully affects risk for the great majority of middle-aged American men.⁸⁰ Other studies have shown that LDL cholesterol is the main contributor to the relationship between cholesterol and CHD whilst HDL cholesterol levels are inversely related to CHD incidence.^{81,82} The association between plasma triglyceride and coronary heart disease have been consistently demonstrated^{83,84} but the interpretation of this relationship has been controversial. Recent re-evaluation has shown that the strength of the association between triglyceride and CHD has been significantly underestimated.⁸⁵

1.21 Low Density Lipoprotein and atherogenesis

Lipoprotein phenotypes are now used extensively to diagnose and classify familial hyperlipidaemia. The mean level of LDL-cholesterol was found to be highest in survivors with familial hypercholesterolaemia, intermediate in those with familial combined

hyperlipidaemia and lowest in those with familial hypertriglyceridaemia.⁸⁶ Support for the atherogenic potential of LDL cholesterol is based on clinical observations in patients with familial hypercholesterolaemia. Such patients have a single, well-defined gene defect involving the LDL receptor, as shown by the work of Goldstein and Brown,⁸⁷ and the premature atherosclerosis, is directly or indirectly, related to their elevated LDL levels, which in turn are due to the deficiency in LDL receptors. Fatty streaks are believed to be the first stage of the atherosclerotic process and consist predominantly of foam cells derived from circulating monocytes.⁸⁸ Although LDL is the major atherogenic lipoprotein generating foam cell-rich lesions, incubations of monocytes/macrophages with LDL did not lead to foam cell formation.⁸⁹ It is now recognised that LDL must undergo some form of modification in its structure and biological properties before it can be taken up by monocyte/macrophages at a rate sufficient to generate foam cells.⁹⁰ A number of chemical modifications have been shown to have this effect^{89,91,92} but the biological evidences is strongest for oxidative modification.^{93,94,95,96} Oxidatively modified LDL is taken up by monocyte/macrophages three to ten times more rapidly than the native LDL and can therefore generate foam cells. The oxidatively modified LDL is chemotactic for circulating monocytes,⁹⁷ inhibits the motility of tissue macrophages⁹⁸ and is cytotoxic, and could contribute to atherogenesis by causing cell injury and cell death.⁹⁹

1.22 HDL and reverse cholesterol transport

HDL is now considered a potentially modifiable risk factor and HDL has been shown to be inversely correlated with CHD risk. A higher HDL level confers a lower risk for CHD. Strong support for this is based on an aggregate analysis of the findings of several large studies i.e. The Framingham Study, The Lipid Research Clinics Coronary Primary Prevention Trial (LRC-CPPT), The Multiple Risk Factor Intervention Trial (MRFIT) and the Lipid Research Clinic Follow-up Study (LRCF).¹⁰⁰ The inverse relation between level of HDL and risk for CHD was found to persist whether the population studied was male or female and whether the focus was on morbid or fatal coronary events. An increase in HDL of 1 mg/dL translates to a 2 to 3 % reduction in CHD risk. The level of HDL cholesterol is thought to reflect the efficiency of reverse cholesterol transport and hence the inverse relationship with CHD. Animal and human studies have shown that cholesterol is removed from atheromata when plasma LDL cholesterol levels are lowered and HDL levels are increased. It is likely that the regression mechanism involves cholesterol removal by HDL.¹⁰¹ In vivo studies of the turnover of HDL cholesterol showed that tissues exchange cholesterol primarily with HDL and not with VLDL or LDL and the HDL cholesterol in turn is rapidly exchanged with the liver cholesterol pool.¹⁰² There is thus a net transfer of cholesterol from tissues to liver via HDL, of similar magnitude to the net transfer of HDL cholesteryl ester through LDL and VLDL to the liver. The nonesterified cholesterol diffuses from the tissue cell membrane to HDL through nonspecific lipid-lipid interactions. However, in adipocytes, macrophages, and fibroblasts, the mobilization of intracellular cholesterol pools and their translocation to the cell membrane are facilitated by the binding of specific HDL apolipoproteins (apo A-I and apo A-IV) to a particular recognition site on the cell surface.^{103,104} The nonesterified cholesterol is then taken up in the plasma by nascent HDL particles containing solely apo A-I, undergoing esterification by lecithin cholesterol acyltransferase (LCAT) within these particles.¹⁰⁵ The cholesterol esters are then removed from HDL by the liver, where the

cholesterol is used for bile acid synthesis.^{101,106} These studies have shown that HDL cholesteryl ester can be removed and delivered to the liver by at least 4 mechanisms. A subpopulation of HDL requires apoE and is recognised by hepatic apo E receptors. HDL without apo E can be endocytosed by hepatocytes. Hepatic triglyceride lipase mediates uptake of cholesteryl esters into liver cells and finally cholesteryl ester transfer protein (CETP) catalyses the net transfer of HDL cholesteryl esters to VLDL, IDL and LDL. Thus HDL not only take up cholesterol from tissues, but also mediate cholesterol exchange into the apoB-containing lipoproteins, VLDL, IDL or LDL, from which cholesteryl esters can be removed when circulating VLDL, IDL and LDL particles are taken up by hepatic apo B and E receptors.

1.23 Role of Triglyceride in atherogenesis

The relationship between elevated plasma triglyceride and an increased risk of coronary heart disease has been consistently demonstrated by epidemiological studies.^{83,84,107} The interpretation of the relationship, however, has been controversial. A recent re-evaluation of the epidemiological data has suggested that the strength of the association has been significantly underestimated because of biological variation of this plasma lipid and shortcomings in the multivariate analytical approach.⁸⁵ It is postulated that increased levels of plasma triglyceride affect atherogenesis both directly and indirectly. Individuals with CHD fail to metabolise triglyceride-rich chylomicrons and VLDL efficiently,¹⁰⁸ resulting in a prolonged residence time in the circulation and accumulation of cholesteryl esters in these lipoproteins resulting from loss of triglyceride into LDL and HDL via CETP and reciprocal transfer of cholesteryl ester. These cholesteryl ester enriched lipoproteins have increased likelihood of being ingested by macrophages¹⁰⁹ at sites of lesion development. Recent work has also indicated that in the general population, LDL structure is strongly influenced by the level of plasma triglyceride.¹¹⁰ Individuals with plasma triglyceride above approximately 1.1 mmol/L were more likely to express the 'Pattern B' phenotype for LDL which is associated with a predominance of smaller particles and higher apo B concentrations. 'Pattern A' with a predominance of larger LDL particles are seen with triglyceride below 1.1 mmol/L. When hepatic triglyceride levels are high, large triglyceride-rich VLDL are produced which are delipidated to smaller VLDL and finally to slowly metabolised LDL. In the presence of a reduced hepatic triglyceride load, smaller VLDL are made which give rise to rapidly metabolised LDL.¹¹¹ Elevated levels of chylomicrons and large VLDL as mentioned previously, also enhances exchange of triglyceride into the core of circulating LDL^{112,113} and these are more suitable substrates for hepatic lipase which through lipolysis causes shrinkage of the particles.¹¹⁴ High plasma triglyceride is also thought to accelerate atherogenesis through its inverse relationship with HDL-cholesterol. In the presence of large triglyceride-rich VLDL particles, increased CETP mediated exchange will generate HDL that is triglyceride enriched and these are a more favourable substrate for HL. This enzyme acts to remove lipid from the core of HDL, reducing its size and results in the conversion of HDL₂ to HDL₃.¹¹⁵ HDL₂ is thought to have a greater cardioprotective role compared to HDL₃^{116,117}

1.24 Very Low Density Lipoprotein subfractions

VLDL particles are known to consist of a core of triglycerides and cholesteryl esters surrounded by a monomolecular film of constant thickness composed of phospholipids and free cholesterol together with protein.¹¹⁸ We now know that VLDL is not homogeneous, but rather consists of metabolically distinct groups.¹¹⁹ The larger triglyceride particles (VLDL₁) of Sf 60-400 are secreted from the liver and undergo rapid hydrolysis via lipoprotein lipase.¹²⁰ The majority of these are converted to smaller remnant particles that are removed from the circulation by receptor-mediated mechanisms¹²¹ and hence make little contribution to the production of low density lipoprotein. The smaller, denser, less triglyceride-rich VLDL₂ particles (Sf 20-60) are also secreted by the liver but the majority are rapidly metabolised by conversion through intermediate density lipoprotein (IDL) to LDL, and hepatic lipase is critical for this conversion¹²². It has also been demonstrated that there is preferential enrichment of the larger VLDL particles with cholesteryl esters,¹²³ the main source of cholesteryl esters being LDL. The VLDL particles enriched with cholesteryl ester are thought to be unable to complete the VLDL to LDL conversion process.¹²⁴ The smaller VLDL₂ population is thought to have relatively more protein and phospholipid than the larger VLDL₁ population.¹²⁵ The relationships between VLDL subfraction and LDL subfraction, and the role of VLDL subfraction in the atherogenic process are examined in this thesis.

1.25 Intermediate Density lipoprotein

Intermediate density lipoproteins are usually defined as lipoproteins of buoyant density of 1.006 to 1.019 g/ml.¹²⁶ Human IDL was initially regarded as a single class of lipoproteins generated from VLDL by lipolysis. However, it has since been shown to consist of two subpopulations from gradient gel electrophoresis of ultracentrifugal fractions from normal subjects i.e. IDL₁ and IDL₂.¹²⁷ Often no distinct transition was seen between the smaller VLDL and IDL₁. The same study also demonstrated that IDL₂ was cholesterol enriched and triglyceride depleted compared with IDL₁. It has also been shown that small VLDL gives rise to particles of intermediate size which, in turn, form products with size and density characteristic of the LDL-II subclass.¹²⁸

1.26 Low Density Lipoprotein Subfractions

Lindgren et al were the first to show that LDL (1.019- 1.063 g/ml) could be fractionated into at least 3 subgroups by preparative ultracentrifugation in a fixed angle rotor.¹²⁹ Subsequently, other investigators have demonstrated that LDL is a heterogeneous group of at least 3 to 7 subclasses.^{130,131,132,133} With increasing density, mean particle diameters decreased progressively, while protein/phospholipid ratio increased.¹³² The non-denaturing, polyacrylamide, gradient gel electrophoresis describes a normal pattern 'A' characterised by a predominance of larger, less dense LDL and an abnormal pattern 'B' consisting mainly of small, dense LDL.¹³⁴ This method of LDL isolation provided a qualitative appraisal of LDL subspecies.¹³² Isolation of LDL subfraction by density gradient ultracentrifugation separated LDL into 3 classes i.e. LDL-I, LDL-II and LDL-III with LDL-I being larger and buoyant LDL particles whilst LDL-III particles dense, less buoyant particles. This later method has the advantage of quantification of individual

species.¹³³ A predominance of small, dense LDL in plasma has been associated with a three to seven fold increase in risk of coronary heart disease.^{135,136} It is postulated that at low triglyceride levels, LDL-I and LDL-II represent the major LDL species and these subfractions are cleared rapidly via receptor mediated mechanisms.¹¹¹ At higher triglyceride levels (>1.5 mmol/L), there is a decrease in LDL-II and a corresponding increase in small, dense LDL-III due to a combination of factors. There could be increased production of large, triglyceride-rich VLDL₁ giving rise to smaller and slowly metabolised LDL through delipidation and /or that the increased levels of triglyceride-rich VLDL in the circulation promote the exchange of cholesteryl esters in LDL for triglyceride, with the resultant action of hepatic lipase converting the larger LDL (LDL-II) into smaller LDL (LDL-III).¹³⁶

1.27 High Density Lipoprotein subfraction

HDL, like the other lipoproteins are also known to be heterogeneous and can be classified into larger, less dense HDL₂ (1.063 to 1.125 g/mL) or smaller, denser HDL₃ (1.125 to 1.21 g/mL).¹³⁷ The major proportion of HDL is normally present in HDL₃ but individual variability in HDL levels in human populations usually reflects different amount of HDL₂.¹⁰¹ Women have also been found to have significantly higher HDL₂ levels but similar HDL₃ levels.^{138,139} The less dense HDL₂ has a higher proportion of cholesterol, cholesteryl ester, and phospholipid content than the denser HDL₃,¹⁴⁰ which has a higher apolipoprotein content in proportion to total mass.¹⁴¹ A third HDL subfraction, HDL₁ has been found within the lowest density range of HDL particles and has a higher cholesterol and cholesteryl ester content than HDL₂ or HDL₃.¹⁴² Cholesteryl esters can be exchanged from HDL₂ into VLDL or LDL by cholesteryl ester transfer protein (CETP). HDL particles have also been classified by apolipoprotein composition¹⁴³ and Apolipoprotein A-I, the essential component of HDL particles is found in all HDL subfractions. Apolipoprotein A-II function is still undetermined, and is most abundant in HDL particles of intermediate density, but is also detected throughout the density range of HDL particles. Most LCAT and CETP activities are confined to Lp (A-I without A-II), suggesting that this apolipoprotein-defined subfraction is the key mediator of cholesterol transport. Unfortunately, this recent classification of HDL based on apolipoprotein content does not correspond to classification based on particle density or size; each classification overlaps with the other.¹⁴³ The action of lipoprotein lipase on triglyceride-rich particles eg. chylomicrons and VLDL, results in the formation of HDL₂ from newly synthesized HDL precursors or from HDL₃.¹⁴⁴ Removal of core cholesteryl ester from HDL₂ by CETP results in the formation of a relatively triglyceride-rich particle that is further catabolized by hepatic lipase,¹⁴⁵ resulting in conversion of HDL₂ to HDL₃.¹⁴⁶ HDL subfraction metabolism is influenced by many factors. Increasing waist to hip ratio is associated with a lower HDL₂¹⁴⁷ levels whilst intensive exercise,¹⁴⁸ oestrogen therapy¹⁴⁹ increases HDL₂ levels. Abstinence from alcohol in moderate drinkers¹⁵⁰ decreases HDL₃ levels but showed a disproportionate decline in HDL₂ compared to HDL₃ levels in alcoholic men.¹⁵¹ However, the additional predictive value of subfraction levels, and the relative epidemiologic importance of HDL₂ over HDL₃ remains controversial.¹⁵²

1.2.8 The Atherogenic Lipoprotein Phenotype

The predominance of small dense LDL obtained by gradient gel electrophoresis has been described as phenotype B by Melissa Austin and is associated with high plasma triglyceride, apolipoprotein B, VLDL and IDL, and low HDL cholesterol. This clustering of risk factors has been described as the atherogenic lipoprotein phenotype (ALP) and appears to be inherited as a single gene trait with a dominant mode of inheritance. Phenotype B is often not expressed in young males and premenopausal women and hence hormonal factors have been suggested to affect the full penetrance of the phenotypic picture. Furthermore, environmental factors such as diet, exercise and the use of lipid altering medications may also affect the expression of the trait.¹⁵³ Interest in ALP is not simply because of clustering of coronary risk factors in families but there is evidence that predominance of small, dense LDL particles appears to be common in the general population, with a prevalence of at least 30%.¹⁵⁴ The presence of small, dense LDL particles is associated with a 3 to 7 fold increased risk of myocardial infarction.^{135,136} Thus a combination of both genetic and environmental factors identifies the individuals susceptible to premature coronary artery disease.

1.3 Insulin Resistance and Lipid metabolism

What then is the link between insulin resistance and lipid metabolism? Available evidence suggest that both insulin resistance and ALP are associated with increased risk of coronary artery disease. Insulin resistance is associated with a number of metabolic disturbances and so is the ALP. Insulin resistant individuals have higher plasma triglyceride, lower HDL cholesterol and small, dense LDL particles,⁷⁴ almost identical to the ALP. The only difference being the disturbance in glucose metabolism in insulin resistant individuals. One might ask whether they are just part of the same spectrum of metabolic abnormality or whether one is a subset of the other? Furthermore the prevalence of small, dense LDL estimated at approximately 30%¹⁵⁴ in the general population is similar to the prevalence of 25% estimated for insulin resistance syndrome.¹³ Insulin suppresses the release of non-esterified fatty acids (NEFA) from adipose tissue and in insulin resistant states, the suppressive effects of insulin on fatty acid release from adipose tissue after a meal is impaired.¹⁵⁵ Insulin resistance is also associated with a decreased responsiveness of lipoprotein lipase to the action of insulin.¹⁵⁶ This is particularly important for postprandial activation of LPL with resultant elevation of plasma TG. Prolonged residence of TG-rich particles in the circulation leads to increased exchange of their TG with cholesteryl ester in HDL through the action of CETP protein.¹⁵⁷ The failure of suppression of NEFA during the postprandial period in insulin resistant individuals aggravates¹⁵⁸ the increase in VLDL TG already present.¹⁵⁹ The elevation in plasma TG concentrations reflects an increased number of the larger TG-rich VLDL₁ particles both in normals¹⁶⁰ and in insulin resistant individuals.¹⁶¹ The insulin resistant individuals with higher plasma TG levels would thus have a predominance of large TG-rich VLDL₁ particles and it is these particles that determines the rate of TG transfer into LDL and HDL since TG-rich VLDL has been shown to be the preferred substrate for CETP action.¹⁶⁰ The TG in such TG-enriched LDL particles may then be removed by HL, leading to small, dense LDL particles.¹⁶⁰ Thus we can see that the link between insulin resistance, hypertriglyceridaemia and decreased HDL cholesterol

concentrations could be mediated through disruption of the normal multifactorial role of insulin in co-ordinating postprandial lipid metabolism.¹⁵⁸ This thesis seeks to explore the link between insulin resistance and the atherogenic lipoprotein phenotype.

Chapter 2 Materials and Methods

Happy is the man who finds wisdom, And the man who gains understanding.

Proverbs 3:13

2.1 Introduction

A great deal of research into the aetiology of coronary heart disease (CHD) has been done over the years and large studies have established the links between an elevated cholesterol level, and in particular LDL-cholesterol, and the development of CHD. Many patients with CHD have more than one risk factor and the aggregation of risk factors in patients have led investigators to explore the relationship between these risk factors. This led to the hypothesis that coronary risk factors may not occur in isolation and they may be linked aetiologically. CHD can no longer be considered as a vascular problem but that it represents an underlying metabolic disorder which culminates in the development of atherosclerosis.

There are also many publications recently on the insulin resistance syndrome (IRS) and the atherogenic lipoprotein phenotype (ALP) as metabolic disorders which accelerate the process of atherosclerosis. Such syndromes were thought to be inherited but subjected to modification by environmental factors. Personal interest in the IRS and current knowledge on the ALP and its relationship with atherosclerosis, had led to the subject for this particular thesis. The relationship between the IRS and the ALP were explored with data obtained from three studies, two of which were conducted in Scotland and the third was performed in Singapore.

Study, Aims and Objectives

The primary aim of the first study in Scotland was to determine the risk profile in family members of patients with coronary heart disease and the extent that these risk factors were influenced by lifestyle and socio-economic class. We were particularly interested in the lipid, lipoprotein and lipoprotein subfraction profile as well as glucose metabolism. These were correlated with markers of general obesity, central obesity as well as the post heparin lipase activity. Normals were recruited from various sources both within the hospital and also from newspaper advertisements and contacts of patients. This group of normals were to establish the reference ranges for lipid and lipoprotein parameters and for an exploration of the relationships between lipids, lipoprotein subfractions and anthropometry.

The second study was performed in Scotland and dealt primarily with the effects of alimentary lipaemia and its various ramifications in a group of middle aged men since many have argued that fasting lipids and lipoprotein profile do not represent the physiological state as most of us take several meals a day. The ability to handle fat meals is dependent on lipase activity, which in turn is modified by other factors amongst which is insulin and these issues are addressed.

The third study carried out in Singapore, involved a group of normal males from different ethnic groups with plasma cholesterol of less than 6.0 mmol/L. The primary aim

was to determine the prevalence of the atherogenic lipoprotein phenotype in normal males aged 30 to 45 years and its relationship to the insulin resistance syndrome in a population with high prevalence of diabetes mellitus. What is unique to the Singapore society is that different races live under fairly similar social and economic conditions and a secondary aim of this third study was to look for ethnic differences with regards to the various lipid and lipoprotein risk markers.

2.2 Materials and Subjects

2.21 Materials

Names and addresses of suppliers of the reagents used in the studies are shown in Appendix 1 together with manufacturers and suppliers of hardware and software used for this work.

2.22 Coronary Heart Disease in Families Protocol (Scotland)

All subjects were required to come to the Glasgow Royal Infirmary for 2 visits and gave written informed consent for each visit (Appendix 3). During the first visit, anthropometric indices and blood pressures were recorded. Fasting blood samples were taken for a full lipid profile, serum urea/electrolytes, thyroid function tests, liver function tests, details of which are given in subsequent sections. In addition, total LDL and HDL fractions, VLDL₁, VLDL₂, IDL and LDL and LDL subfractions were isolated from fresh plasma. This was followed by the conduct of a standard glucose tolerance test (75g glucose) with blood samples for glucose and insulin.

The second visit took place at least 2 weeks after the initial visit. Subjects were assessed for suitability of heparin administration through a questionnaire (Appendix 4). For this visit, 10mL of blood was taken before and 12 minutes after heparin administration into lithium heparin tubes placed on ice. The aliquots were frozen immediately and stored at -70° C prior to analyses. During the second visit, all results of tests done during the first visit were given to the subjects and any abnormalities were explained in detail. Furthermore, all who took part were given general health education with regards to diet and exercises and those found to have specific abnormalities were given appropriate counsel for intervention. Some were found to have high levels of lipids, requiring dietary intervention. These subjects were invited back for further follow up and lipid analyses. In instances where the lipids did not show adequate fall after at least 6 months of good dietary changes, such individuals were then advised on drug therapy and their GPs informed accordingly.

2.23 Recruitment of Patients

The inclusion and exclusion criteria for probands in both patients and controls are listed in Appendix 2. Patient probands were recruited from the Department of Cardiothoracic surgery and Department of Cardiology, from patients who had proven coronary heart disease from coronary angiogram or had coronary bypass operations or angioplasty between 1991 and 1992. All who satisfied our inclusion and exclusion criteria were

invited to take part if they were agreeable. Control probands were recruited from age and sex matched friends of patient probands as well as from those who responded to advertisements put up in the local papers. A total of 13 extended families made up of 27 nuclear families (total of 70 individuals) was finally recruited from patient probands. However, only 6 control families were recruited because of the stringent entry criteria. To make up for the lack of number in normal controls, independent individuals who satisfied the entry criteria were also recruited from staff at the Glasgow Royal Infirmary and friends of staff members. A total of 97 individuals were recruited as normals for the study. This number was added to the 6 control families already recruited. In addition, unrelated spouses of patient families were added into this pool of normal controls giving a total of 137 subjects. The number of normal subjects were increased with the addition of data from normal subjects recruited during previous studies done at the laboratory giving a total of 304 normal subjects.

2.24 Study Questionnaire

All subjects were asked to complete a lifestyle assessment questionnaire (see Appendix 5) during the first visit.

2.25 Blood pressure measurements

Blood pressure was taken by myself with an Accoson mercury sphygmomanometer during visit 1. All subjects were rested for at least 15 minutes after arrival and 5 minutes interval between blood pressure measurements. Korotkov phase V was taken as the diastolic blood pressure.

2.26 Anthropometric indices

Waist was recorded as the smallest circumference between the rib cage and iliac crest whilst the hip was recorded as the largest circumference between the waist and thigh, both being taken in the standing position.

2.27 Fasting blood samples

A total of 70 mL of blood was taken in the fasting state and put into five 10 mL EDTA tubes, one plain glass tube, one 4 mL EDTA tube and one 2.5 mL fluoride oxalate tube.

2.28 Oral Glucose Tolerance Test

75 gram of dextrose monohydrate diluted in 300 mL of water was prepared on the morning of the first visit. Subjects were fasted for at least 12 hours prior to the glucose tolerance test and encouraged to consume the glucose drink within a 5 to 10 minute period. Blood for glucose and insulin were sampled via a gauge 21 venflon into fluoride oxalate tubes and EDTA tubes respectively at half hourly interval. The venflon was flushed with 1 mL heparinised saline (10 units per mL) after each blood sampling to ensure patency. No food was allowed during the two hour period of the test but there was no restriction on clear fluids, coffee and tea taken without sugar or milk.

2.29 Post heparin Lipase sampling

Subjects were also instructed to fast for 12 hours prior to the second visit. Weight was retaken to ensure that heparin dose was according to present weight. A gauge 21 venflon was inserted into the cubital veins and 10 mL of blood was taken into a lithium heparin tube kept on ice. Heparin according to weight (70 u/kg body weight) was given through the venflon and a second 10 mL blood into lithium heparin tube kept on ice was taken after 12 minutes.

2.2.10 Data Analysis

All data analysis was done on the PC version of Minitab Release 10 for windows (Minitab Inc., PA). All variables were assessed for normality by drawing normality plots and in instances where they were not normally distributed, appropriate transformation were performed to obtain one. Models to explain the variability in cholesterol, triglyceride, LDL-cholesterol, HDL-cholesterol, VLDL₁, VLDL₂, IDL, LDL and LDL subfractions were developed with data from normal controls using an analysis of variance General Linear Model. Differences in mean values between groups were compared by Student unpaired t-test, using transformed normalised data where appropriate. A Bonferroni correction was employed to allow for multiple comparisons. Insulin resistance was calculated from fasting plasma glucose and insulin concentration ($IR = \text{fasting insulin} / 22.5 e^{-\ln \text{fasting glucose}}$) using the computer solved homeostasis model assessment (HOMA) method as this method had been shown to correlate with values obtained by the use of euglycaemic and hyperglycaemic clamps.

2.2.11 Alimentary Lipaemia Protocol (Scotland)

Subjects were required to come to the Glasgow Royal Infirmary for 3 visits. During the first visit, subjects were asked to complete a questionnaire and anthropometric indices, blood pressure readings were recorded. This was followed by blood sampling after heparin administration according to methodology described in a preceding section. An oral fat tolerance test was done during the second visit and a modified glucose loading test was conducted during the 3rd visit.

2.2.12 Patient Recruitment

Male patients under the age of 55 with documented coronary heart disease on coronary angiograms were eligible for the study. They must not be previously treated for any form of dyslipidaemia, diabetes mellitus or hypertension. The first 10 patients who were eligible and agreeable to take part in the study were finally recruited. 7 male controls matched for age were also recruited from friends of patients and staff.

2.2.13 Study Questionnaire

Subjects were asked to complete a simple questionnaire on the first visit (Appendix 7).

2.2.14 Anthropometric Indices and Blood pressure

Height, weight, waist and hip circumference were recorded during the first visit. Definitions for waist and hip was as in the previous study. Blood pressure was also recorded during the first visit after a 15 minutes rest and 2 readings were taken.

2.2.15 Visit 2: Fat meal

Subjects were given a standard fat meal after an overnight fast of 12 hours. The meal consisted of 280 ml fresh double cream, 20g sucrose, 20g dried skimmed milk powder and 20 ml of flavoured syrup. They were also instructed to refrain from smoking during the fast and from alcohol intake during the 3 preceding days. Blood was sampled into a 10 mL lithium heparin tube kept on ice, 10 mL EDTA tube and 2.5 mL fluoride oxalate tube at 0, 1, 2, 4 and 6 hours after the fat meal. The bloods were later measured for hepatic lipase, lipoprotein lipase, plasma cholesterol and triglyceride, free fatty acids and insulin at each time point. At the end of the 6th hour, intravenous heparin at 70u/kg were administered and the last sampling done 12 minutes later.

2.2.16 Visit 3: Glucose meal

After an overnight fast of 12 hours, subjects were given a 22 g glucose load carried in a virtually fat free yoghurt (0.1 g fat per 125 g tub). Blood sampling and intervals between sampling was done as in the fat meal.

2.2.17 Data Analysis

All data was subjected to test of normality as in the previous study and in instances where they were not normal, appropriate transformation was done. For the second and third visit, the lipase response, plasma cholesterol and triglyceride response, plasma glucose and insulin response were assessed by the area under the respective response curve. This was computed by using the trapezoid rule.

2.2.18 Coronary risk factors in Normal males in Singapore

The aims of this study are 1.) To establish normality ranges for lipids, Lp(a) and markers of insulin resistance in a group of Singaporean males. 2.) To look at the contribution of elevated triglyceride levels towards excess cardiovascular morbidity and mortality in the same group of normals. 3.) To explore the relationship between elevated plasma triglyceride and the presence of small, dense low density lipoproteins (LDL) subfraction, since the presence of small dense LDL is known to increase CAD risks by 3 to 7 fold. 4) To examine the metabolic links between the atherogenic lipoprotein phenotype and insulin resistance, which had been suggested in some recent research, but especially important in Singapore with a prevalence of diabetes mellitus at 8.6%.

2.2.19 Patient Recruitment

A total of 150 males aged between 30 to 45 years of age were recruited from hospital staff, friends and relatives of hospital staff, as well as advertisements placed in various public places. All subjects must not have had any history of diabetes mellitus, hypertension, coronary heart disease or chest pains, or any other major illnesses. They must also not be on any long term medication which may affect the lipid profile.

2.2.20 Conduct of each phase of the study

All patients were required to fast for at least 10 hours on the night preceding the tests. On the morning of the tests, the following were done.

1. Blood pressure taken after a 5 minutes rest, from the right arm and Korotkoff V taken as the diastolic pressure. The average of two readings was taken as the blood pressure.
2. Waist and hip measurements
3. Height and weight
4. A gauge 21 venula was inserted into the cubital veins, whereby fasting bloods for total cholesterol, HDL, TG, LDL, insulin, glucose, Lp (a) and LDL subfractions was taken.
5. Two further samples at 5 minutes interval were taken for glucose and insulin.
6. LDL subfractions was isolated from fresh plasma by non-equilibrium density gradient ultracentrifugation using a six-step, curvilinear salt gradient. Following centrifugation for 24 hours at 40,000 rpm, at 23°C in a swinging bucket rotor (SW40; Beckman Industries Inc.), the tube contents were eluted by upward displacement and the presence of LDL fractions detected by continuous monitoring at 280 nm. The subfraction areas under the concentration curve can be quantified (Data graphics, Beckman Industries Inc.) corrected for differences in extinction coefficient and expressed as percentage of total LDL.
7. Insulin resistance will be derived from mathematical modelling (Homeostasis model assessment; HOMA) using the 3 fasting samples of glucose and insulin, derived by D R Matthews et al, and this model had been validated against the glucose clamp technique, which is regarded as the gold standard for measurement of insulin resistance.

2.2.21 Data Analysis

Data were analysed using PC version of Minitab for windows version 10. As in the previous studies, all data will be assessed for normality and transformed appropriately if they were not normal.

2.2.22 Ethical Approval and funding

The studies conducted at the Glasgow Royal Infirmary were approved by the ethical Committee of the Hospital and funded in part by a grant from the British Heart Foundation. The study conducted in Singapore was approved by the ethical committees of the Singapore General Hospital and the National Medical Research Council. The funding for the Singapore study was through a grant from the National Medical Research Council of Singapore.

2.3 Methods

The assays and measurements of lipids, lipoproteins, proteins, insulin and other laboratory techniques are described in detail in the subsequent sections.

2.31 β Quantification

Plasma total cholesterol, triglyceride, HDL cholesterol, VLDL cholesterol and LDL cholesterol measurements were performed by a modification of the standard Lipid Research Clinics Protocol. Five mL of plasma was placed in a Beckman Ultra-clear tube (13 x 64 mm) and overlaid with 2mL of d 1.006 g/mL solution. Tubes were capped and centrifuged overnight at 35, 000 rpm (4°C) in a Beckman 50.4 rotor. The supernatant was collected from the top 25 mm into a 3 mL flask, this being the VLDL. The contents of the bottom fraction were transferred to a 5.0 mL volumetric flask. The tube was washed with saline and the wash added to the flask and the final volume adjusted to 5.0 mL with 0.15M NaCl. A 1.0 mL aliquot of this bottom fraction was then placed in a Beckman centrifuge tube and 50 μ L of precipitating agent {9.56 g Mn Cl₂.4H₂O + 1.05 g (approximately 5×10^5 units) heparin sodium salt in 25 mL 0.15M NaCl} were added and mixed. This mixture was kept at 4°C for 15 minutes and then centrifuged at 10,000 rpm for 30 minutes. This would precipitate the LDL fraction leaving the supernatant as the HDL fractions. The cholesterol content of whole plasma, top fraction (VLDL), bottom fractions(LDL + HDL) and heparin/Mn²⁺ precipitated supernatant (HDL) were then determined by enzymatic colorimetric assays as described in the next section. The total plasma cholesterol, triglyceride, LDL cholesterol and HDL cholesterol were measured by kind courtesy of the routine staff of the Lipid section of the Department of Pathological Biochemistry, at the Glasgow Royal Infirmary.

Calculations

Bottom fraction cholesterol - HDL cholesterol = calculated LDL

Total cholesterol - Bottom fraction cholesterol = calculated VLDL

Measured VLDL should agree with calculated VLDL by ± 0.35 mmol/L

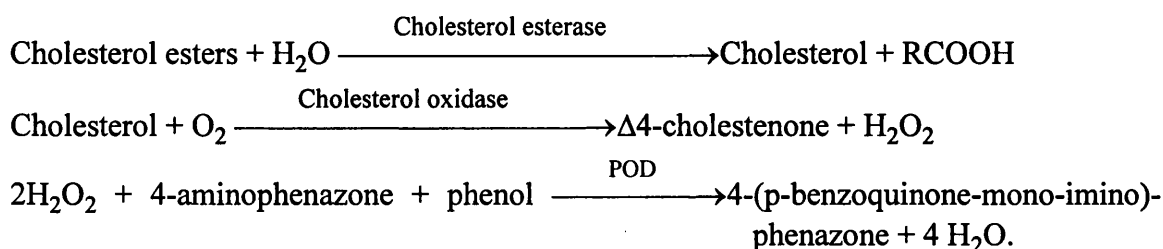
Lipid analysis done in Singapore

The plasma cholesterol, triglyceride and HDL cholesterol were done by dry chemistry methods using the Kodak Ektachem Clinical Chemistry Slide for cholesterol, triglyceride and HDL cholesterol read on the Kodak Ektachem 700 Analyzer. Lipid analysis was done by courtesy of the routine staff at the Pathology department of the Singapore General Hospital.

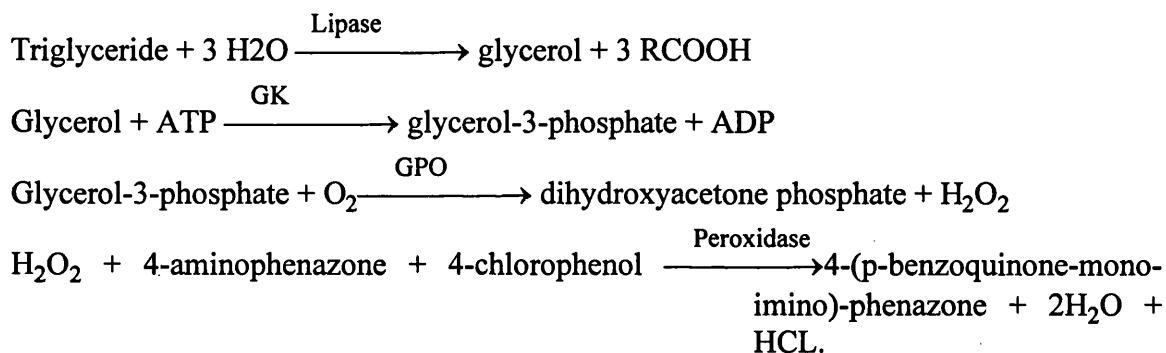
2.32 Compositional Analyses

Total cholesterol and triglyceride were determined in whole plasma and in the lipoprotein fractions by enzymatic colorimetric assays on a Hitachi 717 autoanalyser.

The principal of the cholesterol assay is shown diagrammatically below:



The principal of the triglyceride assay is as follows.



The assay kits used for the cholesterol and triglyceride were Boehringer Kit No.704121 and 704113 respectively and resultant colour changes were measured at 505 nm.

Free cholesterol and Phospholipid were estimated with Boehringer Kit No. 310328 and 691844 respectively using enzymatic colorimetric assays on a Centrifichem Encore centrifugal analyser (Baker instruments).

2.33 Modified Lowry Protein Assay

Protein measurements were performed according to a modified Lowry Protein Assay.

Reagents

1. Stock Reagents

Solution A- 2% Na_2CO_3 in 0.1M NaOH (w/v)

Solution B- 2% NaK Tartrate (w/v)

Solution C- 1% CuSO_4 (w/v)

Folin Ciocalteu Reagent- BDH

2. Working Reagents

Biuret Reagent: 100 mL Solution A, 1 mL Solution B, 1 mL solution C
If sample to be analysed is turbid, add 1 mg/mL sodium dodecyl (Lauryl) sulphate
Dilute stock Folin Ciocalteu 1:1 with deionised water.

Standards

A stock solution of human albumin (Fraction V) sigma A-8763, Lot 127F-9037 1 mg/mL is stored at 20°C.

Working standards in the range 0-50 µg (0, 15, 25, 50 µg) were prepared by taking appropriate volumes of stock standard (0-50 µl) and adjusting the final volume to 400 µl with deionised water.

Quality Control

Bovine serum albumin (Fraction V) Sigma A4503, Lot 17F-0150.

Two stock solutions of bovine serum albumin at 0.15 mg/mL and 0.30 mg/mL were stored at 20°C. 100 µl will be equivalent to 15 µg and 30 µg respectively. The final volume in the assay was adjusted to 400 µl with deionised water.

Sample preparation

Sample requiring dilution were adjusted to a final volume of 400 µl with deionised water. For VLDL₁ and VLDL₂ fractions, 100 µl sample was used whilst 50 µl sample was used for IDL fractions. In LDL fractions, 50 µl sample was diluted to 500µl with distilled water and 100 µl of this diluted sample was used for the assay.

Method

1. 2 mL of Biuret reagent were added to 400 µl standard, control and samples.
2. These were vortexed and then allowed to stand for 10 minutes.
3. 200 µl working Folin Ciocalteu reagent was added with immediate mixing.
4. Allowed to stand for 30 minutes.
5. Read optical density at 750 nm within 2 hours

Sigma Lowry Protein Assay done in Singapore

For the Lowry proteins done in Singapore, the Sigma Diagnostic Protein Assay kit were used but modified for our purposes.

Reagents

Modified Lowry reagent

DOC solution. An aqueous solution of sodium deoxycholate, 1.5 mg/ ml

TCA: Trichloroacetic Acid solution, 72% w/v

Folin & Ciocalteu's Phenol Reagent

Protein Standard, prepared from bovine serum albumin fraction V

Working Folins

18 ml was transferred to a dark bottle and washed with 10 ml water, add 80 ml, stored at room temperature.

Lowry Reagent

40 ml water was added to Lowry reagent and mixed slowly to avoid foaming, then stored at room temperature.

Protein standard

Add volume of water and swill gently, stored at 4° C

Assay standard curve

	µg	µl standard	µl water
	0	0	1000
	10	25	975
	20	50	950
	30	75	925
	40	100	900
	50	125	875
Sample 1:10 dilution	?	100	900

Procedure

- 1 Add sample or standard as above
- 2 Add volume of water
- 3 Add 1 ml Lowry reagent
- 4 Mix
- 5 Stand at room temperature for 20 minutes
- 6 With immediate mixing, add 500 µl Folins
- 7 Read OD at 750 nm (500-800) at 30 minutes

Standard curve should be straight line in this range

2.34 Analytical ultracentrifugation of HDL

Plasma concentrations of HDL₂ and HDL₃ were estimated by analytical ultracentrifugation in a Beckman Model L8-70 ultracentrifuge equipped with an

ultraviolet scanning attachment, (Beckman Instruments) using an AnF rotor with double sector centrepiece. The HDL subfraction masses were estimated by kind courtesy of Mrs Elizabeth Murray (Institute of Biochemistry, Glasgow Royal Infirmary).

2.35 Sequential preparation of IDL, LDL and HDL

Density Solutions

Solution Density = 1.006 g/mL

11.4 g NaCl + 0.1 g EDTA Na₂ + 500 mL H₂O + 1 mL NNaOH

Dissolve solids and make up to 1 litre and add 3 mL additional H₂O. Final NaCl concentration = 0.195 M

Solution Density = 1.182 g/mL

24.98 g NaBr + 100 mL d=1/006 g/mL solution. Final NaBr concentration = 2.44M

Solution Density = 1.478 g/mL

78.32 g NaBr + 100 mL d=1.006 g/mL solution. NaBr concentration = 7.65 M

Solution Density = 1.019 g/mL

8 mL 1.182 + 100 mL saline

Solution Density = 1.063 g/mL

Add d = 1.006 g/mL to d = 1.182 g/mL at 2:1 ratio

Solution Density = 1.21 g/mL

Add d = 1.063 g/mL to d = 1.478 g/mL at 2:1 ratio.

All densities are checked with a digital densitometer DMA 35

IDL 1.019 g/mL

To 4 mL plasma, add 0.32 mL d = 1.182 g/mL solution. This was mixed and overlaid with 1.68 mL d = 1.019 solution to give a total volume of 6 mL. Centrifuged in L7-55 ultracentrifuge at 39,000 rpm at 4°C for 24 hours or at 15° for 16 hours. The top 2 mL is then removed.

LDL 1.063 g/mL

To the remaining 4 mL, 1.47 mL of $d = 1.182$ was added, mixed and transferred to new tubes. This was overlaid with 0.53 mL $d = 1.063$ to give a final volume of 6 mL. Centrifuged in L7-55 ultracentrifuge as above. The top 2 mL is again removed.

HDL 1.21 g/mL

To the remaining 4 mL, add 2 mL $d = 1.478$ solution, mixed and transferred to new tubes. The final volume should again be 6 mL. Centrifuged as above and the top 1.2 mL is removed.

2.36 Method for LDL Subfraction Analysis

Density Solutions

Density solutions $d = 1.006$ g/mL and $d = 1.182$ g/mL were prepared as described in the preceding section. The respective density solutions were then prepared as follows.

Solutions	Volume $d = 1.006$ g/mL		Volume $d = 1.182$ g/mL
1.019	100 mL	+	8.5 mL
1.024	100 mL	+	13.6 mL
1.034	100 mL	+	18.6 mL
1.045	100 mL	+	27.8 mL
1.056	100 mL	+	42.9 mL
1.060	100 mL	+	49.3 mL

All densities were checked with a digital densitometer.

Methods

3 mL of fresh plasma was adjusted to a density of 1.09 g/mL by adding 0.25 g KBr and 0.3 mL of $d = 1.182$ g/mL solution. The sample and 6-step salt gradient were introduced sequentially into polyvinyl alcohol coated polyallomer SW-40 tubes by peristaltic pump. The gradient was prepared and centrifugation carried out at 23°C in a Beckman L8-60. The rotor was accelerated to 170 rpm and then centrifuged at 40,000 rpm for 24 hours. On completion of the run the rotor was stopped with the brake off. After centrifugation, the LDL subfractions were eluted by upward displacement using dense hydrophobic material (Maxidens, 1.9 g/mL, Nyegaard Ltd) by a constant infusion pump (Sage instruments, Orion Research Incorp., USA) at a flow rate of 0.69 mL/min. The eluate was passed through a UV detector (MSE/Fisons, UK) and detected by continuous monitoring of absorbance at 280 nm. In most instances, it was possible to resolve three distinct subfractions i.e. LDL-I, LDL-II and LDL-III. The individual subfraction areas beneath the LDL profile were quantified using Beckman 'Data Graphics' software (Beckman, UK). The detection system measured LDL concentration as absorbance at 280 nm and this was corrected to lipoprotein mass equivalence by applying previously calculated extinction coefficient. LDL-I 1 optical density unit (OD) = 2.63 mg lipoprotein/ml, LDL-II 1 OD = 2.94 mg lipoprotein/ml and LDL-III 1 OD = 1.92 mg lipoprotein/ml. For the LDL

subfractions done in Singapore, the DU650 spectrophotometer was used to resolve the subfractions. However, the data captured on the DU650 (as duf.files) were not compatible with the Beckman 'Data Graphics' software (bsf.files). Hence Beckman USA had to write a conversion program in order to facilitate the conversion of data captured on the DU650 into a format (BSF) which can be read by the existing 'Data Graphics' software. Mrs Muriel Caslake from the Department of Pathological Biochemistry (Glasgow) was also flown in during the setting up of the LDL subfraction system to ensure consistency and compatibility between the LDL subfractions obtained in Glasgow and in Singapore. The integrated areas were corrected for differences in extinction coefficient and expressed as percentage of total LDL concentrations in mg of lipoprotein/dL plasma. The total value for total LDL (d 1.019-1.063) lipoprotein mass (free cholesterol + cholesteryl ester + triglyceride + phospholipid + protein) was then used to generate individual subfraction concentration. Cholesteryl ester was calculated from (total cholesterol minus free cholesterol) times 1.68.

2.37 Very Low Density Lipoprotein Subfraction isolation

VLDL₁ and VLDL₂ were prepared from plasma by a modification of the cumulative gradient centrifugation technique described by Lindgren et al.

Density solutions

The density solutions for the gradient was prepared from d = 1.006 g/mL and d = 1.182 g/mL solutions.

Density (g/mL)	Volume d = 1.006 g/mL		Volume d = 1.182 g/mL
1.0988	25 mL	+	27.89 mL
1.0860	25 mL	+	20.83 mL
1.0790	25 mL	+	17.72 mL
1.0722	25 mL	+	50.05 mL
1.0641	25 mL	+	12.31 mL
1.0588	25 mL	+	10.73 mL

Methods

2 mL of plasma was adjusted to a density of 1.118 g/mL with 0.341 g NaCl. This was mixed well and allowed to stand for a short time before sampling. 0.5 mL of d = 1.182 g/mL solution was pipetted into the bottom of the tube and then plasma and density solutions overlaid in the order and volumes shown below using an AAI pump.

Density in g/mL	Volumes in mL
1.182	0.5
Plasma	2
1.0988	1
1.0860	1
1.0970	2

1.0722	2
1.0641	2
1.0588	2

When the gradient was prepared, centrifugation was carried out in a Beckman SW 40 rotor at 39,000 rpm at 23°C for 1 hour and 38 minutes. The top 1 mL was carefully removed using a long-form glass pipette at the end of the run. This was then replaced with 1 mL $d = 1.0588$ solution and centrifuged at 18,500 rpm for 15 hours and 41 minutes. At the end of this second run, the top 0.5 mL is removed with the long-form glass pipette.

2.38 Insulin assay

The insulin assay used in Scotland was an in-house immunoradiometric assay employing 2 antibody reagents, one of which was labelled and the other coupled to a solid phase matrix. The antibodies are directed at different sites on the protein to be measured and only when both are attached to the protein molecule is a signal generated. The antibodies used are ^{125}I -monoclonal anti-insulin antibody prepared from mouse hybridoma monoclonal and solid phase guinea-pig anti-insulin coupled to sepharose gel. The insulin assays were done by myself with the assistance of a summer student, Ms Balsam Alabassi.

Reagents

1. Stock Buffer

Dissolve 50.5 g of EPPS and 2.0 g sodium azide in approximately 1400 ml of distilled water. The pH was adjusted to 8.0 by adding sodium hydroxide pellets (approximately 5 g) and made up to 21 with distilled water. 4 mL of Tween-20 is added and stored at room temperature (2 weeks).

2. Assay Diluent

The assay diluent was prepared fresh for each assay by adding 500 μl of normal sheep serum per 100 mL of stock buffer. Approximately 100 mL was required for each assay.

3. ^{125}I -Monoclonal Anti-insulin

The antibody (ID1/C10) is a mouse hybridoma monoclonal prepared by Scottish Antibody Production Unit and diluted 1:100 in assay diluent for routine use.

4. Solid-Phase Guinea-Pig Anti-insulin

This antibody was also supplied by Scottish Antibody Production Unit and was coupled to sepharose gel. The preparation was diluted 1:2 in 0.9% NaCl for use. Saline was added to the stock and well mixed and centrifuged for 15 minutes. The supernatant was aspirated and made up to twice the original stock volume with assay diluent e.g. 6 mL stock was made up to final volume of 12 mL.

5. Wash solution

The assay was washed four times with 0.9% saline/0.2% Tween i.e. 9 g NaCl per litre of distilled water with 2 mL of Tween.

Standards

1. Stock standard: MRC Standard 66/304 and one ampoule contains 3IU.
2. Working Stock A: (2U/mL). Dissolve the contents of stock standard ampoule in 1.5 mL 0.01N HCl (0.25% BSA) and stored at -70°C in 100 µl aliquots.
3. Working Stock B: (2mU/mL). Dilute 100 µl of A to 100 mL with assay diluent and store at -70°C in 5 mL aliquots.
4. Working Standard: (100 mU/L). Dilute Stock standard B 1:20 with assay diluent and store in 1 mL aliquots at -70°C. For use in the assay, this standard is diluted serially with assay diluent to give 8 standards: 50, 25, 12.5, 6.25, 3.12, 1.6, 0.8, 0 mU/L.

Quality Controls

Internal

Human serum screened for HIV and Hepatitis viruses was spiked with insulin standards as shown below:

Basal, +10, +20, +40 mU/L

These QCs were stored at -70°C in 600 µl aliquots. QC's were run at the beginning and end of the assay.

External

The UK NEQAS has a scheme for insulin which the laboratory participates in and was run from Guildford, Surrey. Results were routed through the QA officer.

Method

1. Dispense 100 µl of standards, QC, samples in duplicate into plastic tubes
2. Add 100 µl of labelled antibody.
3. Mix and incubate overnight at room temperature.
4. Add 100 µl of solid-phase antibody and shake on a Denley shaker for 1 hour at room temperature.
4. Wash tubes four times with 0.9% saline/Tween using in-house system.
5. Count tubes using Pharmacia gamma counter.

Calculation

Calculation of results was done using the software package RiaCalc and the INS RIA protocol, whilst counting was taking place.

1. Standard curve was presented on screen for approval, editing or rejection.
2. When standard curve was accepted, QC and unknown results were printed.
3. Precision profile and trend analyses were presented.

Insulin assays done in Singapore

This was done by the Abbot Imx® Insulin assay, which is a microparticle enzyme immunoassay (MEIA).

Biological Principles of the procedure

The Imx insulin reagents were added to the reaction cell in the following sequence:

The probe/electrode assembly delivered the sample, anti-insulin (mouse, monoclonal) coated microparticles and the assay buffer to the incubation well of the reaction cell forming an antibody-insulin complex.

An aliquot of the reaction mixture containing insulin bound to the anti-insulin coated microparticles was transferred to the glass fibre matrix.

The matrix was washed to remove unbound materials.

The Anti-insulin: alkaline Phosphatase conjugate was dispensed onto the matrix and binds to the antibody-antigen complex.

The matrix was washed to remove unbound materials.

The substrate, 4-methylumbelliferyl Phosphate, was added to the matrix and the fluorescent product was measured by the MEIA optical assembly.

Reagents

- 1 1 bottle (7ml) anti-insulin (mouse, monoclonal) coated microparticles in buffer with protein stabilisers. Preservative: 0.1% sodium Azide and antimicrobial agents.
- 2 1 bottle (9ml) anti-insulin (mouse, monoclonal) : alkaline phosphatase conjugate in buffer with protein stabilisers. Minimum concentration: 3 µg/ml. Preservatives: 0.1% sodium azide and antimicrobial agents.
- 3 1 bottle (10ml) 4 Methylumbelliferyl phosphate, 1.2mM, in buffer. Preservative: 0.1% sodium azide.
- 4 1 bottle (14ml) assay buffer in calf serum. Preservative: 0.1% sodium azide and antimicrobial agents.

Imx Insulin MODE 1 Calibrator

1 bottle (4ml) MODE 1 calibrator (D). Concentration: 30 µU/mL insulin (human) in buffer. Preservative: 0.1% sodium azide and antimicrobial agents.

Calibrators

IMx insulin calibrators

The 6 bottles (4 ml each) of IMx Insulin calibrators are referenced against WHO insulin IRP at 92.5% of the WHO concentration. The IMx Insulin calibrators contain insulin (human) prepared in buffer at the following concentrations:

Bottle	Insulin concentration ($\mu\text{U}/\text{mL}$)
A	0
B	3
C	10
D	30
E	100
F	300

2.39 Lipase assay

Post heparin plasma was incubated with a ^{14}C -labelled triglyceride/gum arabic emulsion; free fatty acids released by lipase activity were captured by albumin and extracted into a solvent. The ratio of radioactivity in the extracted fraction to the total present in blank incubations provided the basis of calculating the activity of the enzyme source. Selective measurement of Lipoprotein Lipase (LPL) is facilitated by preincubation of post heparin plasma with sodium dodecyl sulphate (SDS) to inactivate hepatic triglyceride lipase (HL) and the inclusion of serum as a source of LPL activator (apo C-II). HL activity is measured in 1.0 M sodium chloride to ensure inactivation of LPL. The lipase assays were done by Mr Michael McConnell at the Institute of Biochemistry, Glasgow Royal Infirmary.

Reagents

Glycerol Tri ($1\text{-}^{14}\text{C}$) oleate in toluene, 250 μCi (Amersham CFA 258)

Triolein	(Sigma T-7140)
Gum Arabic	(Sigma G-9752)
Bovine Serum Albumin Fraction V	(Sigma A-4503)
Trizma Base	(Sigma T-3253)
Sodium Dodecyl Sulphate	(BDH 44244)
Potassium sulfate	(Sigma P-4020)
Thrombin	(Sigma T-4265)

Preparation of stock reagents

Cold Triolein (20 mg/mL in toluene).- Dissolve 0.5 g triolein in 25 mL toluene.

Radioactive Triolein

To 50 μCi (0.5 mL) glycerol tri ($1\text{-}^{14}\text{C}$) oleate add 24.5 mL toluene.
Divide into 7 x 3.5 mL aliquots in round bottom glass flasks.

To each aliquot, add 3.5 mL cold triolein.

Dry under nitrogen.

Wash each flask three times with 3 mL heptane. Dry down under nitrogen between washes and keep the flask in a hot water bath.

Store the tubes dried down under nitrogen and sealed at -20°C.

5% Gum Arabic in 0.2 M Tris-HCl pH 8.4

5 g gum arabic in 100 mL volumetric flask, made up to volume with 0.2 M Tris-HCl (T-1503) pH to 8.4.

Filter through cotton gauze.

Divide into 18 x 5.5 mL aliquots and freeze at -20°C.

10% BSA in 0.2 M Tris-HCl pH 8.4

Weigh out 10 g BSA

Make up 20 mL of 1M Tris-HCl (2.422 g om 20 mL).

To 20 mL of 1M Tris-HCl, add approximately 40 mL of water, add 10 g BSA.

When the BSA has dissolved, adjust pH to 8.4.

Dilute to 100 mL with distilled water and divide into 18 x 5.5 mL aliquots and freeze at -20°C.

Extraction solution

Methanol : 1.41 parts (141 mL)

Chloroform : 1.25 parts (125 mL)

Heptane : 1.00 parts (100 mL)

Extraction Buffer

0.14M potassium carbonate, 0.14M boric acid, pH 10.5

Dissolve 3.8699 g K_2CO_3 and 1.7312 g H_3BO_3 in 200 mL distilled water.

Adjust pH to 10.5 with 2M KOH.

Serum

Collect 100 mL of fasted blood into EDTA from a number of individuals.

Add 0.1% w/v $CaCl_2$ bovine thrombin 1U/mL.

Incubate at 39°C for 30 minutes.

Remove the clot.

Dialyse against 0.15M NaCl in distilled water, pH7.0, using 6,000-8,000 MWCO membrane.

Heat at 56°C for 30 minutes.

Dialyse against PBS.

Store as 1 mL aliquots at -20°C.

SDS reagent

0.2M Tris-HCl, 25 mM SDS (50 mM SDS for horses)
0.360 g SDS, 1.21 g Tris-Base in 40 mL distilled water.
Correct pH to 8.2, then make up volume to 50 mL in a volumetric flask.

Low salt buffer: 0.2M Tris, 0.216M NaCl, pH 8.4

12.11 g Tris Base, 6.31 g NaCl in 500 mL distilled water. This gives a final NaCl concentration of 0.1M in the reaction solution.

High Salt buffer: 0.2M Tris, 2.16M NaCl, pH 8.4
12.11g Tris Base, 63.11 g NaCl in 500 mL distilled water. This gives a final NaCl concentration of 1M in the reaction solution.

Assay Design

Low salt assay (4 tubes)		High salt assay (2 tubes)
Tubes 1,2	Tubes 3,4	Tubes 5,6
-SDS	+SDS	
30 μ l 0.15M NaCl	20 μ l 0.15M NaCl	30 μ l 0.15M NaCl
10 μ l PHP	20 μ l Preincubation mixture	10 μ l PHP

NB: Each sample run in duplicate.
All tubes, samples, substrate and serum kept on ice prior to incubation.
Include two tubes with 40 μ l 0.15M NaCl (no PHP) in each assay half to measure blank (B) and total counts (TC).

Assay Procedure

Preincubation with SDS reagent

Place 0.5 ml post heparin plasma in TMU tube and add 0.5 mL SDS reagent and vortex.
Incubate in the water bath at 26°C for 60 minutes and remove 20 μ l for the low salt assay.
20 μ l is removed for the assay.

Preparation of substrate mixture (30 minutes before required)

Radioactive triolein was placed in a glass flask and 5.5 ml gum arabic solution was added. This was sonicated on ice at 18 microns for 4 minutes so that no fat droplets are visible on the surface. (Sonicator tip 1/2 cm from bottom of flask). Add 5.5 ml of 10% BSA solution, mixed and vortex and keep on ice.

Reaction mixtures

Tubes 1,2,3,4 : To each add 200 μ l substrate
 : To each add 250 μ l low salt buffer (0.2M Tris/0.1M NaCl)

- : To each add 50 µl serum.
- Tubes 5,6 : To each add 200 µl substrate
- : To each add 250 µl high salt buffer (0.2M Tris/1.0M NaCl)
- : To each add 50 µl 0.15 NaCl

Incubation

Cap all tubes and incubate in water bath at 28° C for 60 minutes and return to ice immediately. Add 3.25 ml extraction solution to each tube. Add 0.75 ml extraction buffer to each tube, mix on vortex. Then centrifuged at 4°C, 3K for 30 minutes. Take 1 ml of upper phase for counting in scintillation vial. Add 10 ml Ultima Gold (Packard) scintillation fluid and 200 µl acetic acid. For blank, take 1ml upper phase and for total counts, take 1 ml of lower phase.

Calculation of results

Lipase activity in µmol FFA released/ ml/ hr= $\frac{(\text{CPM samples} - \text{CPM blank}) \times 755.1}{\text{CPM total} - \text{Background}}$

Modification of the lipase assay for the measurement of lipoprotein lipase (LPL) and hepatic lipase (HL) in preheparin plasma

1. Samples were collected into lithium heparin tubes and kept on ice. Plasma should be separated at 4° C and frozen at -70° C within one hour of collection.
2. Samples are assayed in triplicate for LPL, HL and blanks.
3. For the measurement of LPL, plasma was pre-incubated with 35 mM SDS (instead of 25 mM) in 0.2M tris base, pH 8.2 which was made up fresh on the day of assay. Incubation was for 60 minutes at 26° C as per the standard assay.
4. 40 µl of the plasma-SDS mixture (instead of 20 µl) was taken for the LPL incubation: the LPL substrates contains 0.2M NaCl as per the standard assay.
5. The HL incubation contains 20 µl of plasma (instead of 10 µl) with substrate containing 2.0 M NaCl as per the standard assay.
6. Both the LPL and HL incubations were for 1.5 hours (instead of 1 hour).
7. Following solvent extraction, 2 mL of the upper layer (instead of 1 mL) was taken for liquid scintillation counting (added to 10 mL Ultima Gold, 200 µl of acetic acid). Use 1 ml of blank lower phase for total counts.

Calculation of results

$$\text{Lipase activity} = \frac{(\text{CPM samples} - \text{CPM blank}) \times 755.1 \times 1/1.5 \times 1000 \times 0.5}{(\text{CPM Total} - \text{Background}) \times 2}$$

2.3.10 Lipoprotein (a) assay

Lp (a) assay was done with Innotest Lp (a) kit (Innogenetics NV, Belgium), which is an enzyme immunoassay (Elisa) for the quantitative determination of lipoprotein (a) levels in human plasma or serum.

Reagents:

1. 1 sachet containing a strip-holder with 12 x 8 anti-Lp (a) (mouse monoclonal) coated test wells and a silica gel bag as drying agent.
2. 1 vial containing 0.250 ml of a prediluted Lp (a) standard labelled as 100 mg/dl (phosphate buffer with stabilising proteins, containing 0.05% Kathon CG as preservative).
3. 1 vial containing 0.25 ml of prediluted control serum level I (human serum, prediluted in sample diluent + preservatives).
4. 1 vial containing 0.25 ml of prediluted control serum level II (human serum prediluted in sample diluent + preservatives).
5. 1 vial containing 50 ml of concentrated sample diluent (phosphate buffer with stabilising proteins, containing 0.05% Kathon CG as preservative), to be diluted 10 times before use.
6. 1 vial containing 0.4 ml of concentrated conjugate (sheep anti-apo B polyclonal antibody labelled with horse-radish peroxidase, containing 0.05% Kathon CG as preservative), to be diluted 100 times before use.
7. 1 vial containing 20 ml of conjugate diluent (phosphate buffer with stabilising proteins, containing 0.05% Kathon CG as preservative).
8. 1 vial containing 0.3 ml of concentrated TMB substrate solution (tetramethylbenzidine dissolved in dimethyl sulfoxide), to be diluted 100 times before use.
9. 1 vial containing 20 ml of substrate buffer (phosphate citrate buffer containing 0.006% hydrogen peroxide); ready to use.
10. 1 vial containing 60 ml of concentrated wash solution (phosphate buffer containing detergent and 0.17% Kathon CG as preservative) to be diluted 25 times before use.

Procedure

1. Sample diluent

30 ml concentrate was made up to 300 ml with deionised distilled water.

2. Standards

Standards were prepared by serial 2-fold dilution with sample diluent.

100 mg/dl	undiluted
50 mg/dl	100 μ l diluent and 100 μ l 100 mg/dl standard
25 mg/dl	100 μ l diluent and 100 μ l of 50 mg/dl standard
12.5 mg/dl	100 μ l diluent and 100 μ l of 25 mg/dl standard
6.3 mg/dl	100 μ l diluent and 100 μ l of 12.5 mg/dl standard
3.1 mg/dl	100 μ l diluent and 100 μ l of 6.3 mg/dl standard
0 standard	sample diluent

3. First sample dilution 1:200

10 μ l sample and QCs were added to 1990 μ l diluent and mixed.

4. Second sample dilution 1:10

30 μ l diluted samples, QCs and standards was added to 270 μ l diluent and mixed.

5. 100 μ l of diluted samples, QCs and standards were added to each well and incubated at 37° C for 2 hours. The wash solution was placed in the incubator to dissolve any crystals. Then 24 ml of concentrate is diluted to 600 ml with deionised distilled water.

6. After incubation, wash each well 4 times leaving 30 seconds soak for each wash.

7. Conjugate

120 μ l concentrate was made up to 12 ml with diluent. Add 100 μ l to each well using multidispenser and incubate for 1 hour at 37° C.

8. Substrate

120 μ l concentrate TMB substrate was made up to 12 ml with substrate buffer. Each well was washed 4 times as before. Add 100 μ l substrate to each well and incubate for 30 minutes at room temperature, allowing 10 seconds between each addition.

9. After incubation, add 100 μ l 1.5M sulphuric acid to each well to stop the reaction.

10. Read absorbance at 450 nm on Dynatech within 15 minutes.

The Lp(a) done in Singapore uses the Beckman LPA lipoprotein (a) reagent kit.

2.3.11 Non esterified Free Fatty acid assay: Microtitre Method

Reagents and Materials

96 well Microtitre plate

p10 pipette

Gilson micro-pipetter tips

Dynatech MR 5000

BCL 8000 Repeater pipette

Wako NEFA C kit consisting of colour reagents A and B, and diluents for these and 1 bottle of NEFA standard solution

QC material: using seronorm Lipid which gives a value of 0.650 mmol/L, actual range of 0.640-0.680 mmol/L

Method

- 1 Allow standards, samples and reagents to reach room temperature.
Switch on plate mixer/incubator and allow temperature to equilibrate at 37°C
- 2 Standards
In wells A1 to A12, prepare a range of standards from 0 to 1.0 mmol/L in duplicate as follows:

Standard concentration (mmol/L)	Standard volume (µL)	Volume of dH ₂ O (µL)
0	0	5
0.2	1	4
0.4	2	3
0.6	3	2
0.8	4	1
1.0	5	0
- 3 Samples:
Working in duplicate, pipette 5µ of QC and samples into the wells on the microtitre plate.
- 4 To each well add 50µL of solution A using the repeater pipette with a pipette tip attachment touching the side of the well to ensure complete dispersion of the solution.
- 5 Cover with polyethylene plate seal. Immediately place in the plate mixer and incubate at 37°C for 10 minutes exactly.
- 6 Remove from incubator and add 100µL of solution B.
- 7 Place in plate mixer and incubate for a further 10 minutes at 37°C.
- 8 After exactly 10 minutes, remove from incubator and allow plate to equilibrate to room temperature for 10 minutes.
- 9 Remove plate seal cover carefully and read absorbance at 540 nm on the Dynatech MR5000 (stored as test No. 1)

2.3.12 Surface modification of Beckman Ultraclear centrifuge tubes

This was a procedure for coating the interior surface of Beckman ultraclear centrifuge tubes (in particular those used in swing-out rotors) with polyvinyl alcohol. Once coated, salt solutions are then able to run smoothly down the sides of the tubes.

Preparation of coating reagents

10 g polyvinyl alcohol was dissolved in 250 ml distilled water by stirring and heated to a gentle reflux. 250 ml of propan-2-ol is slowly added to the hot solution with stirring and heating until a clear solution is obtained.

The solution was cooled to room temperature. Beckman ultraclear tubes are filled with the solution and left for 15 minutes before the solution was removed. After removing the small amount of solution which collected at the bottom on standing, the tubes were dried overnight.

The tubes were filled with distilled water which was poured out after standing overnight at room temperature. Finally the tubes were briefly flushed with water, tapped to remove excess liquid and left to dry.

In Singapore, the coating reagents were prepared as above at the Department of Biochemistry, National University of Singapore and the use of the reflux system at the department was by kind permission of Associate Professor Kon Oi Lian, Head of the Department of Biochemistry.

Department of Clinical Biochemistry, Singapore General Hospital

All the laboratory tests done in Singapore were carried out at the department of Clinical Biochemistry. the laboratory participates in several quality assurances schemes to ensure a high standard of quality control. These included

- 1 Randox (from UK)
- 2 Murex (from UK)
- 3 WHO (World Health Organisation)
- 4 CAP (College of American Pathologist)
- 5 NQCS (National Quality Control Assurance Scheme)

Grateful thanks also to Dr Chio Lee Foon and Mr Roland Chu for assistance in the laboratory procedures.

Chapter 3 Lipoprotein Subfraction Metabolism

The heart of the prudent acquires knowledge, And the ear of the wise seeks knowledge.

Proverbs 18:15

3.1 Introduction

Plasma lipoproteins are known to play a causative role in atherosclerosis and its clinical manifestation, coronary heart disease (CHD). Raised plasma cholesterol levels, particularly of low density lipoprotein (LDL) cholesterol, correlate positively with the incidence of CHD whilst high density lipoprotein (HDL) displays a negative association. The nature of the risk associated with elevations in plasma triglyceride, carried mainly in very low density lipoproteins (VLDL) is currently controversial. However, it is increasingly clear that one way in which this plasma lipid relates to CHD is through its influence on the structure of LDL and HDL, in particular its relationship to the subfraction distribution within these density classes. Recent improvements in methods for separating and quantifying lipoprotein subclasses have improved our understanding of the role of lipoproteins in the pathophysiology of CHD. The VLDL fraction can be divided into at least two components of differing size, density and metabolic properties.^{162,163} LDL is thought to consist of at least 3 to 7 subpopulations^{132, 133} while the intermediate density class (intermediate density lipoprotein, IDL) has been reported to contain larger IDL₁ and smaller IDL₂ particles.¹²⁷ The heterogeneity observed within these density intervals may be linked since it has been suggested that the conversion of VLDL to LDL does not occur via a single delipidation chain. Rather, parallel processing pathways operate within the delipidation cascade.^{111,164} For example, large VLDL when delipidated give rise to remnants in the smaller VLDL₂ and IDL density intervals and these are inefficiently converted to a class of LDL which is cleared slowly from the plasma. Newly synthesised small VLDL (VLDL₂), on the other hand, is rapidly and almost quantitatively delipidated to LDL which is catabolised rapidly.

Metabolic studies of LDL have been complemented in recent years by examination of its subfraction distribution by the high resolution subfraction techniques of gradient gel electrophoresis and density gradient ultracentrifugation. Austin et al¹³⁵ demonstrated an association between a particular LDL phenotype and CHD risk. The atherogenic lipoprotein phenotype (ALP), defined by the predominance of small, dense LDL ('pattern B'), a moderately elevated plasma triglyceride and a low HDL cholesterol was associated with a 3-fold increase in CHD risk. Using the density gradient technique to quantify individual LDL species, we have translated the ALP into a plasma concentration of LDL-III (d 1.045-1.065 kg/L) of greater than 100 mg lipoprotein/dl plasma,¹³⁶ and shown that this, when present, gives a 7-fold increased risk in a case control study of myocardial infarct (MI) survivors versus normals. The ALP has also been recently been linked to presence of insulin resistance as an underlying metabolic disorder.⁷⁴ This insulin resistance syndrome (IRS), characterised by fasting hyperinsulinaemia and exaggerated insulin response to a glucose challenge¹³ is considered an independent risk factor for CHD. Furthermore insulin resistant individuals, especially those with frank non-insulin dependent diabetes mellitus (NIDDM), have alterations in VLDL structure and metabolism with an abundance of the larger, triglyceride-rich VLDL species.¹⁶⁵ IRS is associated with an increase in central obesity as indicated by increased body mass index

(BMI) and waist/hip ratio (WHR). However, the precise effects of changes in anthropometric indices on plasma lipoprotein subfraction distributions are unknown although it has been linked to an LDL pattern dominated by small dense LDL.⁷⁵ Epidemiological studies have further shown that ALP expression is age and sex dependent.¹¹⁰

In our study of a large group of subjects whose plasma lipid values spanned the normal range, we sought to understand the relationships between apolipoprotein B containing lipoproteins in terms of their overall concentration, composition and subfraction distribution. These parameters were also related to total plasma lipid levels, anthropometric indices and gender. Our principal hypothesis was that the presence of elevated plasma triglyceride (possibly associated with insulin resistance) and the activity of lipolytic enzymes together were responsible for the accumulation of small, dense LDL. Further, the extent to which the male-female difference in CHD risk could be explained by the variation in lipoprotein subfractions profiles were examined.

3.11 Statistical methods

Statistical analysis and manipulations were performed using the PC version of MINITAB Release 10 for Windows (Minitab Inc., PA). All variables were assessed by drawing normality plots and in instances where they were not normally distributed, appropriate transformations were performed to obtain one. The following were subjected to \log_e transformations; BMI, WHR, plasma triglyceride, HDL cholesterol, fasting insulin, LPL, HL, VLDL₁ and VLDL₂, LDL-I, LDL-II and LDL-III concentrations. Plasma VLDL cholesterol was normalised by taking the square root whilst fasting glucose was normalised by squaring it. Associations between variables were tested by calculating the Pearson correlation coefficient and the coefficient of determination (r^2) which was expressed as a percentage, i.e. r^2 gives the percentage of variation in the dependent variable which is explained by variation in the independent variable). The significance of association between pairs of variables was determined by linear regression. Multivariate analysis was employed to determine the extent to which age, sex, general obesity (BMI), central obesity (WHR) and markers of insulin resistance (fasting glucose and insulin) explained the variability in post-heparin plasma LPL and HL activity, VLDL₁, VLDL₂, IDL, LDL and LDL subfraction concentrations. This was conducted using the analysis of variance General Linear Model (GLM) in Minitab which permitted the inclusion of categorical variables (i.e. sex) and multiple regression which generated overall coefficient of determination for a given set of variables. It should be noted that GLM gives r^2 values that related to the independent contribution of variables while the overall r^2 determined by multiple regression is usually higher and takes account of potential interaction between correlated variables. For consistency the same panel of variables (anthropometric indices, markers of insulin resistance and lipases) were included as predictors in all GLM models of lipoprotein subfraction distributions. Plasma triglyceride was added to the models for IDL, LDL and LDL subfractions in order to explore previously identified relationship.

Anthropometric indices, plasma lipids and lipoproteins were compared between males and females, using transformed data where appropriate, by student's unpaired t-tests. A Bonferroni correction was employed to allow for multiple comparisons. Comparison of

slopes for the regression of VLDL₁, VLDL₂ or LDL-III versus plasma triglyceride and LDL triglyceride versus hepatic lipase in males and females was done using the pairwise-slopes routine in Minitab and tested for significance with the Mann-Whitney test. There were 18 peri and post-menopausal women of which four were on hormonal replacement therapy (HRT), and the hormonal status of the remaining 14 was unknown. The effect that the menopause as well as HRT has on lipids and lipases is well documented and hence in each instance, analyses were performed with and without these 18 subjects to ensure that the menopausal state or HRT was not unduly influencing the results. Their inclusion did not affect the overall findings. Ninety-seven of the volunteers came from 19 different families and were related. The results were initially analysed with all related family members excluded and than again with the 97 included. The data was not skewed by the inclusion of these family members and they were present in the final analyses.

3.2 Anthropometric indices and plasma lipids

Age, systolic blood pressure, BMI and hip circumference, plasma cholesterol, LDL cholesterol, VLDL cholesterol and HDL₃ were similar between the sexes (Table 1). Males were generally heavier but this was in keeping with their height since there was no sex difference in BMI. However, men had a significantly greater waist circumference and consequently a bigger WHR. Males had higher plasma triglyceride, plasma cholesterol/HDL ratio and a lower total HDL cholesterol and HDL₂. The mean postheparin HL activity was twice as high in males compared to females but there were no difference in LPL activity.

Table 1. Anthropometric indices, plasma lipids, lipoproteins and lipases in the study group

Variable	Males [§] (n)	Females [§] (n)
Age	35.6±11.2 (140)	37.0±11.0 (164)
Weight (kg)	76.7±9.9 (93)	63.5±10.7 (98)*
BMI	24.9±3.0 (138)	24.6±3.9 (162)
BP systolic (mmHg)	125±13 (93)	121±16 (98)
BP diastolic (mmHg)	81±9 (93)	77±10 (98)‡
Waist (cm)	86±9 (93)	75±10 (96)*
Hip (cm)	98±7 (93)	98±8 (96)
WHR	0.9±0.1 (93)	0.8±0.1 (96)*

Total cholesterol (mmol/l)	5.3±1.1 (139)	5.2±1.0 (164)
Triglyceride (mmol/l)	1.2±0.6 (139)	1.0±0.5 (164)‡
HDL-C (mmol/l)	1.2±0.3 (139)	1.5±0.3 (164)*
LDL-C (mmol/l)	3.5±1.0 (139)	3.2±0.9 (157)
VLDL-C (mmol/l)	0.6±0.3 (139)	0.5±0.3 (157)
Chol/HDL ratio	4.6±1.4 (139)	3.7±1.2 (164)*
HDL ₂ mass(mg/dL)	51±33(133)	91±48(153)*
HDL ₃ mass(mg/dL)	246±60(133)	254±58 (153)
Hepatic lipase (µmolFA/mL/h)	19.5±8.1 (70)	11.4±5.4 (67)*
Lipoprotein lipase (µmolFA/mL/h)	4.4±1.6 (70)	5.0±2.5 (67)

BMI, body mass index; BP, blood pressure; WHR, waist/hip ratio; HDL-C, high density lipoprotein-cholesterol; LDL-C, low density lipoprotein-cholesterol; VLDL-C, very low density lipoprotein-cholesterol; Chol/HDL ratio, Total plasma cholesterol/ high density lipoprotein-cholesterol ratio; HDL₂ mass, high density lipoprotein₂ subfraction mass; HDL₃ mass, high density lipoprotein₃ subfraction mass;

* p<0.001, † p<0.01, ‡ p<0.05 refer to significance of difference between males and females as determined by student's unpaired t-test using transformed data where appropriate, corrected for multiple comparisons. § Means and standard deviations shown were determined on non-transformed data. n refers to number of subjects included in measurement.

3.3 Factors predicting plasma lipids, lipoproteins and lipases

In this normolipaeamic group of men and women, age, BMI, WHR and plasma triglyceride showed significant positive relationships to plasma and LDL cholesterol in univariate analysis (Table 2). In the multivariate model, only age and triglyceride remained significant predictors of plasma cholesterol and LDL cholesterol and together accounted for 44% and 35% of its variability respectively. In univariate analysis, age, BMI, WHR, fasting insulin and LPL showed significant associations with plasma triglyceride. However only age, LPL and fasting insulin remained significant predictors in multivariate analysis, accounting for 20% of the variability in this lipid. BMI, WHR, fasting insulin, LPL, HL and plasma triglyceride all showed significant relationships with HDL cholesterol in univariate analysis. In the multivariate model only fasting insulin, LPL, HL

and triglyceride remained significant predictors and together accounted for 39% of its variability.

Postheparin hepatic lipase activity had significant associations with BMI, WHR, fasting insulin in univariate analysis (Table 3). Using the GLM, sex alone accounted for 28.5% of the variability in HL. When included in a model with age, measures of obesity and insulin resistance, sex was still the most significant predictor (GLM (A) Table 3). The multivariate model (GLM B) in which sex was excluded to reveal the impact of other potential factors, indicated that age and WHR were significant predictors and together accounted for 23% of the enzyme's variability. BMI and fasting insulin had significant association with LPL activity in univariate analysis but none were significant in the multivariate model.

Table 2. Determinants of plasma lipids and lipoprotein concentrations

Variable	n	Plasma Cholesterol		Plasma Triglyceride		LDL-C		HDL-C	
		Uni r ²	GLM r ²	Uni r ²	GLM r ²	Uni r ²	GLM r ²	Uni r ²	GLM r ²
Age	303	22(+)*	11.9*	5.2(+)*	1.9‡	20.1(+)*	9.1*	0.4	1.5
Sex	303	-	0	-	3.1	-	0	-	0.11
BMI	299	10.8(+)*	0	22.7(+)*	0.2	11.4(+)*	0.1	7.7(-)*	0.0
WHR	188	5.6(+)*	0.3	10.8(+)*	2.0	10.3(+)*	0.6	9.0(-)*	0.0
Fasting glucose	186	1.5(+)	0.4	2.4(+)*	0.2	2.2(+)*	0.4	1.7(-)	0
Fasting insulin	186	1.2	0.1	12.0(+)*	2.5‡	2.0	0.2	9.5(-)*	2.7‡
LPL	127	0	1.3	6.1(-)†	3.8†	0.1	0.2	17.8(+)*	5.2*
HL	127	0	0.1	0.5	0	0.8	0.1	19.3(-)*	3.5†
Triglyceride	303	29.9(+)*	10.0*	-	-	20.5(+)*	4.0†	13.9(-)*	3.7†
Overall r ²			44.1		20.0		35.0		39.0

BMI, body mass index; WHR, waist/hip ratio; LPL, lipoprotein lipase activity; HL, hepatic lipase activity; LDL-C, low density lipoprotein-cholesterol; HDL-C, high density lipoprotein-cholesterol; Uni, univariate analysis; GLM, general linear model

* P<0.001, † P<0.01, ‡ P<0.05 refer to the significance of the association r² (correlation coefficient squared) determined by linear regression in univariate analysis or by a multivariate analysis of variance General Linear Model including all of the tabled parameters. In the general linear model r² values provided estimates of the independent contribution of a variable, i.e. when all others have been entered into the model. The overall r², generated by including all variables in the table in a multiple regression analysis, usually exceeded the sum of the individual r² values due to the contribution from interaction between terms. The sign refer to the direction of the relationship. Sex was included as a categorical variable in the General Linear Model.

Table 3. Determinants of lipase activities

Variable	n	Hepatic lipase activity			Lipoprotein lipase activity	
		Uni r^2	GLM(A) r^2	GLM(B) r^2	Uni r^2	GLM r^2
Age	137	1.9(-)	2.3‡	4.8*	0.9	2.3
Sex	137	-	8.6*	-	-	0.2
BMI	137	3.6(+) [‡]	1.6	0	2.8(-) [‡]	0
WHR	137	16.8(+)*	0.5	14.5*	1.9	0.8
Fasting Insulin	133	5.2(+) [‡]	0.5	0.3	2.9(-) [‡]	1.0
Fasting glucose	130	1.1	0	0	0.7	1.2
Overall r^2			31.1	22.6		4.3

BMI, body mass index; WHR, waist/hip ratio; HDL-C, high density lipoprotein-cholesterol; Uni, univariate analysis; GLM, general linear model

* $P < 0.001$, ‡ $P < 0.01$, † $P < 0.05$ refer to the significance of the association r^2 (correlation coefficient squared) determined by univariate analysis or by a multivariate analysis of variance General Linear Model including all of the tabled parameters. The sign refer to the direction of the relationship. Sex was included as a categorical variable in the General Linear Model, but since it is an over-riding determinant of hepatic lipase activity, predictors of HL were sought when it was excluded in GLM (B). For explanation of r^2 values see legend to table 2.

3.4 Chemical compositions of apolipoprotein B containing lipoproteins

The percentage contribution of protein (Table 4) to lipoprotein mass increased steadily from VLDL₁ to VLDL₂ to IDL and LDL in both males and females, with LDL containing almost twice as much protein as VLDL₁. Triglyceride content, on the other hand, decreased from VLDL₁ to LDL. The percentage of cholesteryl ester increased steadily across the lipoproteins and was maximal in IDL. The free cholesterol showed similar patterns with increases in the IDL and LDL. Phospholipid content was relatively consistent across the lipoproteins. These data are in good agreement with previous findings.¹²⁷ Significant male-female differences in compositions were found in the VLDL₁ phospholipid, VLDL₂ phospholipid and LDL triglyceride but only VLDL₂ phospholipid remained significantly different after Bonferroni correction.

Table 4. Chemical compositions of apolipoprotein B containing lipoproteins**Males**

Lipoproteins(n)	Proteins	Triglyceride	Chol esters	Free chol	Phospholipid
VLDL ₁ § (91)	13.0±0.7	57.7±1.5	11.1±0.6	1.3±0.2	17.7±1.9
VLDL ₂ § (91)	15.9±0.5	36.3±1.0	23.8±0.9	3.2±0.3	21.6±1.4
IDL§ (53)	19.6±0.3	12.4±0.6	44.6±0.7	5.9±0.3	17.4±0.7
LDL§ (69)	27.0±0.6	5.1±0.1	34.8±0.7	12.6±0.4	30.6±1.8

Females

Lipoproteins(n)	Proteins	Triglyceride	Chol esters	Free chol	Phospholipid
VLDL ₁ § (98)	14.5±0.6	61.0±1.0	12.5±0.7	1.2±0.2	11.6±1.0 [†]
VLDL ₂ § (98)	17.3±0.5	39.6±0.9	25.0±0.9	2.5±0.2	16.2±0.8 [*]
IDL§ (44)	20.0±0.3	13.0±0.7	42.2±0.7	6.1±0.2	18.8±0.3
LDL§ (83)	28.1±1.3	5.7±0.2 [†]	35.7±0.7	12.7±0.4	33.1±1.4

VLDL₁, very low density lipoprotein₁; VLDL₂, very low density lipoprotein₂; IDL, intermediate density lipoprotein; LDL, low density lipoprotein; Chol esters, cholesteryl esters; Free chol, free cholesterol
 § Values are means ± standard error of means expressed as percent composition. n refers to number of subjects included in measurements. * P<0.001, † P<0.01 refer to the significance of difference between males and females as determined by student's unpaired t-test, before correction for multiple comparisons. Only VLDL₂ Phospholipid remained significantly different (p<0.05) after Bonferroni correction

3.5 Regulation of VLDL subfraction composition and distribution

As plasma triglyceride rises across the normal range, both VLDL₁ and VLDL₂ concentrations increased but the increment in VLDL₁ is much greater than that of VLDL₂ (Fig 3). The ratio of VLDL₁ to VLDL₂ rises from about 1.0 at 0.5 mmol/L to about 2.0 at 2.0 mmol/L plasma triglyceride respectively. The steeper increment in VLDL₁ compared to VLDL₂ is also reflected by a comparison of the regression slopes (VLDL₁= 80.4 TG - 37.9, r²= 64.9% vs VLDL₂= 26.3 TG + 13.5, r²= 32.3%, p<0.001). The relationships were similar in both males and females with the ratio of VLDL₁ to VLDL₂ total lipoprotein rising from about 1.0 at 0.5 mmol/L to about 2.0 at 2.0 mmol/L plasma triglyceride.

In univariate analysis, anthropometric indices like BMI, WHR as well as fasting glucose and insulin levels, LPL and HL were significantly associated with VLDL₁ concentrations (Table 5). Similarly, the same variables with the exception of HL also had significant relationships with VLDL₂, but the addition of age as another factor. In multivariate analysis, only the HL was a significant predictor of VLDL₁ while age and LPL were the significant predictors of VLDL₂ concentration.

A similar preferential rise occurred in VLDL₁/VLDL₂ triglyceride across the plasma triglyceride range (Fig 4). The VLDL₁/VLDL₂ triglyceride ratio rose from 1.7 at 0.5 mmol/L to 3.5 at 2.0 mmol/L plasma triglyceride (Fig 5). Examination of VLDL₁ and VLDL₂ compositions and calculations of the core to coat ratio remained relatively constant across the plasma triglyceride range. Linear regression of core:coat ratios versus

plasma triglyceride revealed regression equations of :- VLDL₁ core:coat ratio =2.74 - 0.4 TG, r= 0.14, NS and VLDL₂ core:coat ratio =1.37 - 0.1TG, r= 0.16, p<0.05). This implied that the increase in VLDL triglyceride as well as VLDL mass was the result of increasing particle number and not particle size. Likewise the cholesterol to protein ratio was unchanged throughout the plasma triglyceride range. Increasing levels of plasma cholesterol were not associated with changes in concentration of VLDL₁ and VLDL₂.

Table 5. Determinants of very low density lipoprotein subfraction concentrations

Variable	n	VLDL ₁		VLDL ₂	
		Uni	GLM	Uni	GLM
		r ²	r ²	r ²	r ²
Age	189	1.6(+)	2.5	2.5(+)	3.3‡
Sex	189	-	0.5	-	0
BMI	189	13.8(+)*	0	5.8(+)*	0.4
WHR	189	9.6(+)*	0.6	10.3(+)*	0.2
Fasting insulin	173	11.5(+)*	2.0	4.4(+)†	0.2
Fasting glucose	169	4.4(+)†	0	2.7(+‡)	0.4
LPL	129	5.4(-)†	2.3	8.0(-)*	7.2
HL	129	10.5(+)*	5.4†	2.2	0.6
Overall r ²			15.5		12.3

BMI, body mass index; WHR, waist/hip ratio; LPL, lipoprotein lipase activity; HL, hepatic lipase activity; VLDL₁, very low density lipoprotein₁; VLDL₂, very low density lipoprotein₂ subfraction; Uni, univariate analysis; GLM, general linear model

*P<0.001, †P,0.01, ‡ p<0.05 refer to the significance of the association r² (correlation coefficient squared) determined by univariate analysis or by a multivariate analysis of variance General Linear Model including all of the tabled parameters. The sign refer to the direction of the relationship. Sex was included as a categorical variable in the General Linear Model. For explanation of r² values see legend to table 2.

3.6 Regulation of IDL composition and concentration

There was a strong positive relationship between plasma cholesterol and IDL concentration (r=0.71, p<0.001)(Fig 6), and the plasma concentration of IDL rose as the plasma cholesterol increased across the normal range. The association between concentration of the lipoprotein and plasma triglyceride levels was weaker (r=0.41, p<0.001, data not shown). In univariate analysis, age, BMI, WHR, fasting glucose, plasma triglyceride were important predictors of IDL concentration (Table 6). Plasma triglyceride and HL were the only significant predictors in the multivariate model and together accounted for 36.2% of its variability.

Increasing plasma cholesterol was due to the increments in both IDL and LDL masses (Fig 7). The IDL core to coat ratio changed little across the range of plasma cholesterol seen in the present study (r=-0.2, p=0.05) implying that the particle decreased slightly in size as the plasma cholesterol rose while the IDL cholesterol to protein ratio showed a steady increase with increasing cholesterol levels (r=0.55, p<0.001). Further analysis showed that as plasma cholesterol increased IDL became enriched in cholesterol and

cholesteryl esters and depleted in triglyceride as illustrated by the change in the cholesteryl ester to triglyceride ratio ($r=0.46$, $p<0.001$) (Fig 8). In contrast, the cholesteryl ester to triglyceride ratio for VLDL₁, VLDL₂ and LDL remained constant across the cholesterol range. Again, these relationships were the same in men and women.

Table 6. Determinants of intermediate density lipoprotein and low density lipoprotein plasma concentration

Variable	n	IDL		n	LDL	
		Uni r^2	GLM r^2		Uni r^2	GLM r^2
Age	97	13.8(+)*	4.0	177	15.0(+)*	6.2†
Sex	97	-	2.3	177	-	1.7
BMI	97	16.1(+)*	0	177	12.7(+)*	0.2
WHR	97	6.4(+) [†]	3.1	176	6.1(+)*	0.4
Fasting insulin	93	1.1	0	174	2.4(+) [‡]	0.2
Fasting glucose	91	7.2(+) [†]	0	170	0.3	1.5
Plasma triglyceride	97	16.8(+)*	7.3 [‡]	177	21.0(+)*	6.2 [†]
LPL	47	3.2	2.6	130	1.0	0
HL	47	5.0(-)	9.9 [‡]	130	0.0	0
Overall r^2			36.2			20.0

BMI, body mass index; WHR, waist/hip ratio; LPL, lipoprotein lipase activity; HL, hepatic lipase activity; IDL, intermediate density lipoprotein; LDL, low density lipoprotein; Uni, univariate analysis; GLM, general linear model

* $P<0.001$, † $P<0.01$, ‡ $P<0.05$ refer to the significance of the association r^2 (correlation coefficient squared) determined by univariate analysis or by a multivariate analysis of variance General Linear Model including all of the tabled parameters. The sign refer to the direction of the relationship. n refers to the number of subjects included in the measurements. Sex was included as a categorical variable in the General Linear Model. For explanation of r^2 values see legend to table 2.

3.7 Regulation of LDL concentration, composition and subfraction distribution

The total LDL concentration showed a significant positive relationship with plasma triglyceride levels (Fig 9) below 1.3 mmol/L ($r=0.30$, $p<0.01$) but no significant relationship above that value ($r=0.17$, NS) in both men and women. There were strong positive relationships between plasma cholesterol and total LDL concentrations ($r=0.73$, $p<0.001$). Age, BMI, WHR and fasting insulin were also significant correlates of total LDL concentration in univariate analyses (Table 6) but only plasma triglyceride and age remained a significant predictor in multivariate analyses, accounting for 20% of its variability. There was little variation in LDL composition as plasma lipid levels changed. The cholesterol:protein ratio (0.81 ± 0.01) and the core to coat ratio (0.46 ± 0.01) remained constant across the plasma cholesterol range in contrast to the changes seen in IDL (see above). It was, however noted that the percent LDL triglyceride content was strongly inversely correlated with HL activity in females ($r=-0.48$, $p<0.001$) but the association in males was less strong $r=-0.29$, $p<0.05$). A comparison of the regression slopes was further support of the male-female differences (LDLTG= 6.0 - 0.04HL vs LDLTG= 8.0 - 0.1HL, respectively, $p<0.001$) (Fig 10).

Age, BMI, WHR, plasma triglyceride, fasting glucose, fasting insulin, LPL and HL all exhibited associations with LDL-I plasma concentration in univariate analysis (Table 7). In the multivariate model, age, sex, LPL, plasma triglyceride and fasting glucose were significant predictors with the last being the most important and when all the parameters in Table 7 were included in a multiple regression analysis, they accounted for 26% of variability in LDL-I. LDL-II exhibited significant correlations with age, BMI, plasma triglyceride but none of these remained significant predictors in the multivariate model. BMI, WHR, plasma triglyceride and HL exhibited the strongest relationships with LDL-III in univariate analysis. Other factors of significance included age, fasting glucose, fasting insulin, and LPL. In the multivariate model GLM (A), sex and plasma triglyceride were the only significant independent. When these together with the other parameters in Table 7, were included in multiple regression analysis, 42% of LDL-III variability was accounted for. When sex was excluded from the model GLM (B), the HL and plasma triglyceride were significant predictors of LDL-III, and this reduced group of parameters still explained 39% of the variability in this subfraction. In an alternative approach to estimating the impact of plasma triglyceride and HL on LDL-III levels, the sexes were divided and separate multiple regression performed with just these two variables. In men, plasma triglyceride alone explained 40% of LDL-III variability ($p < 0.001$) and HL was not a significant predictor. In contrast, in women both plasma triglyceride and HL were significant predictors ($p = 0.002$ and $p < 0.0001$ respectively) and together explained 37% of LDL-III variation. The sex difference can be visualised by examining the relationship between HL and LDL-III concentrations adjusted for plasma triglyceride level. HL activity was an important correlate in females but not in males ($r = 0.38$, $p < 0.001$ and $r = 0.05$, NS respectively)(Fig 13)

Plasma triglyceride was, therefore, a strong predictor of all three LDL subfractions in both univariate analysis and multivariate analysis. When the changes in individual LDL subfraction distribution across the plasma triglyceride range were examined, it was noted that LDL-I exhibited a fall off from mean concentration values of 100 mg/dL at plasma triglyceride of 0.5 mmol/L to 40 mg/dL at a plasma triglyceride of 2.3 mmol/L. LDL-II showed a positive relationship ($r = 0.47$, $p < 0.001$) at triglyceride levels below 1.3 mmol/L and a negative relationship ($r = -0.23$, $p < 0.001$) above 1.3 mmol/L in confirmation with previous findings¹³⁶. However a gender difference was noted in these associations. Specifically, the LDL-II concentration in females showed no significant relationship ($r = -0.1$, NS) with plasma triglyceride above 1.3 mmol/L whilst a significant negative relationship was demonstrated in males ($r = -0.45$, $p < 0.001$) (Fig 11). The concentration of LDL-III was generally low at a mean of 30 mg/dL in the range of plasma triglyceride levels from 0.5 to 1.3 mmol/L. Above the latter value, there was a dramatic rise in LDL-III concentration in males but a smaller increase in females (Fig 12). Linear regression analysis in subjects with plasma triglyceride above 1.3 mmol/L generated equations for LDL-III concentration versus plasma triglyceride of $LDL-III = 119TG - 66.6$, $r = 0.63$ in males and $LDL-III = 47.4TG - 11.0$, $r = 0.49$; the slopes of these lines differed significantly ($p < 0.001$).

Table 7. Determinants of low density lipoprotein subfraction concentration

Variable	n	LDL I		LDL II		LDL III		
		Uni r	GLM r	Uni r	GLM r ²	Uni r ²	GLM(A) r ²	GLM(B) r ²
Age	289	3.6(+)*	3.2‡	5.2(+)*	0.3‡	2.3(+) [†]	0	0.1
Sex	289	-	2.6‡	-	1.4	-	3.0‡	-
BMI	285	1.5(-)‡	0	5.0(+)*	0.5	8.6(+)*	0	0.8
WHR	175	3.5(-) [†]	0.4	0	0	15.7(+)*	0.5	0.2
Plasma Triglyceride	289	4.8(-)‡	2.6*	4.4(+)*	0	34.5(+)*	19.7*	16.9*
Fasting Glucose	169	8.2(-)*	10.4*	0.0	0	2.9(+) [‡]	0	0.5
Fasting Insulin	173	2.6(-)‡	0	0.7	0	4.2(+) [†]	0	0
HL	129	7.6(-) [†]	0.3	0.5	0.1	7.9(+) [†]	1.6	4.0 [†]
LPL	129	5.4(+) [†]	3.6‡	0.5	1.5	3.3(+) [‡]	0	0
Overall r ²			26.1		0		41.5	38.8

BMI, body mass index; WHR, waist/hip ratio; HL, hepatic lipase activity; LPL, lipoprotein lipase activity; LDL-I, low density lipoprotein-I, LDL-II, low density lipoprotein-II, LDL-III, low density lipoprotein-III; Uni, univariate analysis; GLM, general linear model

* P<0.001, † P<0.01, ‡ P<0.05 refer to the significance of the association r² (correlation coefficient squared) determined by univariate analysis or by a multivariate analysis of variance General Linear Model including all of the tabled parameters. The sign refer to the direction of the relationship. Sex was included as a categorical variable in the General Linear Model. For explanation of r² values see legend to table 2.

3.8 The impact of plasma triglycerides on lipids and lipoprotein subfractions

This group of normal subjects were then divided into 2 groups based on a plasma triglyceride level of 1.5 mmol/L. Griffin et al¹³⁶ had previously shown that above the threshold of 1.5 mmol/L, the LDL subfractions were predominantly dense, LDL-III. In order to assess the effects of triglycerides on the lipids and lipoprotein subfractions, the two groups were compared as shown in table 8. It was apparent that the group with triglyceride above 1.5 mmol/L had the more atherogenic profile. The levels of total cholesterol, VLDL₁, VLDL₂ and LDL-III were significantly higher whilst the HDL cholesterol, HDL₂ cholesterol and LDL-I were significantly lower. What is particularly interesting is the lack of difference in HDL₃ mass whilst the HDL₂ mass is significantly lower and it is obvious that apart from the effects of triglyceride on HDL cholesterol, there is significant effects on HDL subfraction distribution. The mean LDL-III concentration in the hypertriglyceridaemic group was above 100 mg/dL, which was previously demonstrated by Griffin et al, to be the level whereby risk for coronary artery disease are increased.

Table 8: Comparing those with high and low triglyceride levels

	TG <1.5mmol/L (mean±sem)	TG >1.5 mmol/l (mean±sem)
Cholesterol (mmol/l)	4.96±0.1	6.04±0.1*
HDL cholesterol (mmol/l)	1.42±0.01	1.13±0.03*
VLDL ₁ (mg/dl)	37.9±2.1	114.8±9.0*
VLDL ₂ (mg/dl)	30.6±1.5	67.4±3.8*
HDL ₂ mass (mg/dl)	82.0±3.2	43.8±3.7*
HDL ₃ mass (mg/dl)	247.0±4.2	261.9±6.3
LDL-I (mg/dl)	76.1±2.7	55.2±4.0*
LDL-II (mg/dl)	168.6±4.3	189.2±10
LDL-III (mg/dl)	42.9±2.3	129.7±11*

*p<0.001 refer to significance of difference between males and females as determined by student's unpaired t-test using transformed data where appropriate, corrected for multiple comparisons.

VLDL₁, very low density lipoprotein₁; VLDL₂, very low density lipoprotein₂; IDL, intermediate density lipoprotein; LDL, low density lipoprotein; HDL, high density lipoprotein

3.9 Differences between males and females

The males produced more VLDL₁ and VLDL₂ when compared to the females and there was a suggestion that the increase in VLDL₁ was more than that of VLDL₂, as demonstrated by the VLDL₁ to VLDL₂ ratio which was 1.53±0.1 and 1.42±0.1 for males and females respectively, although this failed to achieve statistical significance. In the analysis of LDL subfractions, females had significantly higher LDL-I levels and lower LDL-III levels. The females had mean LDL-III levels which were half the levels in the male subjects. There were no significant differences in IDL concentration between males and females. Data in table 1 had also demonstrated that males had significantly lower levels of HDL₂ mass but there was no difference in HDL₃ mass. Interestingly, the mean glucose level was significantly higher in males and the insulin level was also higher but this failed to achieve statistical significance.

Table 9: Differences between males and females

	Males (mean ± sem)	Females (mean ± sem)
VLDL ₁ (mg/dL)	61.5±4.6	49.7±5.2 [†]
VLDL ₂ (mg/dL)	43.2±2.7	35.1±2.4 [†]
LDL-I (mg/dL)	58.6±3.1	81.4±3.2*
LDL-II (mg/dL)	172.1±5.8	174.6±5.7
LDL-III (mg/dL)	87.8±7.1	43.9±2.9*
IDL (mg/dL)	44.9±2.4	42.7±2.4
Glucose (mmol/L)	4.9±0.1	4.6±0.1*
Insulin (mU/L)	8.6±0.5	7.7±0.4

*p<0.001, [†]p<0.01 refer to significance of difference between males and females as determined by student's unpaired t-test using transformed data where appropriate, corrected for multiple comparisons.

VLDL₁, very low density lipoprotein₁; VLDL₂, very low density lipoprotein₂; IDL, intermediate density lipoprotein; LDL, low density lipoprotein

3.10 Discussion

Age was not an entry criterion for this study but due to the nature of the recruitment process the majority of participants were young to middle aged adults. Only a few men and post-menopausal women were over the age of 55 years. The two sexes were well matched for age and exhibited the expected differences in body habitus. It was notable that while the BMI was virtually identical in both groups, the WHR was higher in males as a result of increased central obesity. Plasma lipids were comparable with the lower LDL cholesterol in women being balanced by the higher HDL (and HDL₂) level. Hepatic lipase activity showed a marked sex difference whereas lipoprotein lipase activity did not. The subjects were therefore representative of the general population and an appropriate group in which to seek further the basis of the male-female difference in CHD risk. A number of questions were asked of the data. First, as total plasma cholesterol and triglyceride varied across the range of values seen in the normal population, how did the contents of the lipids in VLDL₁, VLDL₂, IDL and LDL alter? Second, what inter-relationships existed between plasma lipids, VLDL and LDL subfractions? Third, to what extent were changes in lipoprotein levels related to the presence of IRS as revealed by the presence of central obesity and/or raised fasting insulin concentrations? Fourth, did the above associations depend on gender?

Increasing plasma cholesterol from the bottom to top quintiles of the distribution (3.5 mmol/L to 7.0 mmol/L) was associated with a two-fold rise in LDL plasma concentration and a similar increment in IDL. No relationship was seen with VLDL₁ and VLDL₂ concentrations. The rise in LDL from low-normal to high-normal was not accompanied by any perturbation in the composition of the particle, either its core to coat ratio or its cholesterol:protein ratio. That is, the increase was attributable to greater number of particles in the circulation. In contrast to the situation for LDL, we found that IDL exhibited distinct compositional variations in those with differing plasma cholesterol levels. As plasma cholesterol rose, so did the cholesteryl ester:triglyceride ratio, indicating a change in the make-up of the particle core. Further, there was also a slight but

significant downward trend in particle size as judged by the core:coat ratio. Either IDL particles as a whole are being increasingly modified by lipid exchange as their concentration increases in the circulation, possibly because of their longer residence time or, as reported by Musliner et al,¹²⁷ IDL also is heterogeneous and composed of two subfractions, IDL₁ which is triglyceride rich and IDL₂ which is cholesterol rich. It can be postulated that at higher plasma cholesterol levels, there is a selective accumulation of the cholesterol enriched IDL particles.

As plasma triglyceride rose across the range seen in this cross-sectional survey, the plasma concentration of VLDL₁ increased more rapidly than VLDL₂. Closer examination of the scattergrams, suggested that while VLDL₁ and VLDL₂ both increased at the same rate over the plasma triglyceride range 0.5-1.0 mmol/L, above this value VLDL₁ continued to rise but VLDL₂ showed a smaller increment. Kinetic studies¹⁶⁶ of VLDL triglyceride turnover in normal subject revealed that it is the synthesis of the lipid rather than its clearance rate that controls the plasma levels. Thus, we interpret our observations to indicate that in subjects with high-normal plasma triglyceride levels the liver releases larger VLDL₁ in preference to smaller VLDL₂. This allows¹³⁶ the transport of more lipid per lipoprotein particle. As reported in a previous study,¹³⁶ the total LDL concentration exhibited a positive correlation with plasma triglyceride. Again, the relationship appeared biphasic in that a positive correlation was seen at lower plasma triglyceride levels but above 1.3 mmol/L, the association was lost, suggesting that a plateau value had been reached. These associations between VLDL₁, VLDL₂, LDL and plasma triglyceride were present in both males and females and we could not detect any significant gender based differences in the correlation coefficients. What did differ between the sexes, however was the LDL subfraction distribution, as a function of plasma triglyceride concentration.

LDL-I concentration was influenced by a number of factors including age, features of insulin resistance (increased fasting insulin and glucose), increased obesity, plasma triglyceride and the activities of the two lipases. In multivariate analysis, fasting glucose was the most important predictor followed by age, plasma triglyceride, LPL activity and WHR. The metabolic mechanisms underlying these associations are not yet clear. It is possible that the negative association with plasma triglyceride is due to CETP mediated transfer of triglyceride from VLDL to LDL-I which improves its qualities as a substrate for HL. The enzyme then lipolyses the particle to a denser species (LDL-II or LDL-III). LPL activity, on the other hand, demonstrated a positive association with LDL-I possibly because when it is high, the circulating levels of large triglyceride-rich lipoproteins (including chylomicrons which were not represented in the fasting triglyceride measurement) are kept low, thus limiting triglyceride exchange into LDL-I. Alternatively, high LPL activity may favour the conversion of smaller VLDL particles to LDL-I. The impact of factors associated with insulin resistance i.e. higher plasma glucose levels, and WHR, which was present in both sexes is, as yet, inexplicable.

LDL-II in both males and females exhibited as reported previously¹³⁶ a positive association with plasma triglyceride when the latter was less than 1.3 mmol/L (the zenith of the quadratic regression line which was used to fit the LDL-II to plasma triglyceride in men). At higher plasma triglyceride levels (from 1.3 to 3.0 mmol/L) in men, there was fall off in LDL-II as before¹³⁶ with a significant negative correlation and LDL-III which

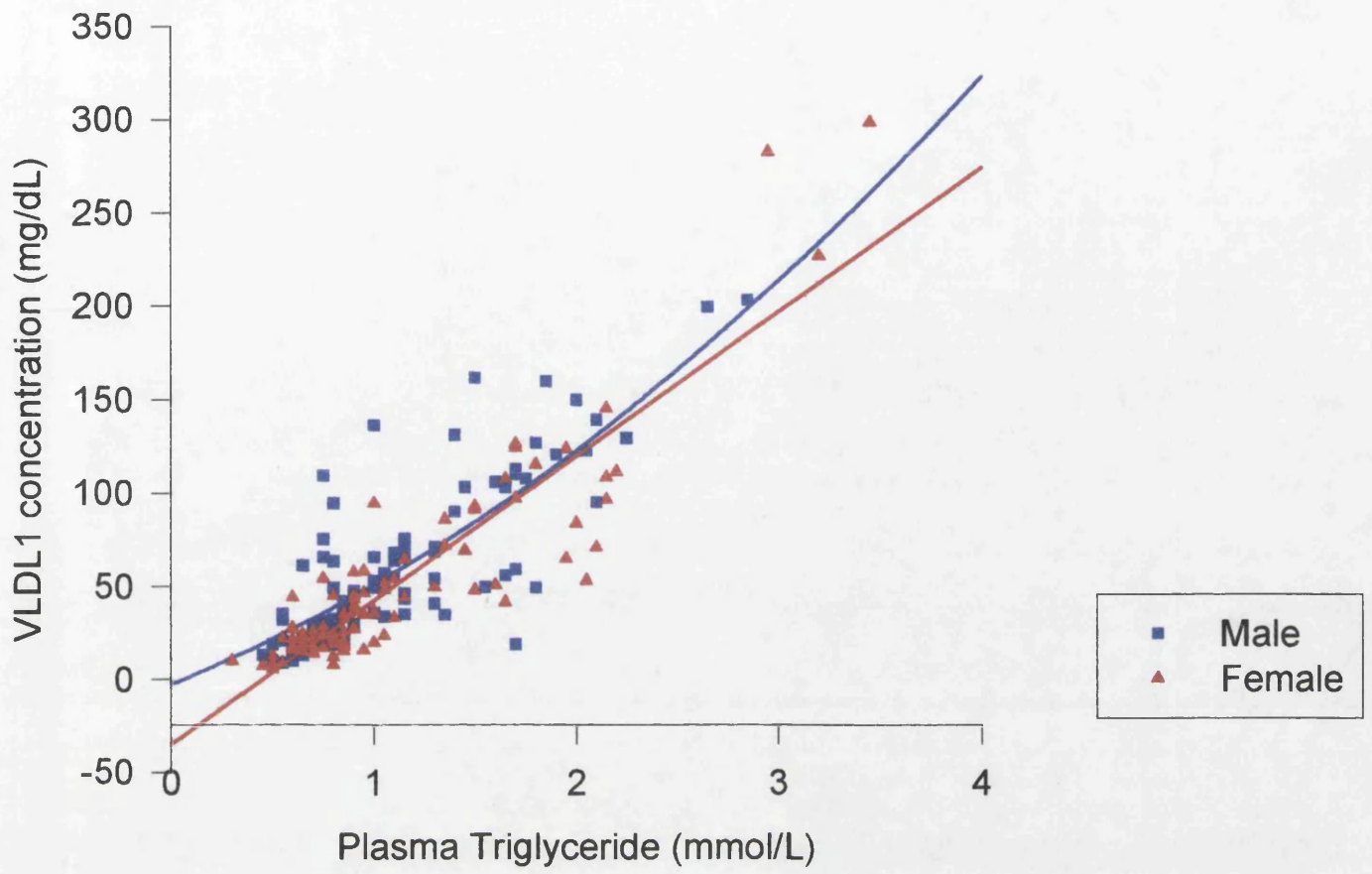
had been low when plasma triglyceride was less than 1.3 mmol/L, rose steeply. The data, therefore, suggest that the formation of LDL-III, possibly by remodelling of LDL-II, is increasingly favoured in men as plasma triglyceride rose above 1.3 mmol/L "threshold". In women, the picture was different. At levels of triglyceride above 1.3 mmol/L, the LDL-II did not fall and little LDL-III was formed. Only 17% of females with plasma triglyceride above the threshold had an LDL-III >100 mg lipoprotein/dl plasma (the level which significant CHD risk occurs) compared to 42% in men.

The sex difference and the findings in Table 7 and 9 strongly suggests that hepatic lipase may play an important role in determining the relative concentrations of LDL-II and LDL-III in plasma. Activity of the enzyme is twice as high in men as women and when LDL-III in the whole group was adjusted for variation in plasma triglyceride, HL activity emerged as the strongest predictor of LDL-III concentration. Generally, a combination of a plasma triglyceride greater than 1.3 mmol/L and a HL of >15 U/L was required to generate LDL-III above the risk level of 100 mg lipoprotein/dL plasma. Further support for the pivotal role of HL activity in LDL subfraction distribution can be gained from examination of the composition of total LDL. The percent LDL triglyceride showed a significant inverse correlation with HL activity which was stronger in females than in males indicating that in the former sex the activity of this enzyme was important in determining the triglyceride content of LDL; women with low HL had relatively triglyceride-enriched LDL. HL is also known to strongly influence HDL cholesterol and HDL₂¹⁶⁷ level, with high activity of the enzyme being associated with a low HDL₂ concentration. These putative HL-driven changes in LDL-III and HDL subfraction distributions go a long way to explaining the risk differences between the sexes with women having less "atherogenic" LDL-III and more "cardioprotective" HDL₂. It is likely that the same mechanism that causes the generation of smaller, denser particles within the HDL operates in the LDL density range. That is, the lipoprotein is first made susceptible to the action of HL by CETP mediated triglyceride transfer and we postulate that it is the VLDL₁ concentration that determines the rate of transfer of triglyceride into LDL since large triglyceride-rich VLDL has been shown to be a preferred substrate for CETP action.¹²³ HL then acts on LDL-II to hydrolyse the triglyceride enriched core and generate LDL-III. On the other hand, if the enzyme activity is low, then LDL-II remains the major species in plasma and is relatively triglyceride enriched. HL is known to be regulated by sex steroid hormones and so this final step is possibly a function of androgen/oestrogen status between and within the sexes. The present findings amplify and set in context the earlier reports of Zambon et al,¹⁶⁸ Jansen et al¹⁶⁹ and Watson et al¹⁷⁰ on the role of HL in determining LDL subfraction distribution. The importance of the enzyme becomes clear only when the subject group exhibit a wide range for its activity e.g. when both sexes are examined.¹⁷¹ The previous data of Zambon et al¹⁶⁸ and Jansen et al¹⁶⁹ were collected in men. The latter study in 326 male subjects reported no overall independent effect of HL on LDL subfraction pattern and our findings agree with this. The former report was on a small group (n=44) with a wide distribution of HL activity that included enough subjects with low HL to see the effect.

IRS has been linked to the appearance of raised plasma triglyceride,¹⁷² lower HDL cholesterol and the presence of small LDL in gel electrophoresis patterns. Here we find features of IRS (WHR, raised fasting insulin or glucose) are correlated with LDL

subfraction concentrations, particularly LDL-I (negatively) and LDL-III (positively). Based on the data in Table 7, we suggest that IRS has its effect primarily by raising plasma triglyceride and VLDL₁ levels since it has¹⁶⁵ been shown previously that VLDL₁ but not VLDL₂ synthesis is influenced by insulin. It is thought¹⁶⁵ that the hormone acts to inhibit hepatic triglyceride release in the form of large VLDL. Furthermore, it would appear that the males are more insulin resistant because of the higher glucose and insulin levels (Table 9). Resistance to insulin mediated glucose disposal would result in higher fasting glucose levels and a compensatory hyperinsulinaemia. We also know that LPL activity can be influenced by insulin sensitivity and this may contribute to the VLDL subfraction distribution being predominantly the larger VLDL₁ particles in males. Conversely, the higher LPL activity in females who are less insulin resistant, would favour the conversion of smaller VLDL particles to LDL-I. The impact of insulin resistance on coronary risk may thus be mediated through several pathways. Firstly, resistance of LPL activities to insulin may result in higher triglyceride levels, especially in the postprandial phase. Second, the insulin itself may inhibit the release of large VLDL particles and insulin resistance (akin to a lack of insulin) may thus result in a failure of this inhibition and favour a shift towards release of larger VLDL particles. Third, the resultant hypertriglyceridaemia can modulate the LDL subfraction distribution, favouring a predominance of dense LDL particles. In conclusion, the generation of small, dense LDL may in many subjects, be viewed as the result of two hormonal effects. First, failure of insulin action leading to higher VLDL₁ levels and increased neutral lipid exchange with LDL-II. Second, high androgen activity leading to enhanced HL activity and the conversion of LDL-II to LDL-III. It is noteworthy also that WHR was correlated with HL activity and that IRS itself and the presence of central obesity can lead to changes in androgen/oestrogen balance.¹⁷³

Fig 3: VLDL1 concentrations vs Triglyceride



VLDL2 concentration vs Triglyceride

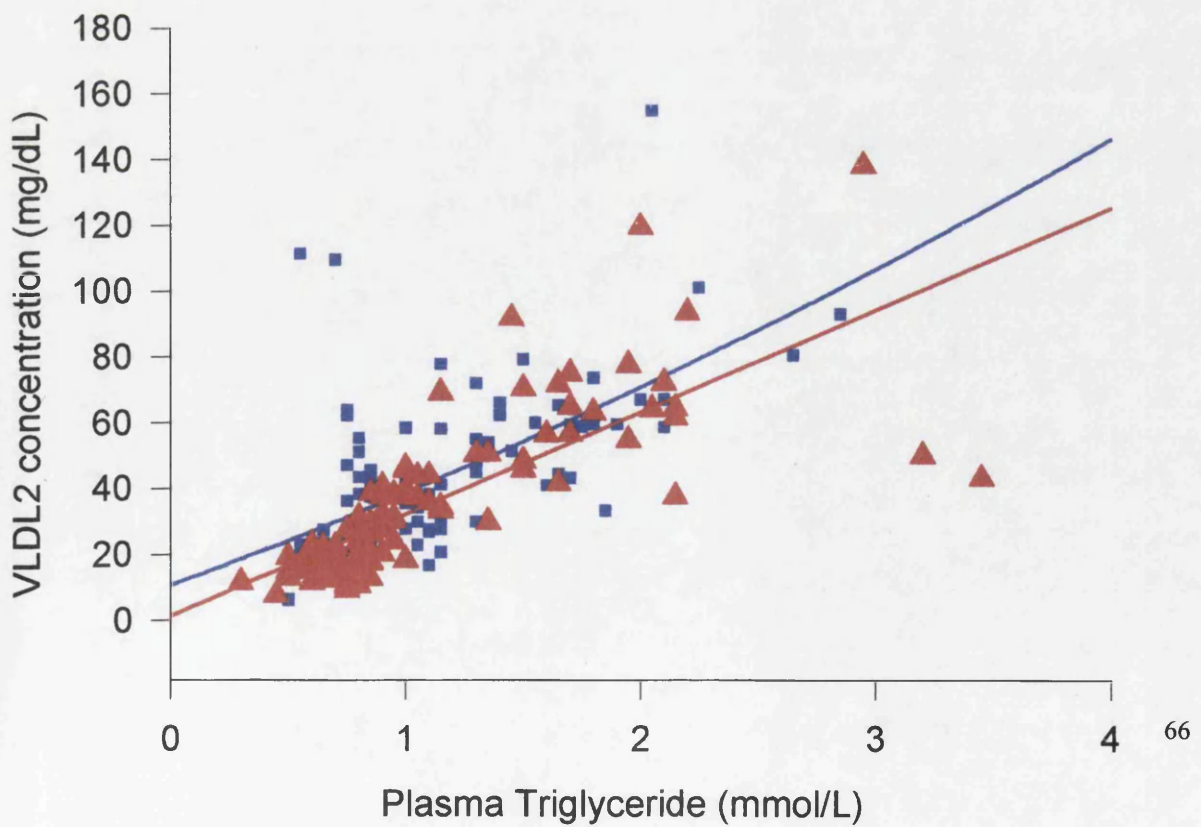
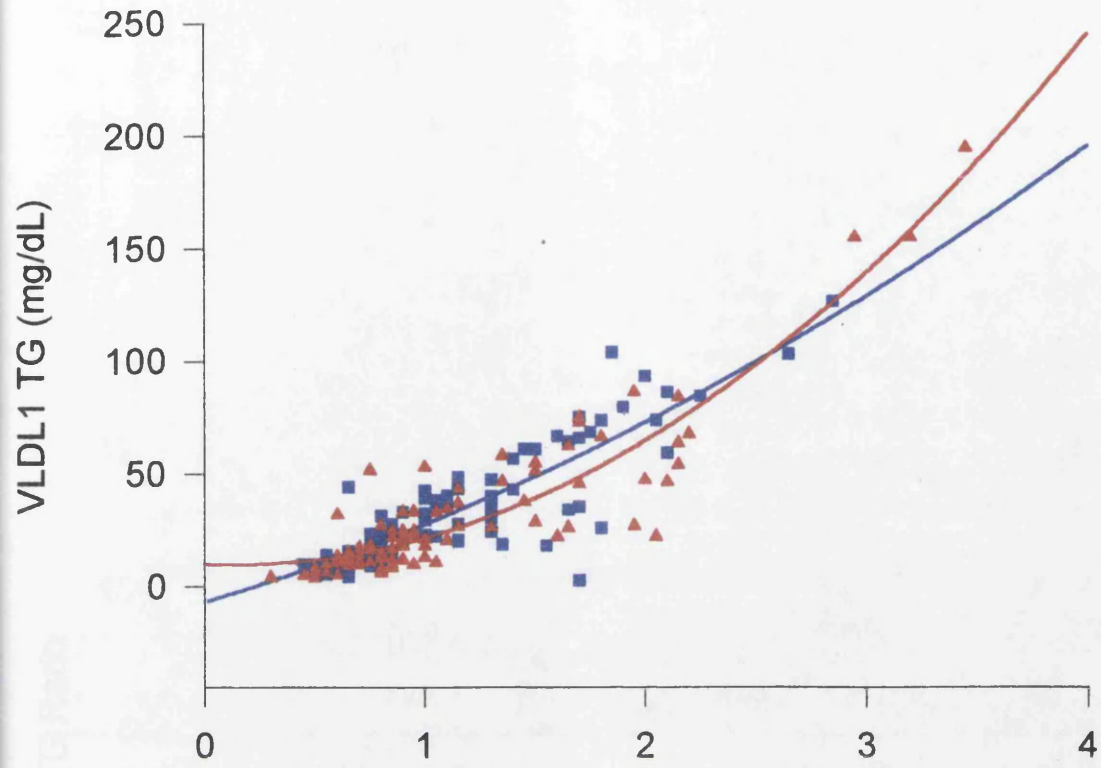


Fig 4: VLDL1 TG vs Triglyceride



VLDL2 TG vs Triglyceride

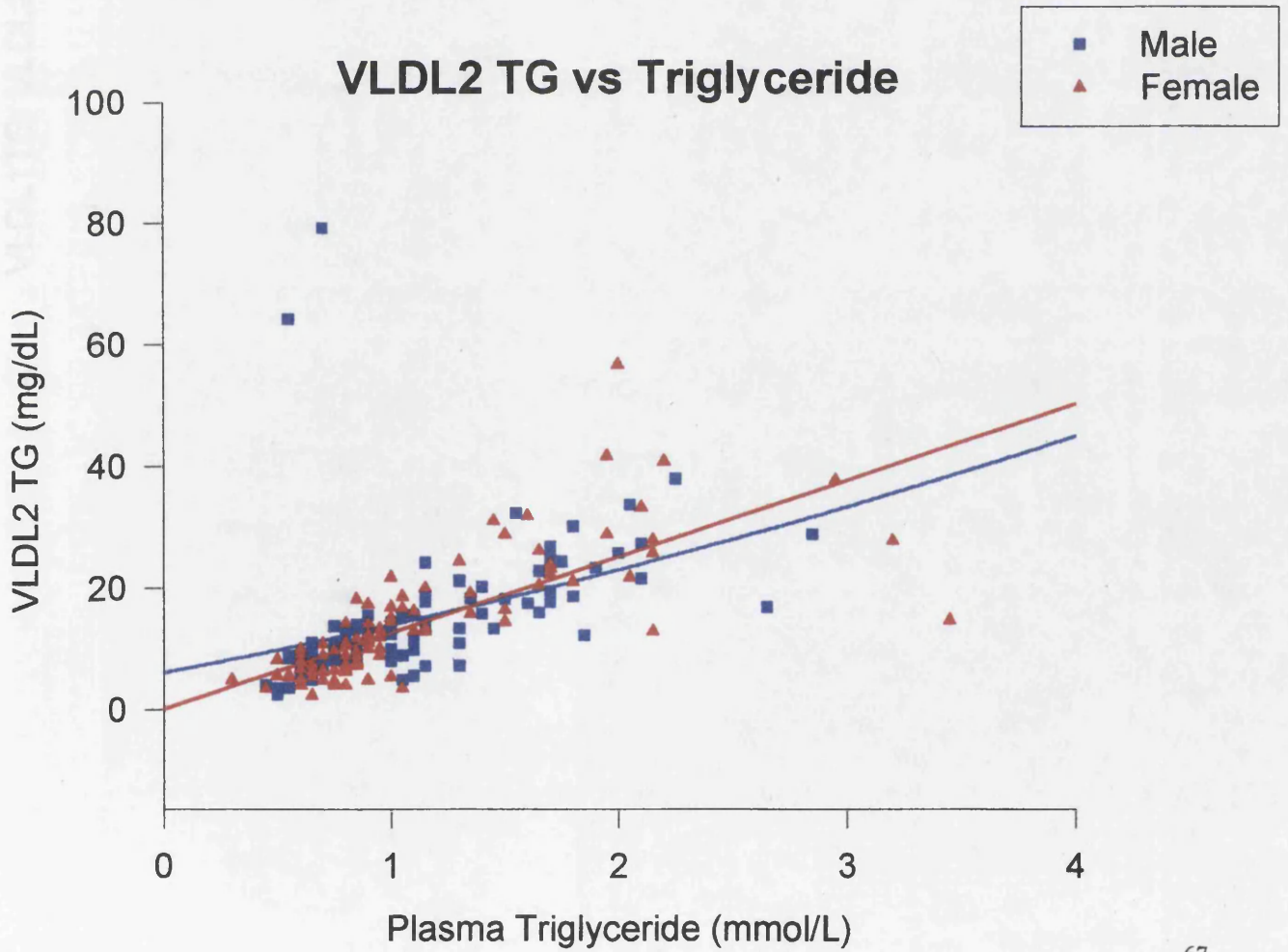


Fig 5: VLDL1 TG /VLDL2 TG Ratio vs Triglyceride

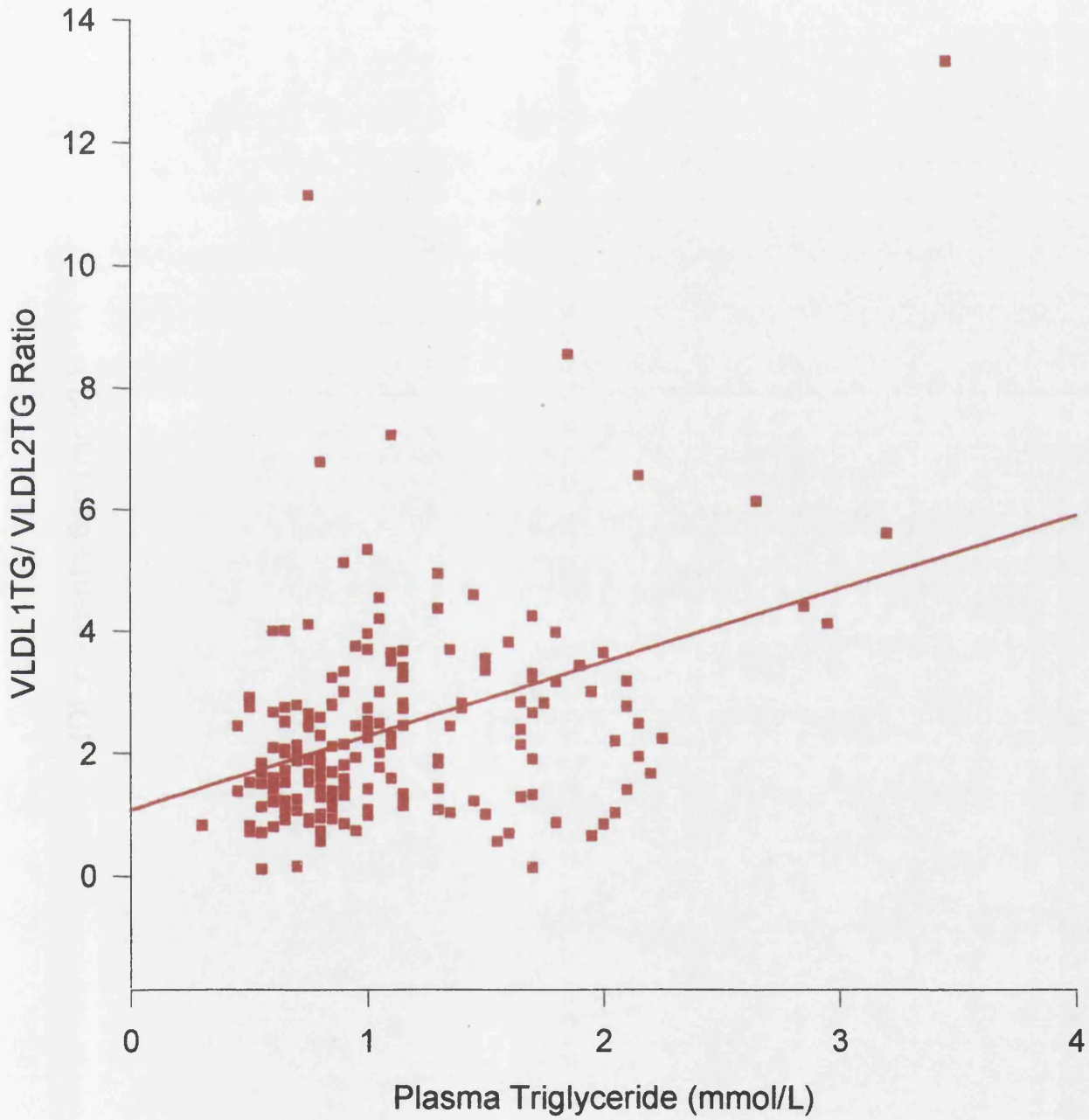


Fig 6: IDL concentration vs Cholesterol

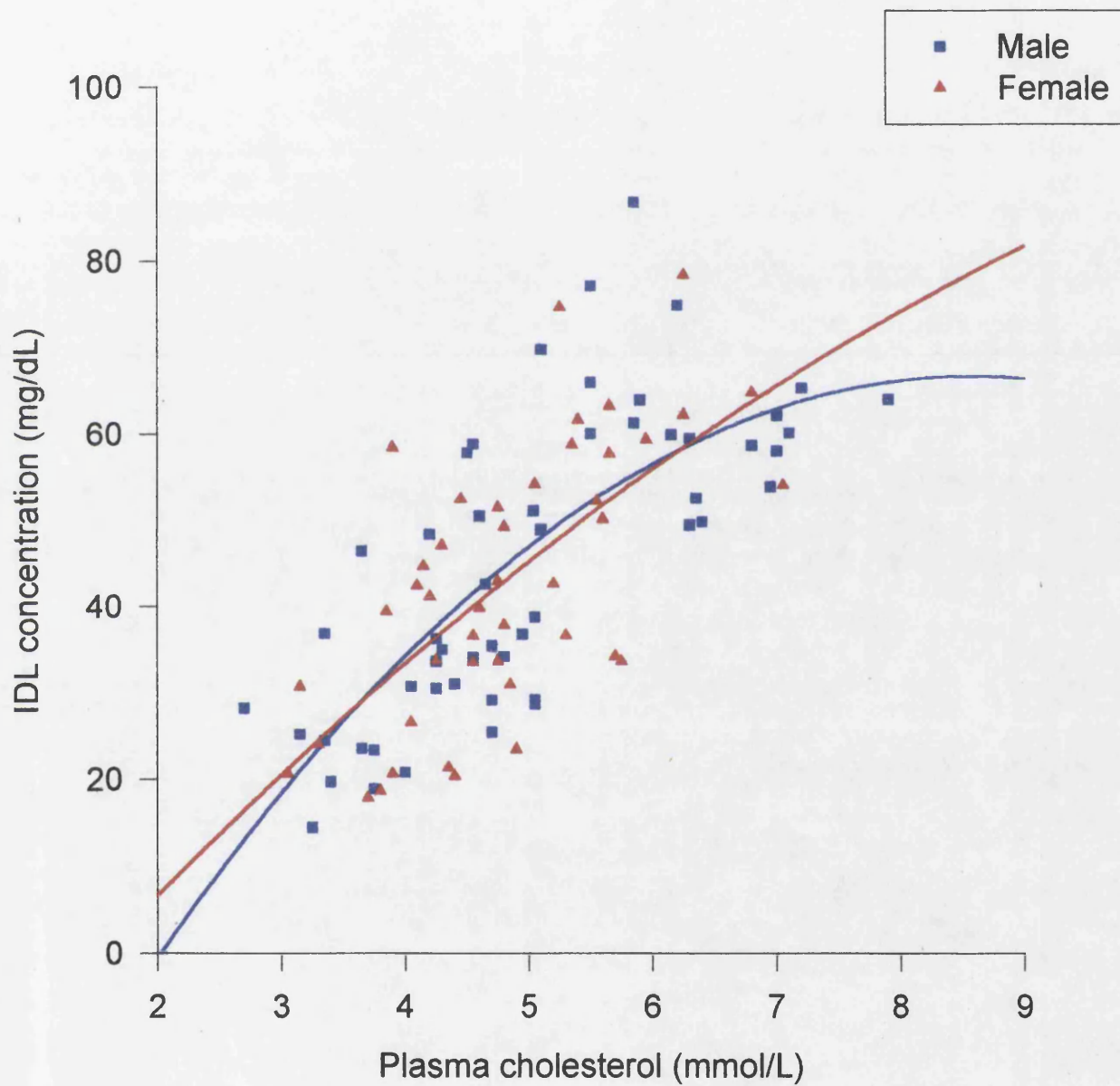


Fig 7: LDL concentration vs Cholesterol

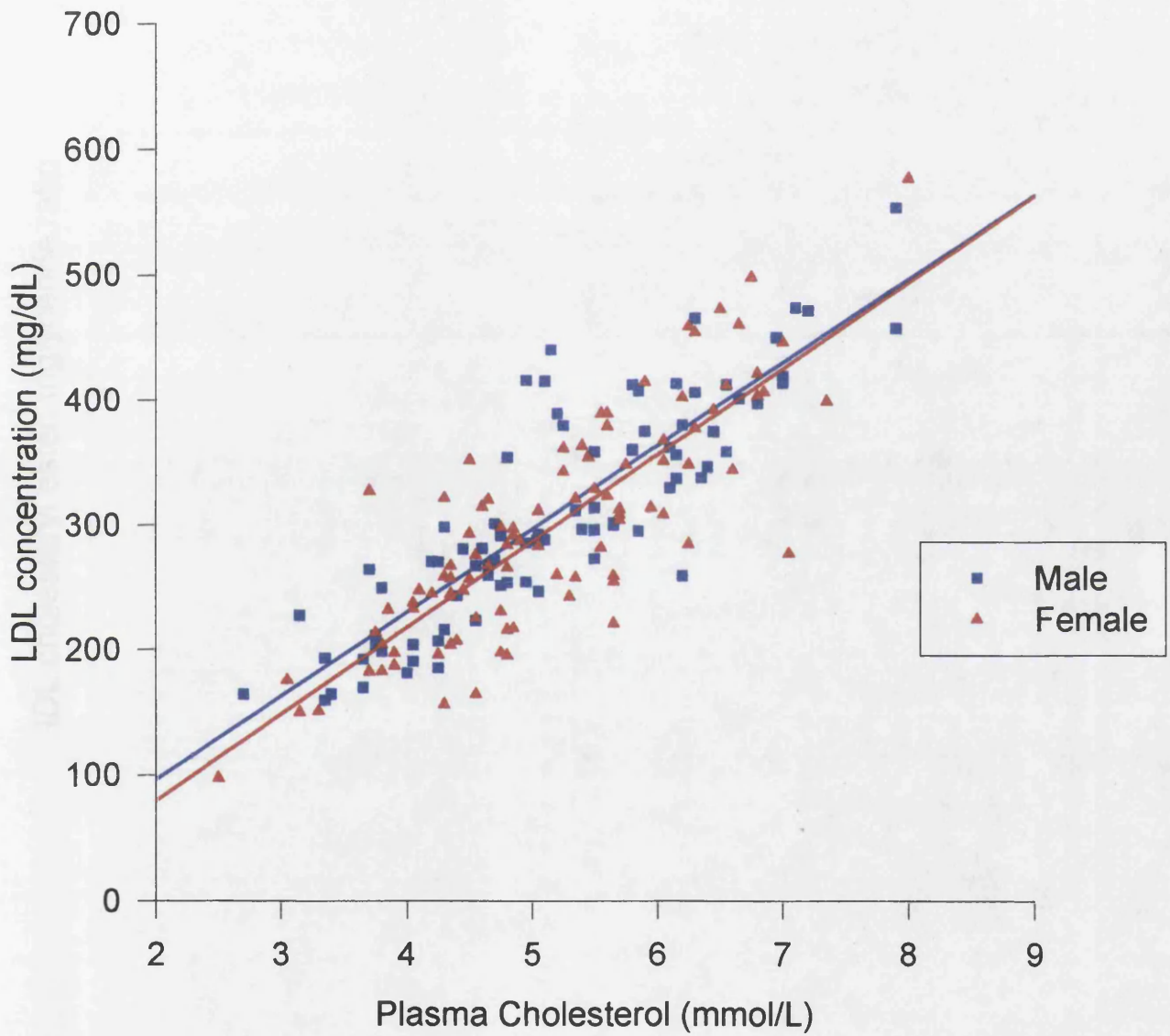


Fig 8: IDL cholesteryl ester: TG ratio vs Cholesterol

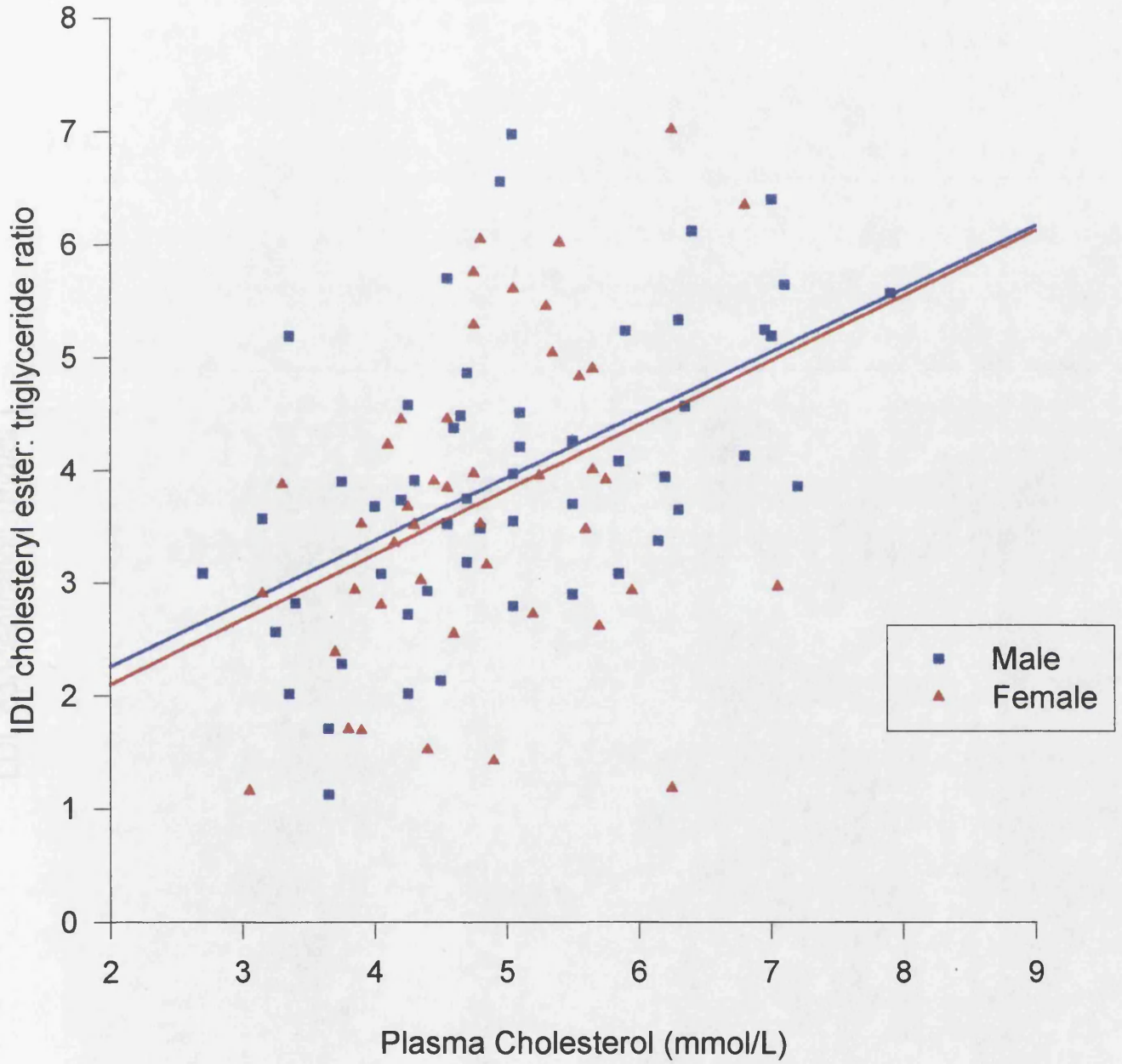


Fig 9: LDL concentration vs Triglyceride

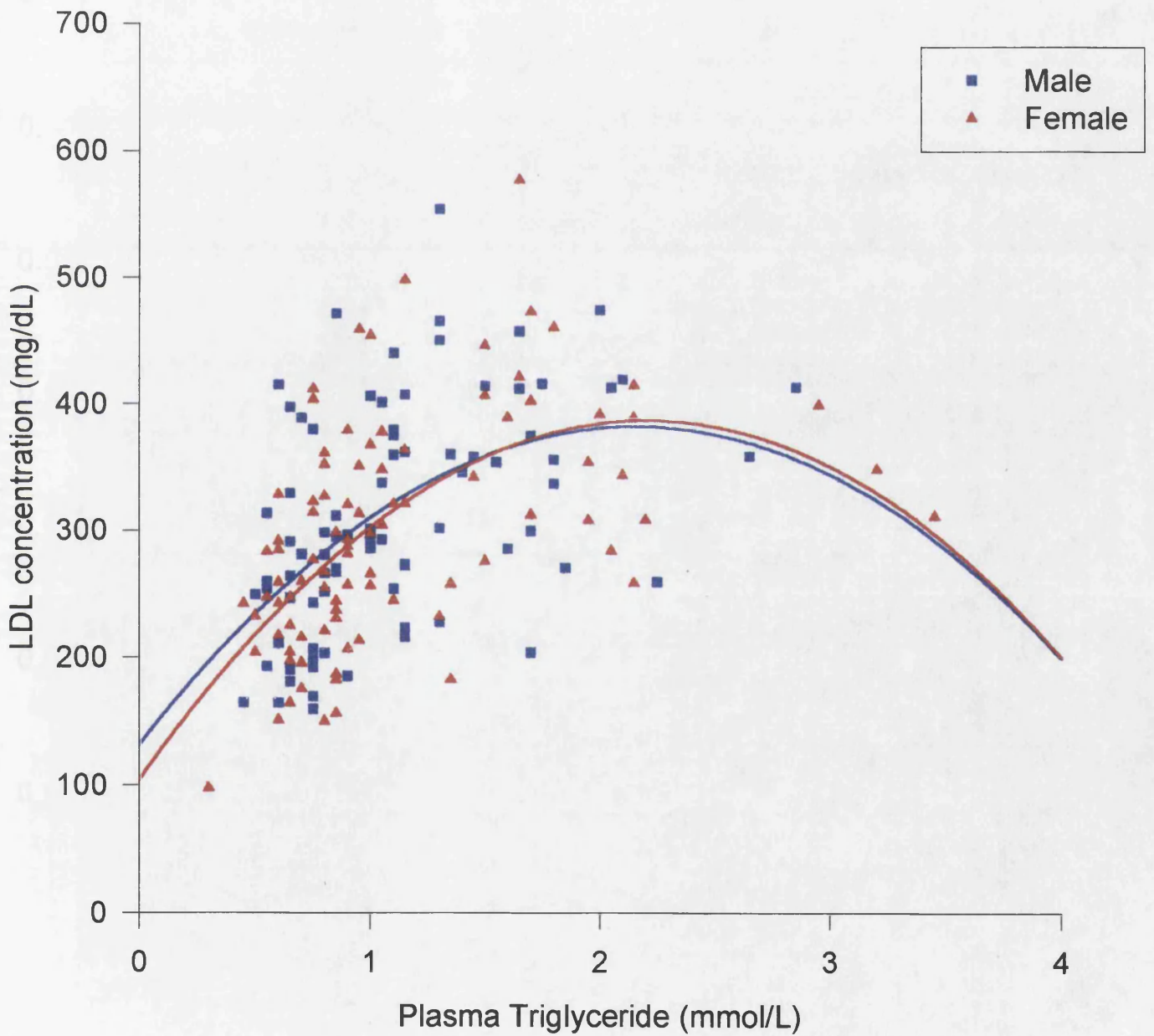


Fig 10: Percentage LDL Triglyceride content vs HL activity

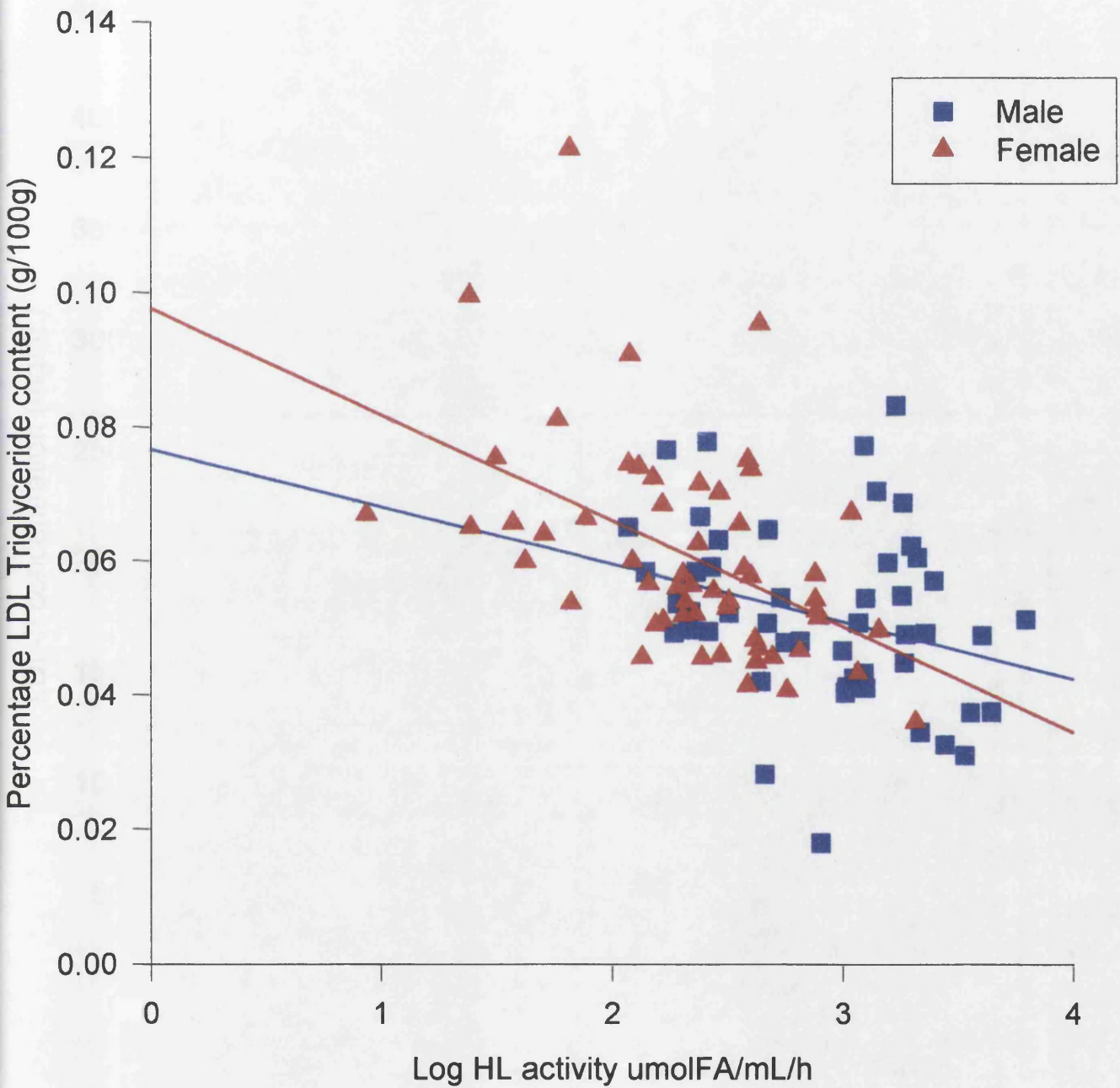


Fig 11: LDL-II concentration vs Triglyceride

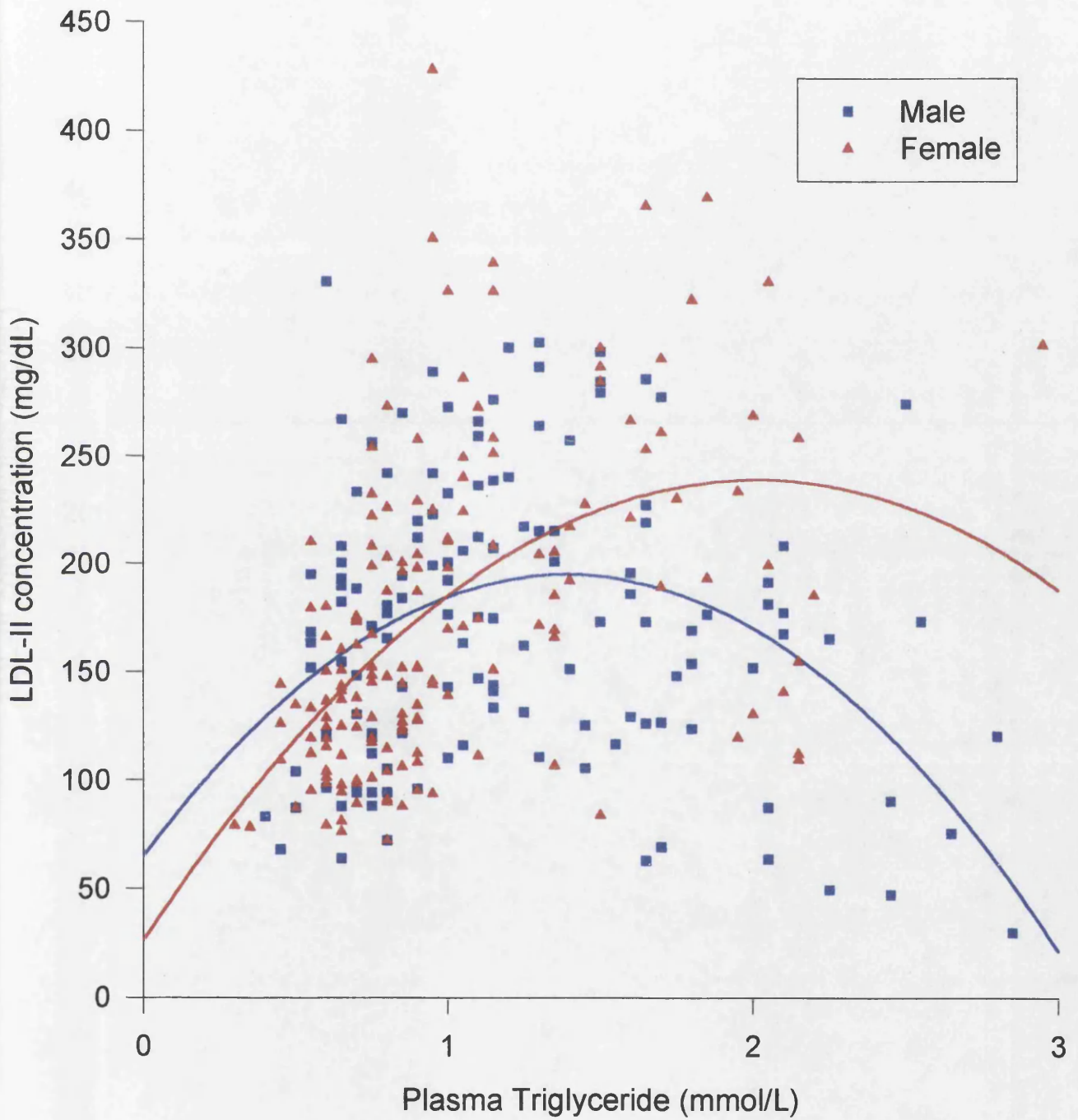


Fig 12: LDL-III concentration vs Triglyceride

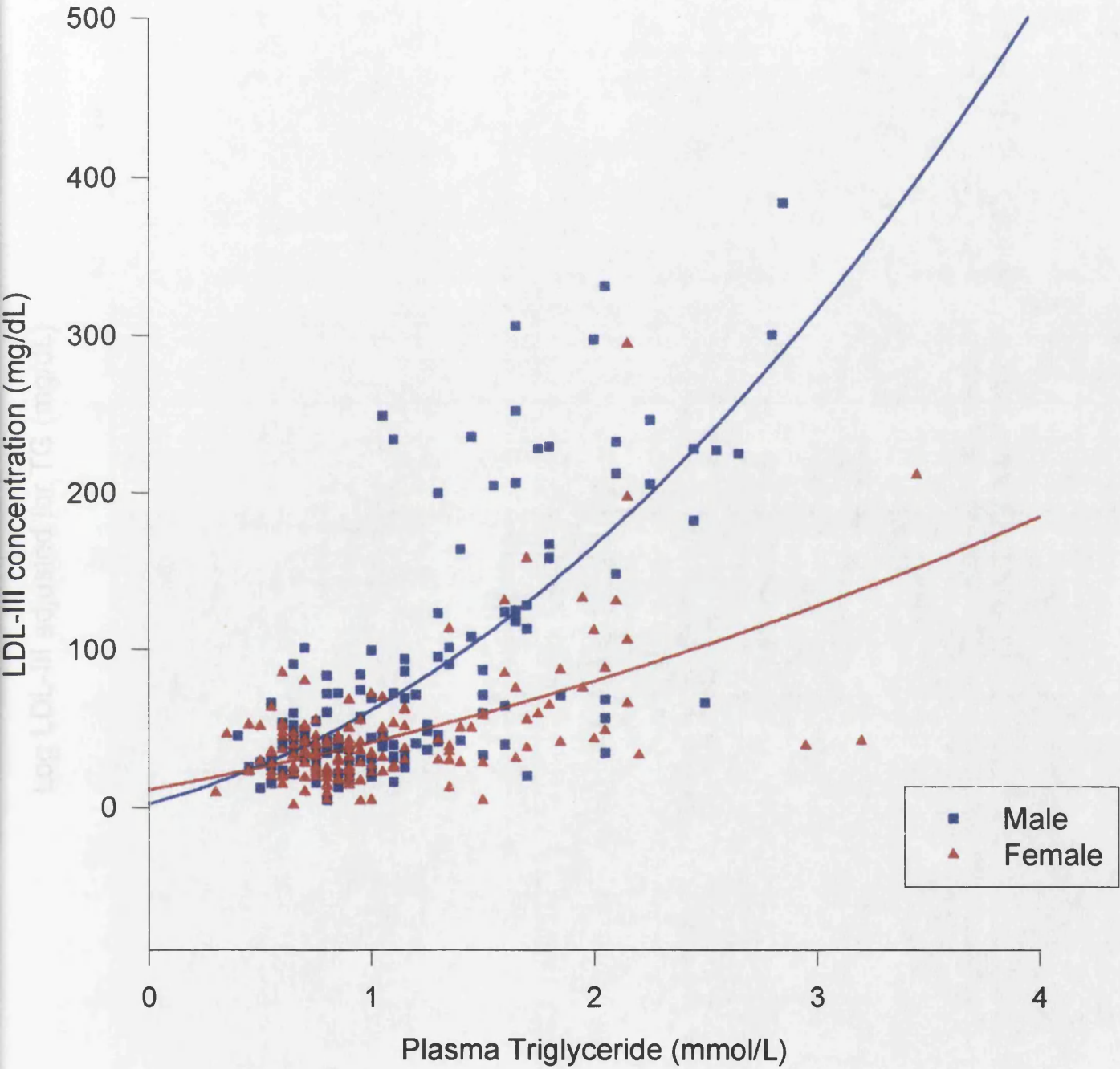
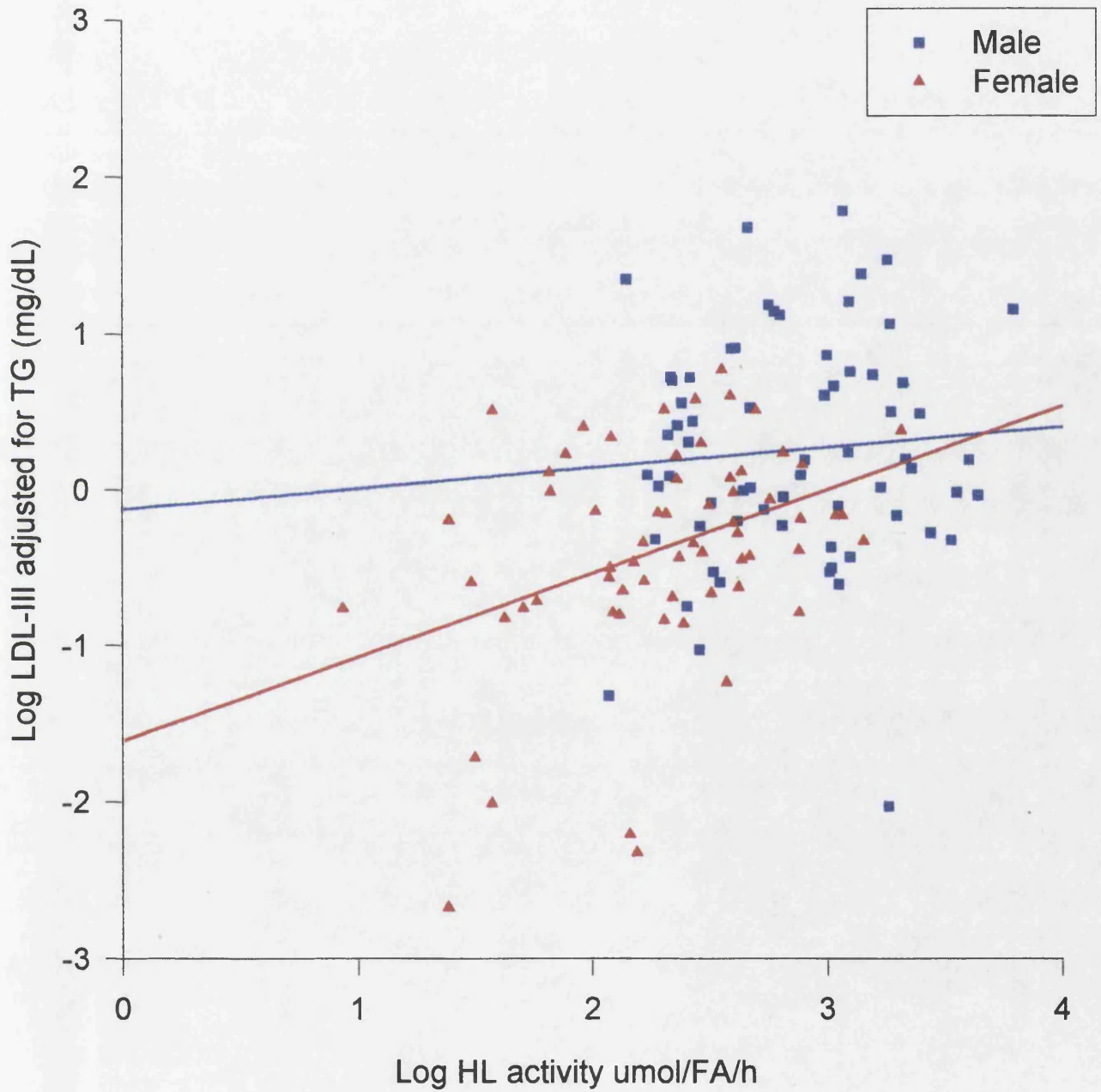


Fig 13: LDL-III concentration adjusted for TG vs HL



Chapter 4 Relationship between insulin resistance, anthropometry, lipids, lipoproteins and lipases

A wise man is strong. Yes, a man of knowledge increases strength

Proverbs 24:5

4.1 Introduction

Insulin resistance is closely associated with non-insulin dependent diabetes mellitus, hypertension, obesity, premature atherosclerosis, and dyslipidaemia.¹³ Two major factors known to influence the appearance of the disorder are age and obesity²⁰. It had been recognised for many years that ageing is associated with declining glucose tolerance and begins at about 40 years and progresses throughout adulthood.⁷⁷ The reason for this declining function is target tissue unresponsiveness to the action of insulin.^{174,175,176} The underlying cause of insulin resistance, however, remains uncertain. Some have suggested it may be attributable to age related changes in body composition and level of physical activity rather than to age itself.¹⁷⁷ For example, it has been shown that insulin sensitivity in men until around 60 to 70 years of age appears to be determined principally by body fat.¹⁷⁸ It is also known that weight loss and physical training improves the body's sensitivity to insulin.^{179,180} Nonetheless, all studies agreed that insulin resistance was a hallmark of obesity.^{181,182} Various markers of obesity have been used and there are no consensus on what constitutes an ideal choice. In the past, markers of obesity took into consideration the height, weight and body mass index (BMI) but recent evidences suggest that the distribution of obesity may be more important than obesity per se.^{183,184} Both environmental and genetic factors contribute to the development of obesity. In some individuals, the obesity and related insulin resistance are related to excessive caloric intake, whilst in others the obesity results from an inherited disturbance in thermogenesis or intermediary metabolism.¹⁸⁵

A close relationship between hyperinsulinaemia and hypertriglyceridaemia has been described in population based studies in healthy normal-weight subjects.²⁰ There is much evidence to suggest that insulin resistance, working through hyperinsulinaemia, enhances hepatic VLDL synthesis and contributes to the elevated plasma triglyceride levels observed in normal-weight healthy subjects, obese nondiabetic subjects, and NIDDM subjects.¹⁸⁶ It is now recognised that the insulin-resistant hyperinsulinaemic state is not associated with substantial changes in LDL-cholesterol concentrations, but rather with increases in LDL apolipoprotein (apo) B levels.¹⁸⁷ Studies have also shown that hyperapo B condition is frequently associated with hypertriglyceridaemia in the general population.¹⁸⁸ which is likely to be due to a high proportion of small, dense (i.e. lipid poor) LDL particles in the circulation.¹⁸⁹ The presence of the dense LDL phenotype is associated with hypertriglyceridaemia and low HDL-cholesterol levels⁸⁵ as well as with insulin resistance⁷⁴ and abdominal obesity.⁷⁵ Furthermore, it is of interest to note that the prevalence of the dense LDL phenotype is quite similar to the insulin resistance syndrome (30% versus 25% respectively).^{13,85}

Lipoprotein lipase(LPL) serves two major functions in the body, the assimilation of triglyceride fatty acid into tissues and the regulation of plasma concentrations of lipoprotein classes.¹⁹⁰ LPL activity in the adipose tissue has been shown to be influenced

by hormonal factors such as insulin¹⁹¹, insulin/glucose ratio, insulin-like growth factors and thyroxine.¹⁹² Animal studies suggest that the increase in LPL activity by insulin and/or glucose in rat adipose tissue is due to the activation of a proenzyme to an active enzyme within the adipose tissue.¹⁹³ Type I diabetic patients with poor glucose control have depressed levels of adipose tissue LPL,¹⁹⁴ with chronic administration of insulin and adequate glycaemic control, the enzyme activity is normal.¹⁹⁵ Likewise, type II diabetics have also been shown to have lower adipose tissue LPL activity during periods of suboptimal glycaemic control.^{196,197} Simsolo et al had shown that the improved glycaemic control was associated with an increase in LPL immunoreactive mass and LPL synthesis but no change in LPL mRNA levels.¹⁹⁸ They suggest that LPL regulation in these diabetics occurred at a translational level or through a possible change in degradation. LPL activity in skeletal muscle has also been shown to be related to insulin sensitivity.¹⁹⁹ Insulin deficiency or a relative insulin deficiency, such as that which occurs in insulin resistance, could affect LPL activity in both adipose tissue and skeletal muscle. This link between insulin and a major lipolytic enzyme provides a potential mechanism for the observed lipoprotein abnormalities seen as part of the insulin resistance syndrome. This chapter seeks to explore the impact of insulin resistance on lipids, lipoproteins and the lipolytic enzymes as well as the relationship with the various markers of obesity. The subjects and data were from the large group of normal previously described in chapter 3.

4.2 Anthropometric indices

It had been observed that both men and women are susceptible to weight gain. However, when men become obese, they deposit fat around the waist, resulting in what is termed as central or "apple-shaped" obesity. When women become obese, they deposit fat around the hips and that is termed "pear-shaped" obesity. Such descriptions are not merely for cosmetic reasons for studies have shown that apple-shaped obesity is associated with a higher risk for coronary heart disease whilst the pear-shaped obesity have lower coronary risk.^{200,201,202,203} It is believed that the differences in body fat distribution may be due to the variation in adipose tissue LPL activity. There is little variation in adipose tissue LPL activity in men²⁰⁴ but women have been shown to have higher adipose tissue LPL activity in the gluteal and femoral depots compared to the abdominal depots.²⁰⁵ These regional differences in adipose tissue LPL activity are thought to lead to the preferential accumulation of fat in the gluteal-femoral region in women whereas men tend to accumulate excess body fat in the abdominal region.^{206,207} The differences in body fat distribution and the regional differences in adipose tissue LPL activity are thought to be associated with the sex hormone balance²⁰⁸ associated with the plasma lipoprotein-lipid profile.²⁰⁹ Many markers of obesity have been used and associated with increased coronary risk. The commonest, BMI is derived from the weight in kilograms divided by the square of the height in metres. Most data which relate the level of fatness to total mortality have shown a U-shaped risk curve i.e. greatest risk of death is experienced by the very lean and the very fat.²¹⁰ BMI of between 25 to 30 kg/m² is deemed overweight and those above 30 kg/m² as obese.²¹¹

However, the use of BMI may not truly represent the anthropometric risk since obese women have lower risk than obese men. Various parameters have been devised to

indicate the type of obesity present. The WHR is one such measure that has been used extensively. Many definitions for measuring waist and hip have been suggested but for purpose of the present work a commonly accepted definition has been employed, namely: waist is defined as the narrowest circumference below the subcostal margin and the anterior superior iliac crest. Hips on the other hand is defined as the widest circumference below the waist. Males should have an ideal WHR of 0.9 or below whilst females should have WHR of 0.85 and below. Individuals with WHR above 0.9 and 0.85 in males and females respectively, were deemed to be centrally obese. The insulin resistance syndrome first described by G M Reaven, encompasses central obesity, insulin resistance, hypertension, raised triglyceride and low HDL cholesterol. The syndrome is associated with higher than normal risk of developing CHD. Reaven postulated that the underlying problem was insulin resistance syndrome and that all others features of the syndrome are derived from this metabolic problem.

The gold standard for measuring insulin resistance is the insulin clamp technique, either the euglycaemic clamps or hyperglycaemic clamps for diabetic patients. Due to constraints of manpower and time, I have used other indices of insulin resistance including the fasting levels of insulin, glucose, and the parameter calculated by the HOMA model (as discussed in chapter 1), as well as presence of low HDL, high triglyceride and dense, LDL-III.

4.3 The impact of body fat distribution on plasma lipids

BMI, WHR and waist circumference and their association with lipids, lipoproteins, lipases and markers of insulin resistance were explored and results are tabulated tables 10, 11 and 12 respectively. For BMI, normal subjects were divided into two groups, i.e. those with BMI below 27 kg/m^2 and those equal or above 27 kg/m^2 . A BMI of 27 kg/m^2 and above was taken as indicative of obesity. Likewise, a WHR of 0.9 and above was taken to signify central obesity and again the normals were divided into two groups based on the WHR of 0.9. As for waist circumference, it was difficult to decide what was normality. The mean waist circumference for all subjects was about 80 cm and if mean plus one standard deviation was taken as normal, then the upper limit of normality for waist circumference would be about 90 cm. Hence, I have opted to divide the normals into two groups using a waist circumference of 90 cm and above as a second index of central obesity.

It was interesting to note that in the obese group, whether one used BMI, WHR or just waist circumference, the total cholesterol, LDL cholesterol, triglycerides were higher and HDL cholesterol were lower when compared to the non-obese group. Likewise when for all 3 parameters of obesity, the obese group had significantly lower HDL₂ mass but no significant differences in HDL₃ mass. All three parameters of obesity were also associated with differences in postheparin lipoprotein lipase(LPL) activity and hepatic lipase(HL) activity. Lipoprotein subfractions showed significant differences between the obese and non obese groups. The obese groups had higher VLDL₁, VLDL₂ as well as LDL-III, and hence a more atherogenic profile. The mean levels of LDL-III concentration in the obese group (BMI $>27 \text{ kg/m}^2$) or centrally obese group (waist $>90 \text{ cm}$) were also noted to be above 100 mg lipoprotein/dL plasma, the level at which significant atherogenesis is

believed to occur.¹³⁶ LDL-I on the other hand, tended to be lower in the obese group, although this failed to achieve significance, except in the group with waist > 90 cm.

The group with BMI > 27 kg/m² had a higher fasting insulin and IR but did not show any difference in fasting glucose. Using WHR, there were no difference between the groups whilst the group with waist circumference > 90 cm showed higher levels of insulin, IR and glucose, and these were highly significant. The data suggested that waist circumference was a better discriminator of central obesity compared to WHR since there seemed to be greater distinction between the groups with respect to the lipids and lipoprotein profile as well as markers of insulin resistance.

The differences in lipids and lipoproteins between the obese and non-obese groups (using BMI and waist circumference as indices) were then explored for possible gender differences. Results showed that the differences between the groups were similar for both males and females (data not shown), with the exception of VLDL₂ and HL which failed to show a statistically significant difference in the females between those with BMI above and below 27 kg/m² although maintaining the trend seen in the whole group analysis. The LDL-III concentration and HL did not show any statistical difference in females with waist circumference above and below 90 cm. The mean concentration of LDL-III and levels of HL activity however, continues to be higher in those with waist circumference greater than 90 cm, similar to that seen in the whole group analysis. The HL in males did not show any difference between groups with waist circumference above and below 90 cm. The differences between the groups with and without obesity were maintained when analysed by gender.

Waist circumference showed significant positive correlations with VLDL₁ and VLDL₂ in both males and females (Fig 14 and Fig 15). The regression of VLDL₁ with waist circumference was highly significant in both sexes ($r^2 = 11.7\%$, $p < 0.001$ for males and $r^2 = 19.8\%$, $p < 0.001$ for females). As the waist circumference increased, the concentration of LDL-III increased in both males and females (Fig 16). The regression of LDL-III with waist circumference was highly significant with $r^2 = 13.9\%$, $p = 0.001$ and $r^2 = 10.5\%$, $p = 0.002$ for males and females respectively.

Table 10: Effects of Body Mass Index on Lipids, Lipoproteins, lipases and Insulin resistance

Variable	BMI<27kg/m ² (n)	BMI ≥ 27kg/m ² (n)	p
Total cholesterol (mmol/L)	5.06±0.07 (233)	5.73±0.14 (57)	<0.0001
Triglyceride (mmol/L)	0.99±0.03 (233)	1.57±0.07 (67)	<0.0001
LDL cholesterol (mmol/L)	3.20±0.06 (229)	3.84±0.13 (64)	<0.0001
HDL cholesterol (mmol/L)	1.40±0.02 (233)	1.19±0.04 (67)	<0.0001
HDL ₂ mass (mg/dL)	78.6±3.1 (218)	52.6±5.5 (65)	<0.0001
HDL ₃ mass (mg/dL)	250.4±42 (218)	251.0±6.7 (65)	ns
VLDL ₁ (mg/dL)	46.6±3.3 (151)	90.3±9.8 (38)	<0.0001
VLDL ₂ (mg/dL)	36.2±2.1 (151)	50.0±3.3 (38)	0.001
HL (µmolFA/ml/h)	14.47±0.7 (112)	19.05±1.8 (28)	0.05
LPL (µmolFA/ml/h)	4.94±0.21 (112)	3.38±0.18 (28)	<0.0001
Fasting Insulin (mU/L)	7.37±0.32 (149)	10.86±0.73 (38)	<0.0001
Fasting glucose (mmol/L)	4.77±0.04 (146)	4.79±0.09 (38)	ns
Insulin resistance	1.60±0.08 (144)	2.34±0.16 (37)	<0.0001
LDLI (mg/dL)	73.3±2.8 (224)	63.9±4.4 (63)	ns
LDLII (mg/dL)	168.7±4.5 (224)	186.5±9.8 (63)	ns
LDLIII (mg/dL)	52.8±3.7 (224)	101.8±10.0 (63)	<0.0001

p value refers to the significance of difference between groups as determined by student's unpaired t-test
 LDL; low density lipoprotein, HDL; high density lipoprotein, VLDL; very low density lipoprotein, HL; hepatic lipase, LPL; lipoprotein lipase

Table 11: Effects of waist to hip ratio on lipids, lipoproteins, lipases and insulin resistance

Variable	WHR<0.9 (n)	WHR ≥ 0.9 (n)	p
Total cholesterol (mmol/L)	5.04±0.10 (135)	5.37±0.14 (54)	<0.05
Triglyceride (mmol/L)	1.02±0.04 (135)	1.32±0.08 (54)	<0.001
LDL cholesterol (mmol/L)	3.21±0.09 (134)	3.66±0.13 (54)	<0.001
HDL cholesterol (mmol/L)	1.38±0.03 (135)	1.19±0.04 (54)	<0.001
HDL ₂ mass (mg/dL)	73.5±4.1 (122)	44.0±4.8 (54)	<0.0001
HDL ₃ mass (mg/dL)	240.8±5.2 (122)	235.3±8.2 (54)	ns
VLDL ₁ (mg/dL)	49.7±3.8 (134)	69.2±7.4 (54)	<0.001
VLDL ₂ (mg/dL)	36.7±2.1 (134)	44.0±3.6 (54)	<0.05
HL (μmolFA/ml/h)	13.04±0.7 (93)	19.91±1.3 (44)	<0.0001
LPL (μmolFA/ml/h)	4.95±0.24 (93)	3.97±0.20 (44)	<0.01
Fasting Insulin (mU/L)	7.71±0.35 (132)	8.77±0.61 (54)	ns
Fasting glucose (mmol/L)	4.77±0.05 (129)	4.82±0.06 (54)	ns
Insulin resistance	1.68±0.09 (127)	1.91±0.14 (53)	ns
LDLI (mg/dL)	70.9±3.8 (124)	65.3±5.5 (52)	ns
LDLII (mg/dL)	175.6±6.5 (124)	165.0±9.6 (52)	ns
LDLIII (mg/dL)	46.3±4.4 (124)	97.9±12.0 (52)	<0.0001

p value refers to the significance of difference between groups as determined by student's unpaired t-test
 LDL; low density lipoprotein, HDL; high density lipoprotein, VLDL; very low density lipoprotein, HL; hepatic lipase, LPL; lipoprotein lipase

Table 12: Effects of Waist on lipids, lipoproteins, lipases and insulin resistance

Variable	Waist <90 (n)	Waist ≥ 90 (n)	p
Total cholesterol (mmol/L)	4.96±0.09 (149)	5.81±0.16 (39)	<0.0001
Triglyceride (mmol/L)	0.98±0.04 (149)	1.57±0.10 (39)	<0.0001
LDL cholesterol (mmol/L)	3.15±0.08 (148)	4.05±0.15 (39)	<0.0001
HDL cholesterol (mmol/L)	1.38±0.03 (149)	1.11±0.04 (39)	<0.0001
HDL ₂ mass (mg/dL)	71.7±3.8 (140)	36.0±4.4 (35)	<0.0001
HDL ₃ mass (mg/dL)	237.7±5.0 (140)	243.0±8.6 (35)	ns
VLDL ₁ (mg/dL)	45.9±3.3 (148)	91.5±9.2 (39)	<0.0001
VLDL ₂ (mg/dL)	34.8±1.9 (148)	54.1±4.0 (39)	<0.0001
HL (μmolFA/ml/h)	14.66±0.8 (107)	17.36±1.3 (30)	<0.01
LPL (μmolFA/ml/h)	4.81±0.22 (107)	4.00±0.23 (30)	<0.01
Fasting Insulin (mU/L)	7.27±0.32 (143)	10.68±0.64 (39)	<0.0001
Fasting glucose (mmol/L)	4.70±0.04 (146)	5.08±0.07 (39)	<0.0001
Insulin resistance	1.55±0.08 (141)	2.44±0.15 (38)	<0.0001
LDLI (mg/dL)	72.8±3.6 (140)	55.8±5.7 (63)	<0.01
LDLII (mg/dL)	168.8±5.9 (140)	187.8±13.0 (63)	ns
LDLIII (mg/dL)	48.8±4.7 (140)	113.0±14.0 (63)	<0.0001

p value refers to the significance of difference between groups as determined by student's unpaired t-test
 LDL; low density lipoprotein, HDL; high density lipoprotein, VLDL; very low density lipoprotein, HL; hepatic lipase, LPL; lipoprotein lipase

4.4 Insulin Resistance and Lipases

LPL is present mainly in adipose tissue and to some extent in other tissues. It is the rate limiting enzyme for tissue uptake of plasma triglyceride.^{212,213,214} In a number of animal studies, adipose tissue LPL has been shown to be sensitive to caloric and hormonal balance and particularly dependent on availability of glucose and insulin.^{215,216} The lipoprotein lipase hydrolyzes triglycerides in chylomicrons and VLDL whilst HL hydrolyzes triglycerides in IDL and HDL, as well as phospholipids in HDL. Together with lecithin:cholesterol acyltransferase (LCAT), these enzymes participate in reverse cholesterol transport, which results in movement of cholesterol from peripheral tissues to the liver. LPL and HL function whilst bound to the luminal surface of endothelial cells in peripheral tissues and the liver. Studies have suggested that HL may play a role in chylomicrons remnant removal. Cell-surface LPL can also play a role in the binding of VLDL and LDL to the cells, similar to the HL-remnant interactions.²¹⁷ However the activity of LPL in human tissues have usually been estimated only indirectly as postheparin plasma lipolytic activity, as in our study. In most instances, but not all, low postheparin plasma lipolytic activity has been associated with impaired triglyceride removal and hypertriglyceridaemia. However we must bear in mind that total postheparin plasma lipolytic activity may not be an accurate index of adipose tissue LPL, which is the major determinant of peripheral triglyceride removal.

Data taken from the group of normal individuals showed that the postheparin LPL activity had a weak but significant negative correlation with body mass index ($r^2=3.3\%$, $p<0.05$) and waist ($r^2=2.9\%$, $p<0.05$) but no significant correlation with WHR. HL on the other hand showed a strong positive correlation with markers of central obesity i.e. WHR ($r^2=16.8\%$, $p<0.001$) and waist ($r^2=13.1\%$, $p<0.001$). There was a weaker but significant correlation with general obesity, BMI ($r^2=3.7\%$, $p<0.05$). LPL activity also had a weak but significant negative correlation with fasting insulin ($r^2=2.9\%$, $p<0.05$) but no correlation with fasting glucose, glucose area under curve or insulin area under curve or insulin resistance (IR) as calculated by HOMA model. HL activity had a strongly significant positive correlation with fasting insulin ($r^2=5.2\%$, $p<0.01$) as well as IR ($r^2=5.4\%$, $p<0.01$). The group of normal individuals were next divided into two groups based on their levels of insulin resistance. The mean fasting insulin for the whole group was noted to be 8.0 mU/L whilst the IR was noted to be 1.7. Hence the group was divided into two groups based on the fasting insulin and IR above and below the mean, as shown in table 13. There were no difference between the two groups in the postheparin LPL activity, whether fasting insulin or IR were used as the marker of insulin resistance. However, the two groups showed significant difference in postheparin HL activity, with the hyperinsulinaemic or insulin resistant group having higher HL levels.

Table 13: Insulin resistance and the lipolytic enzymes

	Lipoprotein lipase ($\mu\text{molFA/ml/h}$)	Hepatic lipase ($\mu\text{molFA/ml/h}$)
$I0 \leq 8.0$ (mU/L)	4.8 \pm 0.3	13.9 \pm 0.8
$I0 > 8.0$ (mU/L)	4.4 \pm 0.3	17.1 \pm 1.2 \ddagger
$IR \leq 1.7$	4.7 \pm 0.3	13.6 \pm 0.8
$IR > 1.7$	4.3 \pm 0.3	17.9 \pm 1.2 \ddagger

\ddagger $P < 0.01$, \ddagger $p < 0.05$, refers to the significance of difference between the two groups using student's unpaired t-test.

I0; fasting insulin, IR; insulin resistance as derived by HOMA model.

4.5 Insulin Resistance and VLDL subfractions

Indices of insulin resistance such as fasting insulin, fasting glucose as well as IR, all showed significant positive univariate correlation with the VLDL subfractions. It was also interesting to note that the correlation with the larger VLDL₁ subfraction were stronger than the correlation with VLDL₂ subfraction. The regression of VLDL₁ and VLDL₂ were shown in Figure 14 with regression equations of VLDL₁= 28.4 + 15.8 IR and VLDL₂= 30.3 + 5.27 IR. A comparison of the regression slopes showed that the difference was statistically significant ($p < 0.001$).

Table 14: Univariate correlation of markers of insulin resistance with VLDL subfraction

Variable	VLDL ₁ (r ²)	p	VLDL ₂ (r ²)	p
I0 (mU/L)	9%	<0.001	3.2%	<0.05
G0 (mmol/L)	4.7%	<0.01	4.5%	<0.01
IR	10.4%	<0.001	4.2%	<0.01

r² (correlation coefficient squared) was determined by linear regression in univariate analysis. P refers to the significance of r². I0; fasting insulin, G0; fasting glucose, IR; insulin resistance.

4.6 Insulin Resistance and LDL subfraction

Indices of insulin resistance (Table 15) showed a significant negative univariate correlation with LDL-I subfraction concentration and a positive univariate correlation with LDL-III. There were no significant correlation with LDL-II subfractions (data not shown). The insulin resistant individuals were characterised by a predominance of dense LDL-III subfractions in preference to the buoyant LDL-I subfractions. When the group of normals were divided into the atherogenic group, who carried a predominance of LDL-III subfractions (defined as LDL-III above 100 mg/dL), and the non atherogenic group (Table 16), it was noted that the atherogenic group were more insulin resistant as reflected by the fasting insulin and insulin resistance. The atherogenic group also had higher postheparin HL activity and lowered LPL activity.

Table 15: Univariate correlation of markers of insulin resistance with LDL subfractions

Variable	LDL-I(r ²)	p	LDL-III(r ²)	p
I0 (mU/L)	2.5%	<0.05	4.2%	<0.01
G0 (mmol/L)	4.3%	<0.01	3.0%	<0.05
IR	3.8%	<0.01	5.1%	<0.01

r² (correlation coefficient squared) was determined by linear regression in univariate analysis. P refers to the significance of r². I0; fasting insulin, G0; fasting glucose, IR; insulin resistance.

Table 16: Comparisons of markers of insulin resistance between those with and without dense LDL-III

Variable	LDL-III<100 mg/dl	LDL-III≥ 100 mg/dl	p
I0 (mU/L)	7.6±0.34	10.53±0.93	<0.01
IR	1.64±0.08	2.33±0.23	<0.01
HL(μmolFA/ml/h)	14.5±0.75	18.75±1.7	<0.05
LPL(μmolFA/ml/h)	4.7±0.2	3.85±0.24	<0.01

Concentrations of LDL-III above 100 mg lipoprotein/ dL plasma gave a relative risk of 7.0 for myocardial infarction and the group was divided based on this concentration of LDL-III.¹³⁶

p value refers to the significance of difference between groups as determined by student's unpaired t-test. I0; fasting insulin, IR; insulin resistance, HL; hepatic lipase, LPL; hepatic lipase.

4.7 Comparing the normoinsulinaemic and hyperinsulinaemic

The results in table 17 gives a summary of the differences between groups with normal fasting insulin and the groups with hyperinsulinaemia. Hyperinsulinaemia was defined by the mean fasting insulin \pm 2 standard deviation and this was calculated to be 13.4 mU/L. The insulin resistant group had higher triglyceride, VLDL₁, LDL-III and lower HDL cholesterol and HDL₂. This was supportive of the hypothesis that insulin resistance confers a more atherogenic profile in affected individuals.

Table 17: Comparing the normoinsulinaemic and hyperinsulinaemic

	Normal insulin (mean\pmsem)	High insulin (mean\pmsem)	p
Cholesterol (mmol/L)	5.11 \pm 0.1	5.30 \pm 0.3	ns
Triglyceride (mmol/L)	1.07 \pm 0.04	1.48 \pm 0.16	<0.05
HDL-C (mmol/L)	1.35 \pm 0.03	1.13 \pm 0.04	<0.01
HDL ₂ (mg/dL)	67.0 \pm 3.6	44.3 \pm 8.8	<0.05
HDL ₃ (mg/dL)	239 \pm 4.5	230.7 \pm 14	ns
VLDL ₁ (mg/dL)	52.2 \pm 3.5	80.8 \pm 14.0	<0.05
VLDL ₂ (mg/dL)	38.1 \pm 2.0	46.5 \pm 5.3	ns
LDL-I (mg/dL)	70.4 \pm 3.4	61.9 \pm 7.1	ns
LDL-III (mg/dL)	58.6 \pm 5.3	87.6 \pm 16.0	<0.05

HDL; high density lipoprotein, VLDL; very low density lipoprotein, LDL; low density lipoprotein
P values refers to the significance of difference between groups using student's unpaired t-test.

4.8 Discussion

Obesity is usually a descriptive term for excess body fat. Assessment of the presence and extent of obesity is often subjective and influenced by cosmetic and cultural considerations. In recent years, both the extent and pattern of obesity has received increasing attention because of the increased mortality and morbidity associated with both. Obesity is clearly associated with hypertension, hypercholesterolaemia, non-insulin dependent diabetes mellitus, excess of certain cancers and other medical problems. Obesity is defined as a body fat content greater than 25% of total body weight for men and greater than 30% for women. Methods such as underwater weighing can accurately determine percent body fat but are expensive and tedious. Other methods such as body impedance meters have been shown to correlate well with other laboratory methods. Body fat contents can also be estimated by skin fold thickness using callipers but this method is often time consuming and imprecise. The most simple and commonly used method is the body mass index, using height and weight for calculation. However the use of BMI is fraught with errors as the heavily muscled athlete will have high values using this index whilst having a low body fat content and the sedentary individual may not be overweight but have decreased lean mass and consequently greater metabolic disturbance. Recently, it has been recognised that the pattern of distribution of adipose tissue throughout the

body affects metabolic consequences and may be a more important factor than total adipose tissue mass. Kissebah et al demonstrated that a person with fat located predominantly in the abdominal region may be at greater risk of insulin resistance, hypertension, heart disease and diabetes than another individual with a greater total amount of adipose tissue that is located predominantly in the gluteal region.²¹⁸ In our group of normals, obesity conferred a greater coronary risk by affecting all lipid parameters. Three indices of body fat distribution were used to create sub-groups, i.e. BMI, WHR and waist circumference. Whichever index was used to identify the obese group it was associated with higher plasma cholesterol and triglyceride, higher LDL cholesterol and lower HDL cholesterol. Furthermore, obesity affected the subfraction distribution of both LDL and HDL with a predominance of the dense, atherogenic, LDL-III subfraction and lower HDL₂ mass. The mean LDL-III concentration of those with obesity, as determined by BMI and waist circumference, was consistently above 100 mg lipoprotein/dL plasma. Hence, obesity not only increased the LDL-cholesterol but also shifted the subfraction distribution towards the more atherogenic dense LDL. This would further contribute to the coronary risk in those with obesity. The data further showed that obesity decreased HDL₂ but did not appear to affect the HDL₃ mass. Thus apart from the reduction in HDL cholesterol, the reduction in HDL₂ compared to HDL₃ aggravated the atherogenic potential as HDL₃ is not as efficacious in reverse cholesterol as HDL₂. The obese group was also noted to have higher VLDL₁ concentration, probably associated with the higher plasma triglyceride. As the waist circumference increased, the concentration of VLDL₁, VLDL₂ and LDL-III increased (fig 14 to fig 16). The rise in both VLDL₁ and VLDL₂ subfractions with increasing waist circumference occurred in both males and females. LDL-III concentrations in males showed a significant rise with increasing waist circumference. Females demonstrated a similar correlation with waist circumference but the gradient of the regression curve in males was greater than that demonstrated in the females. This difference between the gender is probably due to HL activity which differed between the sexes.

General obesity as reflected by BMI appeared to decrease LPL activity whilst central obesity, as reflected by WHR and particularly waist circumference, had greater effect on HL activity. From the data, it would appear that waist circumference was a better reflection of central obesity compared to WHR in that there was greater distinction with regards to lipids, lipoproteins, lipases and insulin resistance, when the waist circumference was used to separate the 2 groups as compared to using WHR. This is consistent with others who have also concluded that different indicators of body fat distribution have different levels of association with parameters of lipid and carbohydrate metabolism.¹⁸² There are limitations to the use of WHR as a marker of regional adiposity distribution in that a high ratio may be obtained in an individual with small hip circumference or a low ratio in an individual with large hip circumference. These two individuals may not necessarily differ with regard to the absolute amount of abdominal fat and in their risk profile, but may be falsely concluded on the basis of their WHR. The correlation of WHR with total body fat and visceral fat is too low to allow the use of WHR as a surrogate for the other two fatness variables. Perhaps the use of WHR should be abandoned in favour of more direct and absolute measurements of truncal-abdominal obesity, such as those provided by truncal and abdominal skinfolds or by the waist circumference.

The effects of insulin resistance on the lipolytic enzymes were explored in detail. Whether one uses fasting insulin or the insulin resistance as derived by the HOMA model, there were no apparent effect on the postheparin LPL activity, although there was a negative but weak univariate correlation with fasting insulin. There was a suggestion that the insulin resistant group may have significantly higher postheparin HL activity and this was further supported by the stronger univariate correlation with fasting insulin and IR. The lack of correlation of insulin resistance with LPL levels could be explained by several reasons. Firstly, the use of fasting insulin or IR as a marker of insulin resistance, has its limitations. It is not a direct measure of the insulin resistance, unlike the clamp studies. Second, the range over which insulin was measured in fasting sample was small and the results depended on the precision of the insulin radioimmunoassay. This can be further compounded by the pulsatility of insulin secretion,²¹⁹ uncertainty of whether proinsulin is being measured as insulin,²²⁰ and the effects of stress²²¹ and exercise,²²² which could all affect interpretation of assay results. It is necessary to measure the fasting plasma insulin over a 15 minute period in subjects who are rested, to overcome the effects of pulsatile release and stress. In this study, however, only one single plasma insulin level was taken and therein lies the limitations in interpretation of insulin resistance. Despite the imprecision which limits the clinical application of HOMA estimates from a single blood sample, the significant correlation of HOMA estimates with independent measurements of B-cell function and insulin resistance supported the use of HOMA as a means to explore the effects of an insulin resistant state. It would appear that the insulin resistant individual tended to be obese, particularly central obesity, and had lowered LPL activity and higher HL activity. A lower LPL activity would delay triglyceride clearance and hence cause elevation in the level of plasma triglyceride and the VLDL₁ concentration. The larger VLDL₁ particles, are more likely to undergo neutral lipid exchange with LDL and HDL, resulting in the accumulation of more atherogenic dense LDL-III subfraction as well as the less efficacious HDL₃ subfraction.

Both the VLDL₁ and VLDL₂ had significant univariate correlation with all the markers of insulin resistance i.e. fasting insulin, fasting glucose and IR. This is probably mediated through the effects of insulin resistance on triglyceride. Likewise, there was significant negative univariate correlation between fasting insulin, glucose and IR with LDL-I and a positive correlation with LDL-III. The results shown in table 16 makes clear the relationship between the atherogenic lipoprotein phenotype and insulin resistance. It is assumed that the group with dense, LDL-III of greater than 100 mg lipoproteins/ dL plasma carried the atherogenic lipoprotein phenotype. It can also be seen that the group with the atherogenic lipoprotein phenotype had significantly higher levels of fasting insulin and IR. Consistent with arguments put forth in chapter 3, the group with atherogenic lipoprotein phenotype also had significantly lower levels of postheparin LPL activity and higher HL activity. There was obviously a close relationship between ALP and IRS but whether the IRS resulted in the ALP or vice versa, is a question which cannot be resolved with present data. Furthermore, whether they represent different manifestations of the same metabolic disorder or different spectrum of the same disease remains unclear.

There appears to be a great overlap between the IRS, first proposed by Reaven, and the Atherogenic Lipoprotein Phenotype (ALP) first proposed by Austin. Perhaps they represent a spectrum of a greater underlying metabolic disorder and the full syndrome should include 1) raised triglyceride, 2) raised LDL-III concentration, 3) lowered HDL cholesterol and HDL₂ mass, 4) raised VLDL₁ mass, 5) lowered lipoprotein lipase activity, 6) raised hepatic lipase activity, 7) central obesity and 8) insulin resistance.

Fig 14: VLDL Subfractions vs Insulin Resistance in Males

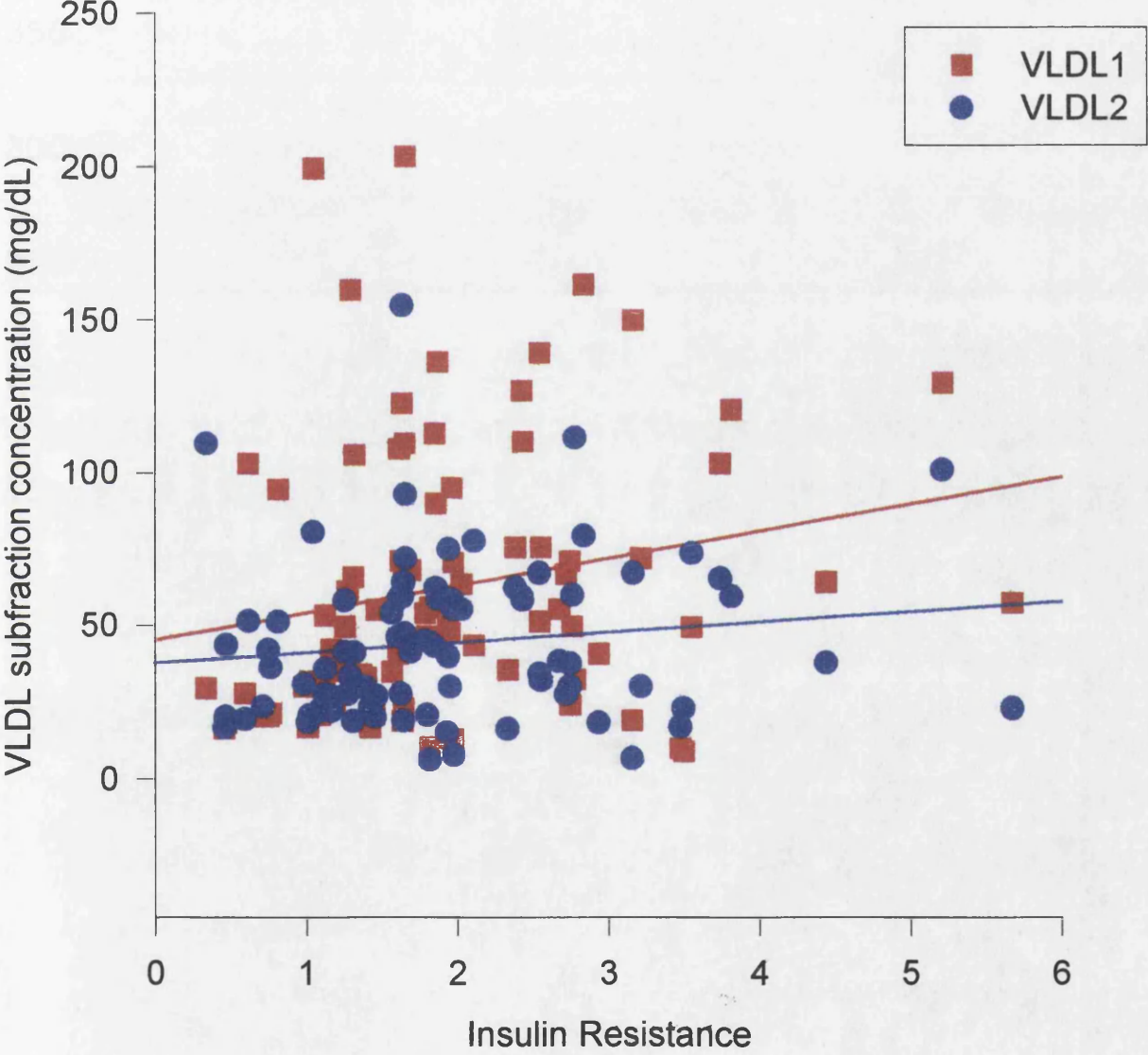


Fig 15: VLDL Subfractions vs Insulin Resistance in Females

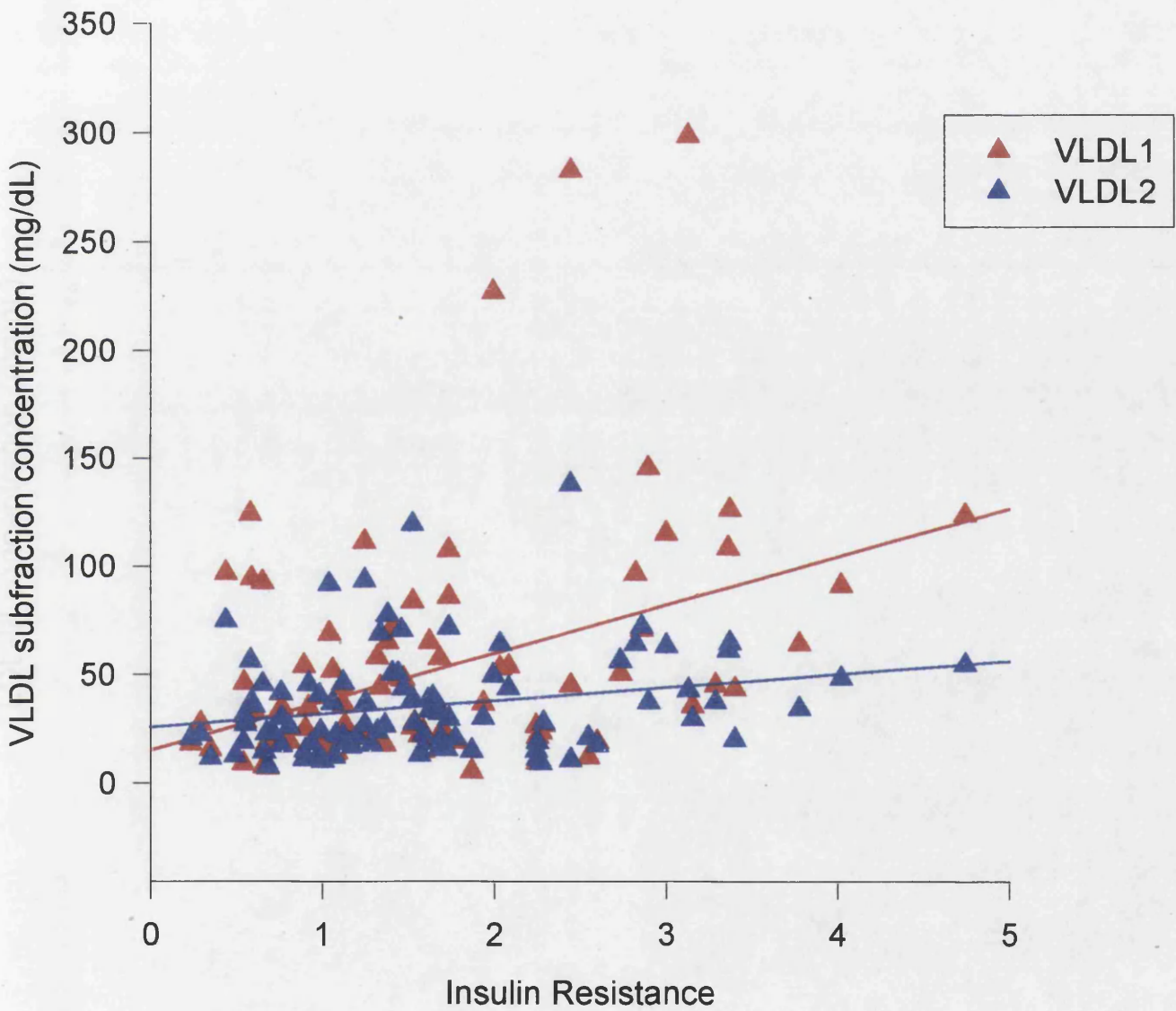
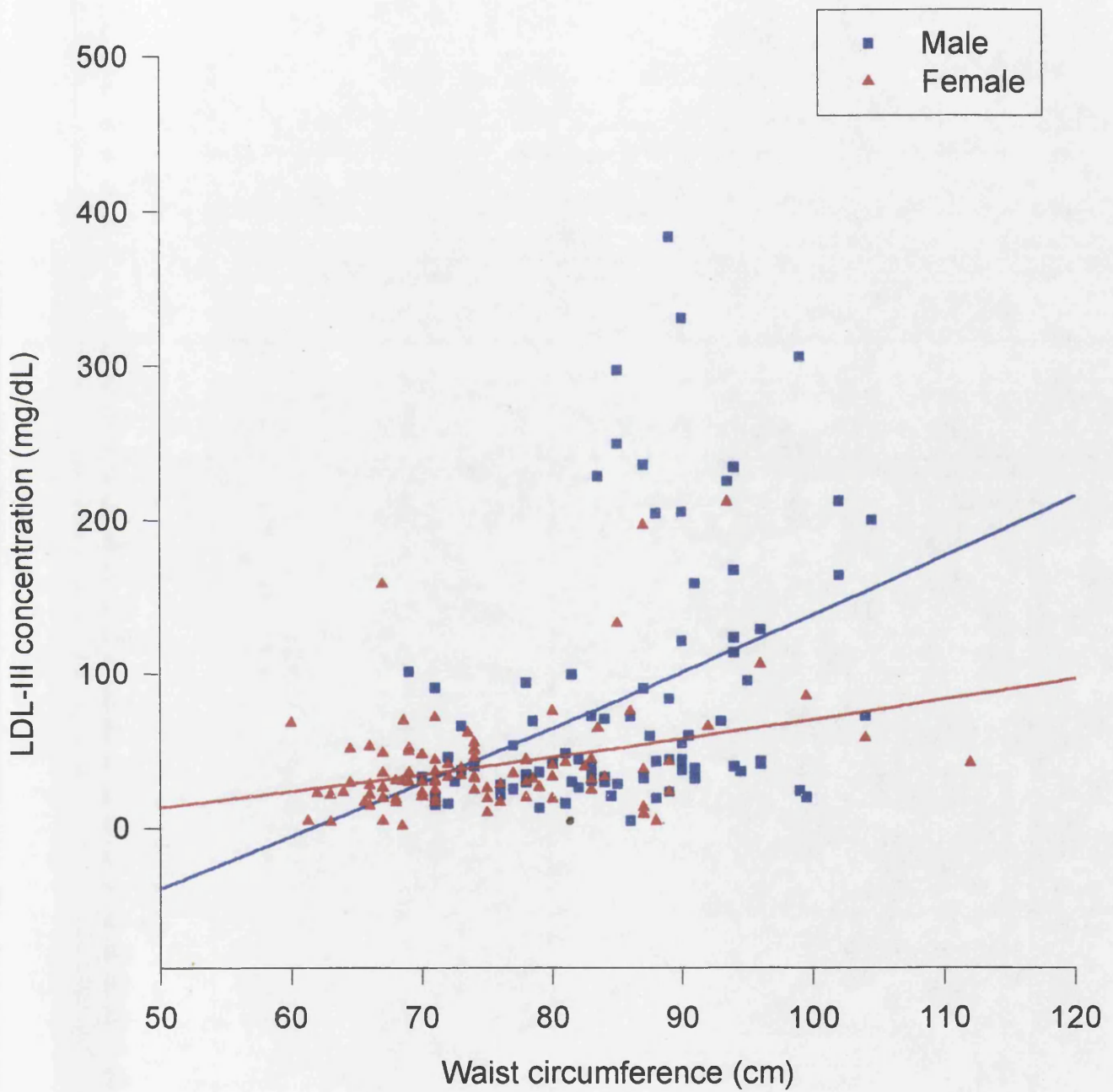


Fig 16 : LDL-III concentration vs Waist



5.1 Introduction

The concept of ischaemic heart disease (IHD) as the product of genetic and environmental factors is generally accepted but the relative contributions of heredity and environment have remained more obscure. A genetic-epidemiologic study undertaken in white Colorado population of 207 patients who had a myocardial infarction before age 55 years showed that heritability of IHD was 63% when monogenic forms of hyperlipoproteinaemia were included and 56% when they were excluded.²²³ Another study carried out in Finland showed that familial hyperlipidaemia was twice as common whilst familial hypertension was three times as common in families with premature coronary heart disease (CHD). This study concluded that familial aggregation of CHD was mediated by familial aggregation of hyperlipidaemia and hypertension.²²⁴ Family studies have consistently shown that there is a two and a half fold increase in risk of coronary deaths among first-degree relatives of coronary patients.^{225,226}

Cross sectional and epidemiological surveys have identified a number of genetic and environmental factors that predisposes subjects to CHD. These include raised plasma cholesterol (particularly the low density lipoprotein fraction), raised plasma triglyceride, reduced concentration of high density lipoprotein cholesterol and insulin resistance. In a typical study, middle-aged adults with these traits exhibit an increased incidence of disease over a 6 to 10 year period of observation. Studies on families with premature myocardial infarction showed 38% aggregation of premature CHD and 58% clustering of hyperlipoproteinaemia in siblings. Recent investigators, however, including the EC sponsored European Atherosclerosis Research study (EARS) project have indicated that lipid risk markers are expressed even in young adults (18- 25 years) in families where the father had a history of premature MI (< 55 years). Surprisingly, the inheritability of coronary risk factors has never been formally explored in families "at risk".

It is accepted that CHD is often an inherited condition but with the exception of familial hypercholesterolaemia, little has been done to discover what characterises the family at risk. Determination of lipid and apolipoprotein kinetics permit the identification of possible key components that when aberrant give rise to lipid-associated risk. Advice and therapy can then be offered in a rational and targeted fashion. The metabolic changes that underlie the plasma lipoprotein abnormalities seen in subjects with CHD have not been well defined and are in fact largely unexplored. This is due in part to the inherent difficulty of conducting metabolic experiments and the requirement until recently to use radioactive substances to measure kinetics. New advances in the use of stable isotope labelled tracers permit more rigorous examination of the inheritability of not just plasma lipid steady state levels but also lipid and lipoprotein production and elimination rates.

The hypothesis to be tested in the study described here are:

- a. that aberrations in lipid and glucose metabolism characterises the family at risk and are present in early adult life and
- b. the defects of lipid and glucose metabolism are synergistic and could possibly be due to a common inherited genetic defect (e.g. insulin resistance).

A total of 13 extended families consisting of 27 nuclear families with 70 related family members were recruited from probands with premature atherosclerosis. Similarly, a total of 6 families were recruited from friends of probands with premature atherosclerosis. Independent normals were also recruited from staff and friends of staff, as well as newspaper advertisements, giving a total of 137 normals.

5.2 Socio-economic Profile

There was no difference in the gender distribution between the families with CHD (all subsequent reference to such families will be known as "at risk" families) and the normal controls. Likewise, there was also no significant difference in the distribution of age groups between the two groups. The predominant education level in both groups was at secondary levels. There was an apparent larger proportion of the normals with tertiary and post-graduate education but this did not reach statistical significance. The distribution in education levels concurred with the distribution in the occupation, with the "at risk" families having the largest proportion of clerical staff whilst the control groups had a larger proportion of professionals. Again, the difference failed to achieve statistical significance.

The only socio-economic factor that differed significantly between the two groups were the smoking habits. The "at risk" families had a significantly higher number of smokers compared to the normal control groups. The normal controls also had a significantly larger proportion who had given up the smoking habit. Not only were those from "at risk" families more likely to smoke, but they were also likely to be heavier smokers. Individuals from "at risk" families smoked at least 13 cigarettes per day, whilst the normals smoked an average of 4 cigarettes per day. There was no difference with regards to the alcohol consumption nor the dietary habits. The lack of difference in alcohol consumption was not surprising, as the level of consumption was generally high and many do not view alcohol consumption as a risk factor for CHD apart from causing excessive weight gain.

The study questionnaire (Appendix 5) also surveyed changes in dietary habits over the last six months prior to the study as well as the reasons for the change. It was interesting to note that although at least one family member of the "at risk" families had had an acute myocardial event, yet this failed to result in any significant change in the dietary habits of the rest of the family. The frequency of exercise (as defined by the questionnaire) was also surveyed. The exercise pattern revealed that "at risk" families tended to exercise more frequently than those that came from normal families. There was however no statistically significant difference in the exercise habits between "at risk" families and normal controls. Slightly more than one-third in both groups did not exercise even once a week.

Table 17: Patient characteristics and Socio-economic status

Variable	Families with premature CHD	Normals	p
Sex: Male	48.6%	48.0%	
Female	51.4%	52.0%	
Age	35.6±1.52	37.2±10.8	

$\chi^2 = 1.987, DF = 1, P = 0.2$

Education level			
Secondary	53%	41%	
Collegè	26%	21%	
Tertiary	10%	23%	
Professional courses	10%	10%	
Post-graduate	1%	5%	

$\chi^2 = 2.338, DF = 4, P = 0.2$

Occupation.			
Professional	17%	38%	
Manual worker	24%	13%	
Clerical staff	29%	21%	
Students	10%	5%	
Housewife/unemployed	20%	23%	

$\chi^2 = 6.971, DF = 4, P > 0.5$

Smoking status			
Smoker	40.4%	22.7%	
Non-smoker	15.8%	13.6%	
Ex-smoker	43.8%	63.6%	

$\chi^2 = 7.238, DF = 2, P < 0.05$

No of cigarettes	12.9±1.7	4.2±0.7	<0.001
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Alcohol consumption			
Yes	74%	87%	
No	26%	13%	

Amount of alcohol	9.6u ±1.8	7.72u ±1.5	ns
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Dietary change		
Yes	40%	46%
No	60%	54%

$\chi^2 = 0.71, DF = 1, P = 0.5$

Exercise habits		
<once/ week	37.5%	40%
once/ week	25%	31.4%
2-3 times/ week	37.5%	28.6%

$\chi^2 = 0.863, DF = 2, P > 0.5$

Differences between the two groups were determined by chi-square test.

5.3 Anthropometric indices

For purposes of analysis of data, all the probands from the diseased families as well as the age matched control families were removed from the data pool. The spouses of the families with CHD were considered as normal controls whilst the rest of the family were considered as families at risk. The “at risk” families were found to be significantly shorter than the normal controls. However there was no difference in weight, body mass index, waist, hip circumference. Thus apart from the difference in height, the “at risk” families and the control groups were well matched in terms of anthropometric indices and markers of obesity. The systolic blood pressure did not show any significant difference between the groups but the diastolic blood pressure was lower in families with CHD compared to the normals. The reason for the lower diastolic blood pressure in the “at risk” families was unclear.

Table 18: Anthropometric indices and Blood pressure

Variable	Families with CHD (mean±SD)	Normals (mean±SD)	p
Height (m)	1.65±0.1	1.70±0.01	<0.001
Weight (kg)	67.4±12.1	70.6±12.1	ns
BMI (kg/m ²)	24.7±3.6	24.7±3.5	ns
Waist (cm)	80.0±11.2	80.3±10.3	ns
Hip (cm)	96.7±8.4	98.6±7.5	ns
WHR	0.82±0.1	0.81±0.1	ns
BP sys (mmHg)	123±16	123±14	ns
BP dias (mmHg)	75±10	80±9	<0.001

p value refer to significance of difference between the groups as determined by student's unpaired t-test. BMI; body mass index, WHR; waist to hip ratio.

5.4 Lipids and Lipoproteins in family members

There was no difference in plasma cholesterol level between the "at risk" families and the normal controls. However, the plasma triglyceride was significantly higher in the "at risk" families than in normals. The other adverse atherogenic profile found in the "at risk" families was the cholesterol/HDL ratio, which was significantly higher and the HDL cholesterol, which was significantly lower in such families compared to normals. There was no difference between the two groups with regards to VLDL or LDL cholesterol. In the lipoprotein subfraction distribution, the "at risk" families had significantly lower HDL₂ and HDL₃ levels whilst having higher LDL-II levels. It was also interesting to note that there was no difference in the buoyant LDL-I or the dense LDL-III subfractions. The VLDL₁ and VLDL₂, non esterified fatty acids (NEFA) and Apo B levels were also higher in the "at risk" families. The postheparin lipoprotein lipase (LPL) activity was significantly lower but there was no difference in hepatic lipase activity (HL). The lowered postheparin LPL activity in the "at risk" family members could result in inefficient clearance of post-meal chylomicrons and triglyceride, and thereby increasing risk for CHD. This will be discussed in detail in Chapter 6, when the effects of alimentary lipaemia is dealt with. Men have higher risk for CHD than women, and families with premature CHD are no exception. Hence, it was important to explore the differences between males and females with regards to CHD risks. The mean LDL-III concentration in males from the "at risk" families was 113.3 mg/dl plasma and this was above the threshold level of 100 mg lipoprotein/dL plasma, where Griffin et al had shown a 7 fold increased risk of CHD.¹³⁶ This was not so in the females from the "at risk" families, where the mean LDL-III concentration were similar to those in controls (table 20). There was no gender difference with regards to LDL-I subfractions. The differences in VLDL and HDL subfractions between the two groups remained significant when analysed by gender. The males from "at risk" families had significantly higher postheparin HL activity when compared to males from the controls. There was no difference in HL activity between the groups in the females.

Table 19: Comparing the Lipids and Lipoprotein subfraction Profile

Variable	Families with CHD (mean±sem)	Normals (mean±sem)	p
Cholesterol (mmol/L)	5.28±0.1	5.19±1.1	ns
Triglyceride (mmol/L)	1.26±0.1	1.09±0.1	<0.001
VLDL (mmol/L)	0.54±0.02	0.52±0.02	ns
LDL cholesterol (mmol/L)	3.52±0.1	3.30±0.1	ns
HDL cholesterol (mmol/L)	1.22±0.05	1.38±0.02	<0.001
Chol/HDL	4.6±0.2	4.0±0.2	<0.05

Lipoproteins and subfractions			
	(mean±sem)	(mean±sem)	p
HDL ₂ (mg/dL)	52.8±5.3	77.8±3.1	<0.001
HDL ₃ (mg/dL)	223.3±7.7	257.1±3.8	<0.001
LDL-I (mg/dL)	72.5±5.5	72.2±3.1	ns
LDL-II (mg/dL)	197.1±9.5	170.8±5.5	<0.01
LDL-III (mg/dL)	72.4±9.3	62.8±4.3	ns
VLDL ₁ (mg/dL)	58.4±4.9	53.6±4.6	<0.05
VLDL ₂ (mg/dL)	43.8±2.9	36.9±2.3	<0.01
Lp (a) (mg/dL)	29.8±5.2	27.7±2.5	ns
NEFA	0.50±0.03	0.37±0.02	<0.001
Apo A1	116.8±3.1	121.1±1.6	ns
Apo B	95.2±3.2	88.5±2.4	<0.05
LPL	4.04±0.23	5.30±0.28	<0.001
HL	15.83±1.1	14.56±0.9	ns

p value refer to significance of difference between the groups as determined by student's unpaired t-test
VLDL; very low density lipoprotein, LDL; low density lipoprotein, HDL; high density lipoprotein, LPL; lipoprotein lipase, HL; hepatic lipase

Table 20: Differences in LDL Subfractions and HL between males and female

	Families with CHD (mean±sem)	Normals (mean±sem)	p
Male LDL-I	65.5±9.1	55.7±3.2	ns
Female LDL-I	76.6±7.0	86.2±4.7	ns
Male LDL-III	113.3±20	86.9±8	ns
Female LDL-III	42.5±3.2	48.6±6.6	ns
Male HL activity	23.7±2.0	17.5±1.1	<0.01
Female HL activity	10.2±1.1	11.5±0.7	ns

p value refer to significance of difference between the groups as determined by student's unpaired t-test

5.5 Insulin Resistance in CHD families

The markers of insulin resistance were again indirect measures as in the analysis with the group of normal subjects (chapter 3). These markers included the fasting glucose and insulin, as well as insulin resistance (IR) calculated by the Homeostasis model assessment (HOMA). Only the fasting glucose levels were significantly higher in the "at risk" families. Other parameters of insulin resistance, including fasting insulin and IR failed to distinguish between the two groups.

Table 21: Comparing markers of insulin resistance

Variable	Families with CHD (mean±sem)	Normals (mean±sem)	p
Fasting glucose (mmol/L)	4.53±0.07	4.88±0.05	<0.001
Fasting insulin (mU/L)	8.23±0.57	8.06±0.37	ns
Insulin resistance	1.70±0.13	1.79±0.09	ns

p value refer to significance of difference between the groups as determined by student's unpaired t-test

5.6 Risk scoring

Plasma triglyceride, HDL cholesterol, dense LDL-III subfractions have been shown by others to be important risk factors for developing coronary artery disease and atherosclerosis. Likewise, there are also evidence from population studies²²⁷ that fasting hyperinsulinaemia is a risk marker. Smoking, which has been shown to increase risk for coronary events, is an important social characteristic of those at risk for premature atherosclerosis. Hence, the possibility of using the above 5 risk factors were explored to see if a scoring system could characterise the "at risk" families.

For plasma triglyceride, normality was taken as levels of 1.5 mmol/L and below, since it was shown in a previous chapter that when triglyceride exceeds 1.3 mmol/L, the VLDL and LDL subfraction distributions were affected. As for HDL cholesterol, normality was taken as being 1.0 mmol/L as studies like the Framingham study had shown that when levels are below 1.0 mmol/L in the presence of 'normal' cholesterol levels (less than 5.2 mmol/l), then the risk for coronary events can be comparable to those with cholesterol levels of greater than 7.8 mmol/L. LDL-III concentrations had been shown previously by Griffin et al, that at levels above 100 mg/dl plasma, there is a seven fold increase in coronary risk.¹³⁶ However, there was no available data on normal fasting insulin levels in the Scottish population. Hence, this was determined by taking the control group, removing extreme outliers and then deriving the mean plus 2 standard deviation for the fasting insulin. This was found to be 13.4 mU/L, and this was taken as being the upper limit of normality.

A score of 0 was given for triglyceride, LDL-III and insulin below the limits of normality and for HDL above 1.0 mmol/L. Likewise, scores of 1 were given for smokers and 0 were given for non smokers. A score of 1 was given for levels which exceeded the upper limits

of normality or HDL below 1.0 mmol/L. Using this scoring, the data was used to explore the profile of families at risk and what characterises such families. The probands were excluded (as in the other analysis) in this scoring system as they would accentuate the difference between the groups. The Chi-square test was used to test for differences between “at risk” families and controls. It was apparent that there was a significant difference between “at risk” families and the normal controls. The percentage of “at risk” families with the different scores for risk factors are summarised in Table 22. The majority of normal controls had 0 or only 1 risk factor whilst half of the “at risk” families had 2 or more risk factors. This difference in distribution of risk scores between “at risk” families and normal controls was determined by chi-square to be significant at $P < 0.001$. Risk factors often cluster together and may in fact be synergistic and the clustering of risk factors in such families was explored. Using a score of 3 or more risk factors as predictive of “at risk” families, 21% of such families had high risk score (Table 23). There was significant difference between the controls and “at risk” families. Using a risk score of 2 and above to represent the “at risk” families, there was even greater difference between the two groups. About 50% of family members from the “at risk” families had risk scores of at least 2 and above and this was contrasted sharply with the normal controls where the great majority (83.6%) had only 1 risk factor or none. A clustering of 2 or more risk factors characterises such families at risk and best distinguishes them from normal control groups.

Table 22: Coronary risk scoring between families and controls

Scoring	0	1	2	3	4	5
“At risk” families	29.8	26.3	22.8	10.5	7.1	3.5
Normal controls	63.0	20.7	8.6	6.0	1.7	0

$\chi^2 = 22.559$, $DF = 5$, $P < 0.001$. Difference in distribution of risk scores between the two groups were determined by chi-square test.

Table 23: Distinguishing between families at risks and normals through risk scoring

Scoring	≤ 2 risk factors	≥ 3 risk factors
“At risk” families (%)	79	21
Normal controls (%)	92.2	7.8

$\chi^2 = 6.333$, $DF = 1$, $P < 0.02$. Difference in distribution of risk scores between the two groups were determined by chi-square test.

	≤ 1 risk factor	≥ 2 risk factors
“At risk” families %	56.1	43.9
Normal controls %	83.6	16.4

$\chi^2 = 15.219$, $DF = 1$, $P < 0.001$. Difference in distribution of risk scores between the two groups were determined by chi-square test.

5.7 Discussion

The statistical analysis in this section of the study posed a considerable problem because the family members from the diseased groups were not independent individuals but were linked genetically. Ideally, each family should be analysed as one occurrence rather than each family member accounting for one occurrence. However, that would entail recruitment of large number of families for reasonable statistical analysis and was beyond the resources of one investigator. Furthermore, the normals represented a heterogeneous group with related family members from non-diseased families as well as unrelated independent individuals. Scotland with its high prevalence of CHD presented a problem in recruitment of those without any family history of coronary heart disease. Hence there was a need to include independent individuals with the 'normal' families, to make up a reasonable number of controls. Normals were taken as independent individuals for the purpose of generating reference values for the Scottish population. The "at risk" families were then examined for deviation from this normality. The limitations of interpreting the statistical analysis from this study to the general population are well recognised because of the aforesaid problems. Nonetheless, the data does contribute further to the understanding of risk factors in families with premature atherosclerosis.

There does not appear to be any significant difference between the "at risk" families and the normal controls with regards to the socio-economic status or anthropometry, apart from the "at risk" family members being shorter and having a lower diastolic pressure. There was a suggestion that the normals were likely to have gone further in their education and were likely to be holding some form of professional job. However, this could be due to selection bias during recruitment, as individuals from a better educated background and professionals were more likely to be better informed about coronary risk and hence were likely to respond to newspaper advertisements and other programs to enlist normal volunteers. They were also likely to have the resources to make changes to their lives should there be any abnormalities detected during screening, and hence were more likely to participate in studies of such nature. Whether this be due to selection bias or otherwise, it would not be surprising that the better educated were more health conscious and better informed, and hence have made changes to lifestyle. This was supported by the evidence that the smoking habits continued to be high in "at risk" families. Smoking habit was the only social habit that distinguished between "at risk" families and normal controls. Individuals from "at risk" families were more likely to be smokers and also smoked more heavily. What is even more interesting is the greater proportion of those from the normal control groups who had given up smoking, perhaps a testimony to the effectiveness of health education on the better informed. The converse is also true and those who come from poorer socio-economic background, may not be as responsive to health education. However, when the dietary habits were examined, it was surprising to note that there were no difference in dietary change between "at risk" families and normals. This could be due to the fact that the questionnaires collected information about change in dietary habits over the past 6 months prior to enlistment into the study. For many "at risk families", dietary habits could have changed significantly immediately after the myocardial events e.g. coronary bypass operation or myocardial infarction. Likewise, the control groups may have made changes in their diet beyond the 6 month period and hence were not reflected in the questionnaire. Personal experience with

counselling of the families however, suggested that many individuals from such families made no effort to change their lifestyle despite the occurrence of a major coronary event in the bread winner of the family.

The lipid profile of "at risk" families did not differ with regard to the total cholesterol, LDL cholesterol or VLDL cholesterol. The total cholesterol for both groups were surprisingly close to the recommended ideal limits of 5.2 mmol/L set by the European Atherosclerosis Society, although the mean cholesterol level for the Scottish population had been estimated at about 6.2 mmol/L. This may be due to the entry criteria for the study which effectively excluded probands with cholesterol level greater than 7.0 mmol/L and in so doing, excluded families which were likely to have familial hypercholesterolaemia. However, such "at risk" families had significantly higher plasma triglyceride and lower HDL cholesterol. This was consistent with our hypothesis that the insulin resistance in "at risk" families may account for the lipid abnormality characterising such families. The insulin resistance syndrome is characterised by the presence of central obesity, high plasma triglyceride, low HDL cholesterol and resistance to insulin mediated glucose disposal. Such syndromes are associated with impaired glucose metabolism as well as premature atherosclerosis. Recently, Reaven has also suggested that there may be a link between the insulin resistance syndrome and the atherogenic lipoprotein phenotype, since individuals with insulin resistance were also found to have predominance of dense LDL particles. However, not all individuals with insulin resistance syndrome demonstrated the dense LDL subfractions, and as explained in the preceding chapter, other factors may determine the manifestations of dense LDL subfractions. These include the HL and the need to exceed the triglyceride threshold of 1.5 mmol/L. Many of the offspring in the "at risk" families are young, and may not yet have manifested all the features of the insulin resistance syndrome. It would be interesting to follow up such "at risk" families to determine what proportion of them eventually develop premature atherosclerosis. The fact that the mean plasma triglyceride levels are higher than normals, although still less than 1.5 mmol/L, and the HDL cholesterol are low, suggest that there are early indications of the presence of insulin resistance syndrome in such "at risk" families. Furthermore, the fasting glucose was significantly lower and the fasting insulin was higher (although this failed to achieve statistical significance), indicate that there was presence of hyperinsulinaemia and hence the lower fasting glucose. This is again indirect evidence of impaired insulin mediated glucose disposal in the "at risk" families. Although the Homeostasis model (HOMA) proposed by Matthews et al was used, this failed to detect a difference between "at risk" families and the normals.

The lipoprotein subfraction patterns provided further insights into the "at risk" families. The HDL₂ and HDL₃ subfractions were significantly lower than normals, but this was not unexpected since the total HDL cholesterol was lower in "at risk" families. The VLDL₁ and VLDL₂ subfractions were significantly higher in the "at risk" families and we had demonstrated in the previous chapter that when the plasma triglyceride threshold is exceeded, triglyceride is preferentially carried as the larger VLDL₁ particles. Such triglyceride-rich lipoproteins are then subjected to neutral lipid exchange with LDL, to form dense LDL particles and smaller HDL₃ subfractions. This pattern of VLDL subfraction distribution was not unlike what had been previously demonstrated in non

insulin dependent diabetics. Although the mean LDL-III subfractions was higher than in normals, this failed to achieve statistical significance. The difference in LDL-III concentration was most apparent in the males but not in the females. The gender difference for the manifestations of dense LDL particles had been alluded to in the previous chapter. We know from Austin et al, that the manifestations of dense, LDL subfractions may be inherited in an autosomal dominant manner, but was also age dependent.¹¹⁰ Many of our "at risk" family members are either offsprings or younger siblings of probands. Hence they may not have manifested the full blown atherogenic lipoprotein phenotype yet. This was borne out by the observations that in family members below the age of 30 years, 13% had LDL-III greater than 100 mg/dl plasma whilst in those above 30 years, 31% had LDL-III greater than 100 mg/dl plasma (data not shown). This was contrasted against the group of normals where none of those below the age of 30 years had LDL-III greater than 100 mg/dl plasma. It would appear that with increasing age, many of the "at risk" family members would develop the atherogenic lipoprotein phenotype. What was unexpected from this study was the fact that some of the younger family members were demonstrating high levels of LDL-III at a very young age. Whilst it is true that the manifestations of the atherogenic lipoprotein phenotype is age dependent, those from "at risk" families may manifest the phenotype early.

Postheparin LPL activity was lower in the "at risk" families when compared to normals. How does this translate into increased coronary risk is unclear. One possibility could be that the lowered LPL activity could result in delayed clearance of chylomicrons after a meal and thus lead to higher plasma triglyceride levels. The resultant high triglyceride then leads to shift in the various subfraction distribution i.e. lower HDL cholesterol, lowered HDL₂ and HDL₃, raised LDL-III concentration. The post heparin hepatic lipase activity failed to show a significant difference between "at risk" families and normals although there was a suggestion that the activity was higher in "at risk" family members. This may be due to the fact that both males and females were analysed together and hence clouded the picture. This became obvious when the HL activity were analysed separately by gender. The males from "at risk" families had significantly higher hepatic lipase activity compared to normals. This was not so when the females were analysed and activity levels was similar between the groups. The significance of this differential HL activity and its relationship to coronary risk is presently unclear. Hepatic lipase may not an important rate limiting factor in those with high HL levels but may become a limiting factor in generating LDL-III, such as in the females with low HL activity.

What profiles the "at risk" population?

The characteristics of the family at risk were assessed using scoring systems. Using a score of 0 for risk factors within ideal limits and 1 for those above the limits, it was interesting to note that whether a total risk score of 2 or 3 and above were used, the "at risk" families had significantly higher scores. The difference in distribution of risk scores between the "at risk" families and the control groups were immediately apparent as those from "at risk" families were scattered across the spectrum of risk scores whilst the converse was true of the normal controls, which were mainly in the low end. The majority of normal controls had only one or no risk factors whilst about 50% of the "at risk" families had at least 2 or more risk factors. This difference between the two groups was

highly significant. Although it can be argued that the scoring was selective in that only triglyceride, HDL cholesterol, LDL-III, fasting insulin and smoking habits were used and that other factors may be just as important, it is suffice to say that the aim of this risk score is to try and ascertain whether it may profile the "at risk" families and not claim to be an all encompassing scoring system. Certainly, it left out LDL cholesterol or total cholesterol and markers of obesity. However, in the entry criteria for the study, probands with total cholesterol of less than 7.0 mmol/L was chosen to exclude families with familial hypercholesterolaemia and also to assess families at risk of premature atherosclerosis apart from total or LDL cholesterol. Body mass index and other markers of obesity such as waist or waist to hip ratio were also omitted because the two populations were evenly matched with regards to anthropometry. All socio-economic factors captured by the questionnaire were examined and only smoking habit was significantly different between the two groups. It had been shown previously to be an important coronary risk and was thus included in the risk scoring.

It would appear that in families with premature atherosclerosis (excluding familial hypercholesterolaemia), the constellation of risk factors encompassed in the insulin resistance syndrome or the atherogenic lipoprotein phenotype was a significant contributory factor. Individuals with insulin resistance syndrome often present with low HDL cholesterol, high triglyceride, hyperinsulinaemia or insulin resistance, impaired glucose metabolism and increased coronary risk. This was not unlike what had been demonstrated in some of the family members. The increased risk scores of "at risk" families would support the concept of an inherited element but what was even more interesting was that young adults in their early twenties from such families were manifesting high concentration of dense LDL-III and other traits of the atherogenic lipoprotein phenotype. It would be interesting to follow up members of such "at risk" families and document at a later stage, those that eventually develop coronary heart disease.

The data showed that those from families with premature atherosclerosis were more likely to be smokers and were also more likely to smoke heavily. Smoking is a learned social behaviour conditioned by our external environment. In population with high prevalence of smokers e.g. Japan, one would expect high incidence of coronary events. This is not so as borne out by the Japanese population, this is sometimes also known as the Japanese paradox. This could be due to the fact that the mean cholesterol levels of the Japanese population are within acceptable 'normal' limits and hence despite the high prevalence of smokers, they do not have high incidence of coronary heart disease. The Japanese suffer from other problems such as strokes and chronic lung problems as a result of the smoking habits but they do not have high incidence of coronary heart disease. It would appear that smoking in the presence of high mean cholesterol levels could then be extremely harmful and this may be so in the Scottish population with mean cholesterol levels at 6.2 mmol/L. Other social habits such as exercise habits and dietary changes did not differ significantly between the "at risk" families and the control groups. questionnaire. It was surprising that there was no difference in the exercise habits of the two groups. More than half of both groups claimed to exercise at least once a week and does suggest that the Scottish population as a whole may be more physically active and thus may not discriminate between "at risk" families and the normal controls. It would

appear that in families with higher lipids and lipoprotein risk factors, the effects of smoking may be an important contributory factor to the development of premature atherosclerosis. In summary, the family members from those with a history of premature atherosclerosis may have a clustering of risk factors that contribute to the overall risks. Such may not have very high levels of one risk factor but the different risk factors could act synergistically to contribute to overall risk.

5.8 Health Education and Lifestyle modifications

Family members with increased coronary risk required lifestyle modifications and these were usually done in the context of the family unit. Diseased probands were counselled together with their spouses and children regarding lifestyle and dietary changes. There was greater motivation for change in both probands and family members when they share similar risk profile. Changes in dietary habits and lifestyle within such "at risk" families must take place early. Making changes in adult life may be too late as the premature atherosclerosis may already be firmly established.

Chapter 6 Alimentary Lipaemia and Coronary Heart Disease

How much better it is to get wisdom than gold! And to get understanding is to be chosen rather than silver.
Proverbs 16:16

6.1 Introduction

The topic of the present chapter is the mechanism by which insulin controls postprandial triglyceride metabolism in particular postprandial lipaemia. The hypertriglyceridaemic response of an individual to a fatty meal is believed to play a key regulatory role in governing HDL cholesterol concentration and subfraction distribution.²²⁸

Lipoprotein lipase (LPL), the major enzyme catalysing the triglyceride hydrolysis in the circulation is reported to have a low activity in coronary heart disease patients.²²⁹ This would give rise to increased VLDL levels and an impaired ability to clear chylomicrons following a dietary fat load test, a phenomenon observed in previous studies of CHD patients from our laboratory.¹⁰⁸ LPL in adipose tissue and skeletal muscle is under strong hormonal control by insulin. A failure of insulin action in insulin resistance may lead to less LPL being elaborated in tissues, a reduced lipolytic capacity and the consequently exaggerated alimentary lipaemia.

Protocol

Subjects with coronary heart disease and age, sex and weight matched controls were administered a standardised fat meal to elicit a lipaemic response. Postheparin LPL activity was measured at the first visit in the fasting state and at the second visit, 6 hours after the ingestion of the fat meal. The measurement of LPL required the injection of heparin to displace the enzyme from its normal site attached to heparan sulphate on the capillary endothelial surface. This manoeuvre which interfered with triglyceride metabolism needed to be performed well before and at the end of a fat load test. The increment in LPL activity "6 hours post prandial minus fasting baseline" was compared to the extent of alimentary lipaemia, and between control and coronary heart disease patients. It was also compared to the insulinaemic response during the fat load test and to the insulinaemic response to an equivalent glucose load during a glucose tolerance test (containing the same amount of glucose as the fat meal) to ascertain if insulin alone elicited a rise in LPL, and if this rise was blunted in coronary heart disease patients. LPL and HL was also assayed in the non-heparinised state to monitor the natural release of the enzyme from capillary sites after the fat and glucose loads.

A total of 10 male, coronary heart disease patients and 7 matched controls were recruited for the study and they attended for three visits. The characteristics of the patients and controls are shown in Table 24. It can be seen that both the CHD patients and the controls were well matched with regard to their age, markers of obesity as well as the blood pressure. There were no difference between the two study groups when the baseline lipids and lipoprotein subfraction patterns (Table 25) were examined. Although the CHD patients tended to have higher LDL-III levels, this did not achieve statistical significance. The two groups also had similar baseline lipids and lipoprotein levels. Since normal subjects were recruited from friends of probands, it was assumed that they would be well

matched in socio-economic factors. These were not known to have any coronary heart disease nor have any symptoms pertaining to ischaemic heart disease. A rather unexpected finding was the high concentration of LDL-III amongst the control group but this may be a reflection of the plasma triglyceride which were equally high amongst this group of normal controls (see chapter 3). The small sample size could also account for this finding. The similarity in baseline triglyceride levels facilitates comparison of the fat load responses between the CHD and control groups since the fasting triglyceride level is otherwise a major determinant of postprandial lipaemia.

Table 24: Profile of the study groups

	CHD patients (mean ± SD)	Controls (mean ± SD)	p
Age	49.3±3.8	44.9±5.9	ns
Body mass index (kg/m ²)	28.7±4.0	26.8±4.9	ns
Waist (cm)	96.3±12.3	93.4±10.6	ns
Waist hip ratio	0.99±0.1	0.94±0.1	ns
Systolic BP (mmHg)	134±16	126±13	ns
Diastolic BP (mmHg)	80±10	84±10	ns

p value refers to the significance of difference between groups as determined by student's unpaired t-test

Table 25: Baseline Lipids and Lipoprotein Subfractions

	CHD patients	Controls	p
Cholesterol (mmol/L)	6.4±0.2	5.8±0.4	ns
Triglyceride (mmol/L)	1.8±0.3	1.7±0.4	ns
HDL cholesterol (mmol/L)	1.1±0.1	1.1±0.1	ns
HDL ₂ (mg/dL)	43.7±7.4	40.3±7.4	ns
HDL ₃ (mg/dL)	273.7±13	233.7±2.6	ns
LDL-I (mg/dL)	47.7±10	52.8±39	ns
LDL-II (mg/dL)	225.4±27	240.0±54	ns
LDL-III (mg/dL)	126.3±26	104.7±36	ns

HDL; high density lipoprotein, LDL; low density lipoprotein

p value refers to the significance of difference between groups as determined by student's unpaired t-test

6.2 Comparing the fat meal and the glucose meal

The standardised fat meal had been studied extensively by other investigators and is now increasingly accepted as one of the tools for profiling the patients at risk of premature atherosclerosis. The fat meal is believed to elicit a rise in circulating LPL levels as the binding of LPL to the endothelium is thought to be weakened by local fatty acid accumulation that may result from lipolysis of plasma triglycerides.²³³ Whether this rise in plasma LPL levels during the fat meal is of any physiological significance remains to be proven. The fat meal is also known to elicit an insulin response and it is still unclear

whether the hyperinsulinaemic response that occurs in CHD patients during the oral fat challenge, could be responsible for the rise in plasma LPL levels. Hence, this study sought to compare the response of the standardised fat meal to an equivalent amount of glucose in a non-fat meal (i.e. fat free yoghurt). This permitted an exploration of the effects of both free fatty acids, glucose and insulin on the responses observed.

In the standardised fat meal and the glucose meal pre-heparin lipases were measured to enable serial measurements during short term metabolic studies. However the metabolic relevance of pre-heparin lipases is unclear and were first studied by Eckel et al²³⁰ who found that pre and post heparin activities of LPL and HL were related and that the activities of both enzymes rose after an oral glucose load. Subsequent studies²³¹ confirmed that pre and post-heparin activities of HL but not LPL were correlated with each other and showed that the pre-heparin activities of both enzymes were associated with the LDL cholesterol levels. It was suggested that the enzymes bound to lipoprotein remnants.

The response between the standardised fat meal and glucose meals were first compared between CHD patients and normals. This did not show any significant difference between the two groups in any of the parameters and hence they were analysed together and results summarised in Table 26. The response for each of the parameter was plotted against time and the area under the response curve was calculated using the trapezoid rule. There were no significant difference in incremental areas (i.e. total area minus baseline area) and total areas, and in all subsequent discussions, area under the curves referred to total area obtained by the trapezoid rule. The fat meal obviously induced a much higher triglyceride response than the glucose meal. There was no difference in the glucose area under curve between the fat meal and the glucose meal presumably because the amount of glucose in the glucose meal was made equal to that found in the fat meal i.e. 22 g glucose. Despite the similarity in the glucose levels, it was interesting to note that there was a much higher insulin response with the fat meal than with the glucose meal, suggesting that the fat meal was a more potent stimulator of insulin secretion than glucose. The difference in insulin AUC achieved statistical significance. Circulating LPL was also significantly higher with the fat meal than with the glucose meal. This was presumably due to the weakening of binding of LPL to the endothelium as a result of accumulation of fatty acids or the presence of triglyceride rich particles. That there were no significant difference in free fatty acid levels between the meals does suggest that other mechanisms such as hyperinsulinaemia, may play a role in the rise in LPL levels. When the individual time points during the fat challenge were examined, there were again significant difference between the oral fat challenge and the glucose challenge. The insulin response did not differ between the two meals until the 2nd hour, and thereafter, the difference in insulin levels persisted until the 6th hour (Fig 17). The insulin response was also consistently higher throughout the oral fat meal than the glucose meal. The glucose on the other hand did not show any significant difference until the 4th and the 6th hours, where the glucose levels after the oral fat challenge remained higher (Fig 18). The preheparin LPL levels did not show any difference between the meals until the 4th hour and this persisted to the 6th hour (Fig 19). Free fatty acid levels were significantly different between the meals at the 1st and 2nd hour, but thereafter were similar between the meals (Fig 20). It would appear

that the LPL response patterned after the insulin response rather than the free fatty acid response.

Table 26: Differences between the fat meal and the glucose meal

	Fat meal (mean ± sem)	Glucose meal (mean ± sem)
AUC TG (mmol/l/hr)	22.1±2.4	11.2±1.3*
AUC Chol (mmol/l/hr)	37.2±1.4	36.8±1.7
AUC Insulin (mU/ml/hr)	157.1±18	90.7±16*
AUC Glucose (mmol/l/hr)	33.9±0.9	31.6±0.9
AUC LPL (nmolFA/mL/h ²)	108.5±9.7	78.8±8.7‡
AUC HL (nmolFA/mL/h ²)	301±30	302±33
AUC FFA	2.9±0.3	2.6±0.2

AUC: Area under curve, TG: Triglyceride, Chol: Cholesterol, LPL: Lipoprotein lipase, HL: Hepatic lipase, FFA: Free fatty acids

‡ p<0.01, *p<0.001, p value refers to the significance of difference between groups as determined by student's unpaired t-test.

6.3 Postheparin plasma lipases following fat and glucose meals

We have previously found that there was absence of any significant correlation between pre and post-heparin LPL activities²³² and indicates that the plasma and endothelial pools of enzyme are not in equilibrium. This dysequilibrium arises because the levels of LPL free in the circulation are kept low by avid uptake and degradation of the enzyme by the liver. Furthermore, the addition of heparin alters the enzyme's apparent specific activity,²³³ either by post-translational activation of the enzyme or by increasing the fraction of enzyme transferred to the vascular endothelium at the expense of that degraded at the site of synthesis.²³⁴ The results of the study (Table 27) showed that there were no significant difference in the post-heparin lipoprotein and hepatic lipases activities between that taken in the fasting state, after the oral fat challenge and after the glucose meal. Although the pre-heparin LPL were significantly higher after the fat loading, this did not affect the post-heparin LPL which were similar across all three visits. This is consistent with our previous finding that there is a lack of correlation between the pre and post-heparin lipase activities. The magnitude of pre-heparin lipoprotein lipases released during meals is obviously small compared to that during post-heparin lipase release.

Table 27: Comparing Post-heparin lipases between meals

	Fasting (mean±sem)	Post fat meal (mean±sem)	Post glucose meal (mean±sem)
LPL (µmolFA/mL/h)	24.6±2.5	27.3±2.8	23.5±2.1
HL (µmolFA/mL/h)	8.4±1.3	8.3±0.8	7.2±0.7

The results in Table 28 showed that the CHD patients had significantly higher post-heparin HL levels and lower LPL levels than the controls even in these small groups. In chapter 2, we demonstrated that there were significant differences in post-heparin hepatic lipase levels between males and females, that males had about twice the levels found in females. The hepatic lipase activities may be important determinants of the LDL subfraction distribution.

Table 28: Differences in post-heparin lipases between CHD patients and normals

	CHD patients (mean ± sem)	Controls (mean ±sem)	p
Hepatic lipase	30.6±2.6	16.0±2.6	<0.001
Lipoprotein lipase	10.4±1.9	5.6±0.9	<0.05

p value refers to the significance of difference between groups as determined by student's unpaired t-test

6.4 Differences between CHD patients and normals during the fat meal

A delayed clearance of postprandial lipoproteins from the plasma may play a role in the aetiology of premature coronary atherosclerosis. Disturbance in the removal of chylomicron remnants from plasma would expose the vascular bed more intensively to the atherogenic lipoproteins and accelerate the atherogenic process. Table 29 showed the difference in metabolic response to an oral fat loading between the patients with documented coronary artery disease and a group of matched controls. Bearing in mind that the baseline lipids and lipoproteins between the two groups were similar, the dynamic response to a fat meal challenge may give further clues to the atherogenic potential in patients with premature CHD. When the area under the triglyceride curve was examined, there was no significant difference between the two groups. When the triglyceride levels between the two groups were examined at each time point during the oral fat loading, there was also no significant difference. This may be explained by two possible reasons. Firstly, it could be because of the small sample size and thus failed to detect a difference between the groups. However, the second and most likely reason is that metabolic study was terminated at the end of 6 hours whilst other studies which demonstrated a difference between CHD positive and negative patients did so after the 6th hour and this difference often persisted to the 12th hour. The reason this metabolic study was terminated at the 6th hour was because the aim of the study was predominantly to assess the effects of the fat meal on the lipases and its interaction with insulin secretion rather than on triglyceride levels per se.

The free fatty acid levels were significantly higher in the CHD patients than in the normals. The levels of FFA were similar until the 4th hour where normals began to show declining levels whilst that in CHD patients remained elevated (Fig 21). The difference in FFA levels persisted even at the 6th hour. The pre-heparin LPL levels were also significantly different between the CHD patients and normal controls (Fig 22) but there were no difference in hepatic lipase levels. Interestingly, there were also significant difference in glucose levels between the two groups. Although the area under curve for insulin did not show any difference between the groups, the levels of insulin did differ

significantly at the 4th and the 6th hour (Fig 23). The higher glucose levels, together with insulin levels which were higher at every time point during the oral fat meal (Fig 23), suggest that the CHD patients were insulin resistant and hence demonstrated hyperinsulinaemic response. Resistance to insulin mediated glucose disposal would also result in higher glucose levels.

Table 29: Differences between CHD patients and normals during oral fat loading

	CHD patients (mean±sem)	Normals (mean±sem)
AUC TG (mmol/L/h)	23.7±2.8	19.6±4.2
AUC Chol (mmol/L/h)	38.2±1.6	35.8±2.7
AUC Insulin (mU/ml/h)	179.2±26	125.5±20
AUC Glucose (mmol/L/h)	35.8±0.9	31.3±1.0 [‡]
AUC FFA	3.75±0.3	1.70±0.2*
AUC LPL (nmol/FA/ml/h ²)	89.9±11	135.1±12 [‡]
AUC HL (nmol/FA/ml/h ²)	66.8±9.5	95.9±15

[‡]P<0.01, *P<0.001, p value refers to the significance of difference between groups as determined by student's unpaired t-test

6.5 Differences between CHD patients and normals during the glucose meal

The standardised fat meal is a convenient way of testing the triglyceride metabolic capacity but it contains a substantial amount of glucose. In the standardised fat meal the amount of glucose contained in the meal was about 22 gm. Eckel at al had previously shown that LPL rises even with a glucose load and not just a fat meal. If the mechanism of rise in pre-heparin LPL levels during an oral fat meal is due solely to the accumulation of free fatty acid disrupting the binding of LPL to the vascular endothelium, then this rise should not be demonstrated during a glucose meal. However, we know that LPL is also modulated by insulin, and in individuals with insulin resistance, such as those with premature atherosclerosis, the rise in insulin during an oral fat meal can be substantial, even if the amount of glucose contained in the fat meal may not be equivalent to the standard glucose tolerance test i.e. 75 g,. The results in Table 30 again showed that the CHD patients were insulin resistant, as they had hyperinsulinaemic response to the glucose meals, and the area under insulin curve were almost twice that in normal controls. Furthermore, the glucose area under curve in the CHD patients were also significantly higher than the normals (Fig 24), suggesting resistance to insulin mediated glucose disposal. Apart from the insulin and glucose, there were no difference between the two groups in the triglyceride, cholesterol, free fatty acid, and the lipases. The preheparin LPL levels were consistently lower in the CHD patients throughout all the time points during the glucose meal although this failed to achieve statistical significance. (Fig 25)

Table 30: Differences between CHD patients and Normals during glucose meal

	CHD patients (mean±sem)	Normals (mean±sem)
AUC TG (mmol/L/h)	12.2±1.5	9.7±2.1
AUC Chol (mmol/L/h)	37.9±1.6	34.9±3.5
AUC Insulin (mu/L/h)	116.3±23	54.1±10‡
AUC Glucose (mmol/L/h)	33.3±1.2	29.1±0.8†
AUC FFA	2.7±0.2	2.3±0.3
AUC LPL (nmol/FA/ml/h ²)	66.8±9.5	95.9±15
AUC HL (nmol/FA/ml/h ²)	281.5±31	332.0±69

† P<0.05 ‡ P<0.01, p value refers to the significance of difference between groups as determined by student's unpaired t-test

6.6 Discussion

Fasting plasma triglyceride levels have been associated with increased risk of coronary artery disease. However, its role has been shrouded in controversy because in multivariate analysis, its inverse association with HDL cholesterol eliminates triglyceride as a risk factor for coronary artery disease. The individual's triglyceride metabolic capacity, defined as the magnitude of lipaemia which occurs after a standardised oral fat load, is a strong determinant of his or her plasma HDL cholesterol, especially the HDL₂ cholesterol level. Patsch et al²³⁵ have suggested that the metabolism of triglycerides is a critical determinant of cholesterol metabolic routing and that a challenge of triglyceride metabolism such as the postprandial lipaemia may be a better indicator of coronary risks than fasting triglyceride per se. Whether a 'triglyceride tolerance test' should become a routine risk factor screening, particularly in those with premature atherosclerosis but with "normal" cholesterol and triglyceride level remains unclear.

This chapter set out to explore several issues, some of which still remain unclear after completion of the study. There was no doubt that the plasma triglyceride rose to a higher level and remained high for a longer period of time than age matched normal controls. The results of the present study supported this finding although statistical significance was not achieved because of the small sample size and also because of the termination of test at the 6th hour. We did not set out primarily to look at triglyceride levels postmeal but rather at other aspects of the "triglyceride tolerance test". One of the important areas explored in this study was the relationship between insulin resistance and the individual's capacity to handle triglyceride. The results suggested that the CHD patients were insulin resistant whether one looks at the area under the glucose and insulin curve or individual time points during the oral fat meal challenge. CHD patients also had significantly higher glucose and insulin levels during the glucose challenge carried in an almost fat-free yoghurt meal. The hyperinsulinaemic response and the consequently higher glucose suggest a resistance to insulin mediated glucose disposal, although this can only be inferred and not conclusively drawn, since glucose clamp studies or other parameters for directly assessing resistance were not carried out. During the fat meal, it was also

observed that the CHD patients had a significantly higher glucose levels compared to matched normals. The insulin response showed a parallel hyperinsulinaemic response although this did not achieve statistical significance except at the 4th and 6th hour. This is further support that the CHD patients are insulin resistant. Further, the greater insulin response during the fat meal suggested that free fatty acid and/or triglyceride was a more potent stimulator of insulin release than glucose alone.

The difference in postprandial lipaemia response between the CHD patients and normals revealed other interesting features. It was observed in Table 29, that the free fatty acid levels in CHD patients were more than twice that of normals, and this difference achieved statistical significance. On the other hand, the pre-heparin lipoprotein lipase levels were significantly lower in the CHD patients. If the rise in pre-heparin LPL levels was due only to dissociation from binding to vascular endothelium by fatty acids, then it would be obvious that the LPL levels must be higher in the CHD patients because of the greater accumulation of free fatty acid. This was however, not the case and other mechanisms must be at work to affect the pre-heparin lipoprotein lipase levels. Bearing in mind that the CHD patients were insulin resistant and had hyperinsulinaemic response to the fat meal and that insulin modulates LPL activities, it is possible that resistance of LPL activity to hormonal influence of insulin could result in lower levels during alimentary lipaemia. This would then contribute to the higher plasma triglycerides seen in CHD patients post fat meal challenge.

What then is the role of the standardised fat meal challenge in understanding lipid and lipoprotein metabolism? Notwithstanding the limitations mentioned above, I think that it is a useful tool in determining an individual's triglyceride metabolic capacity. The individual with an impaired ability to clear chylomicrons and triglyceride post meal would be at increased risk of premature atherosclerosis. The accumulation of triglyceride-rich lipoproteins would affect the lipoprotein subfraction distribution whilst the prolonged exposure of such lipid moiety would increase the chances of atherogenicity in the vascular endothelium. The category of patients who should be subjected to the "fat tolerance test" should thus include those with documented atherosclerosis but without hypercholesterolaemia or hypertriglyceridaemia.

Fig 17: Comparing Insulin response between meals

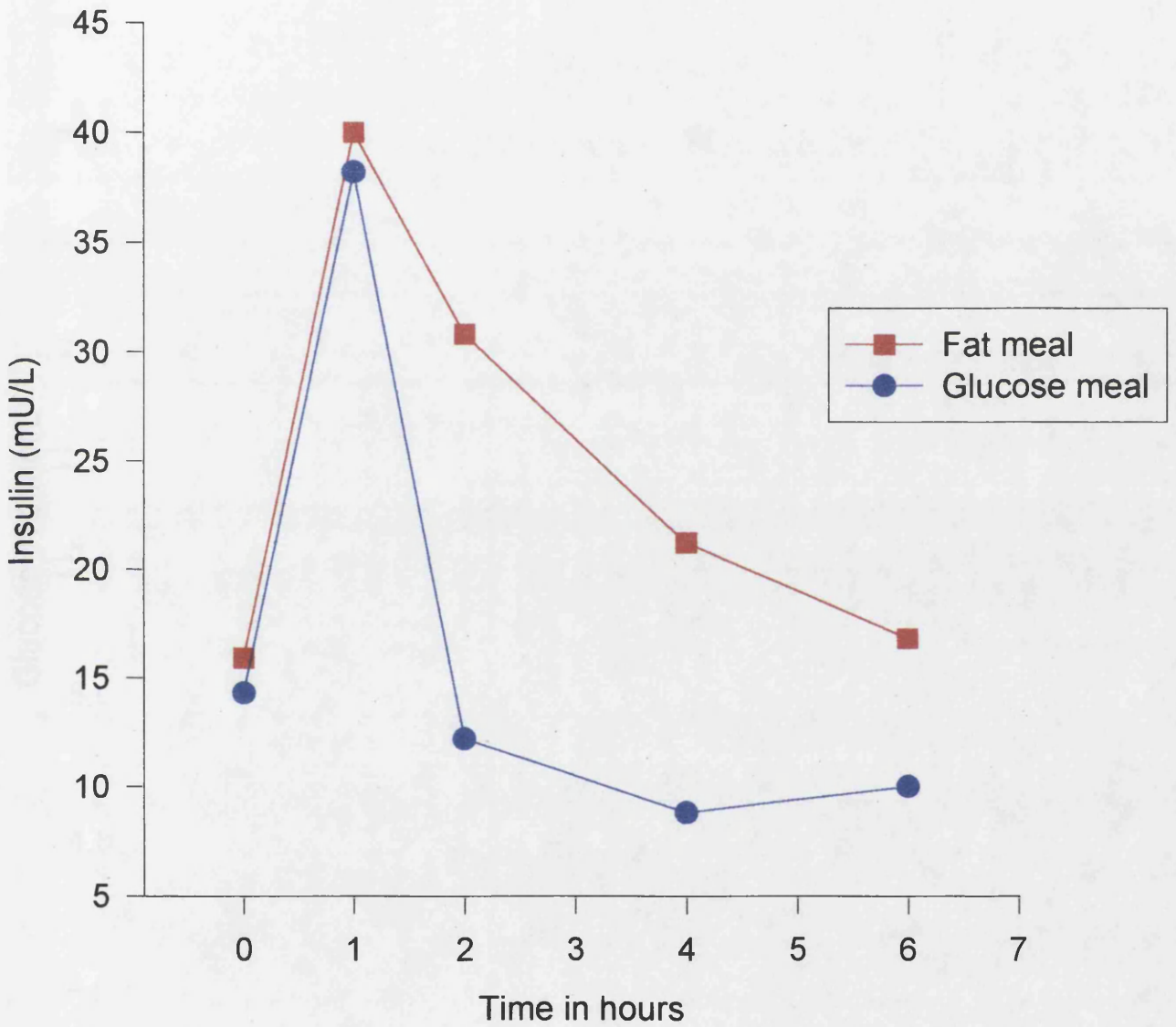


Fig 18: Comparing Glucose response between meals

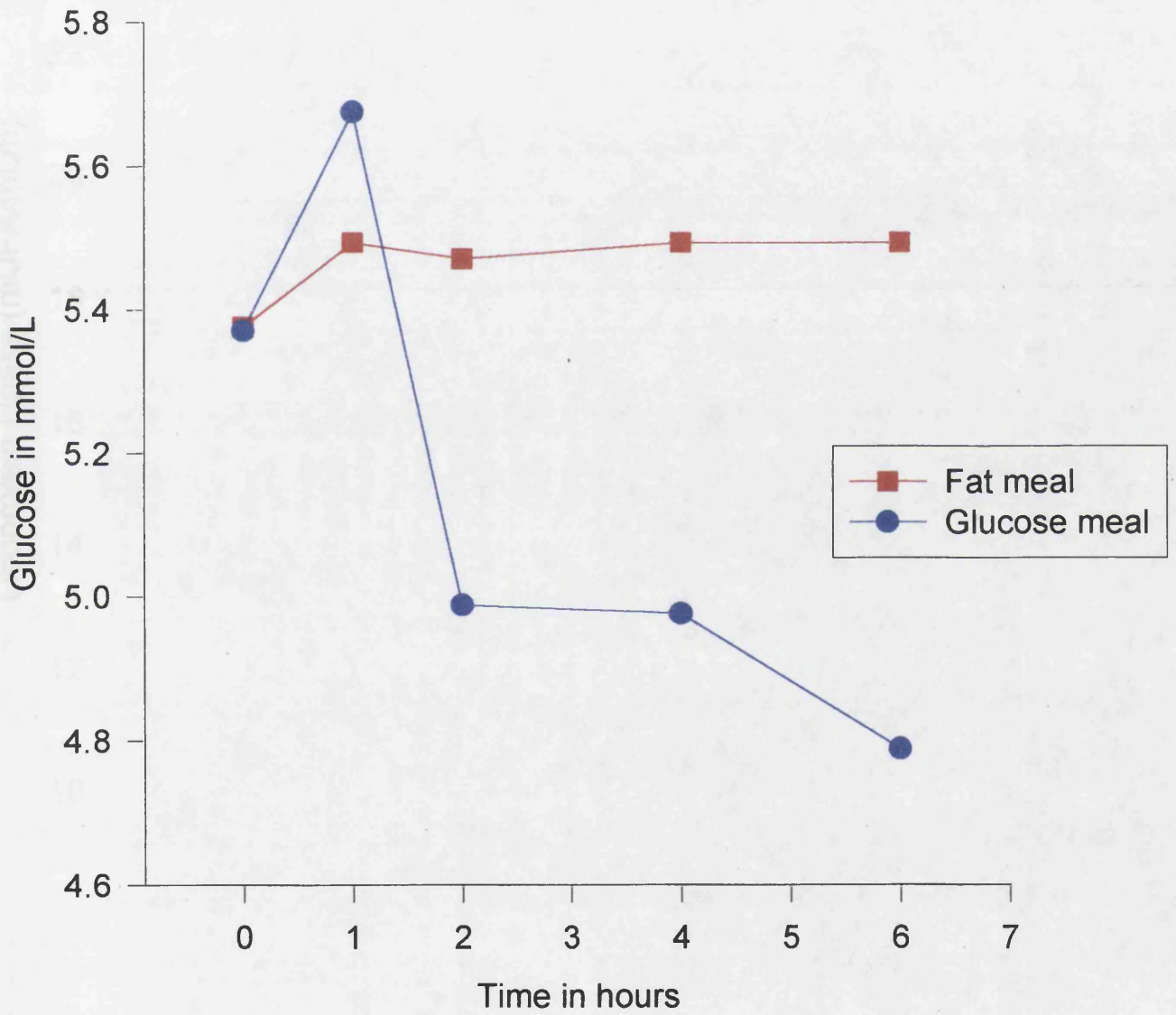


Fig 19: Comparing LPL response between meals

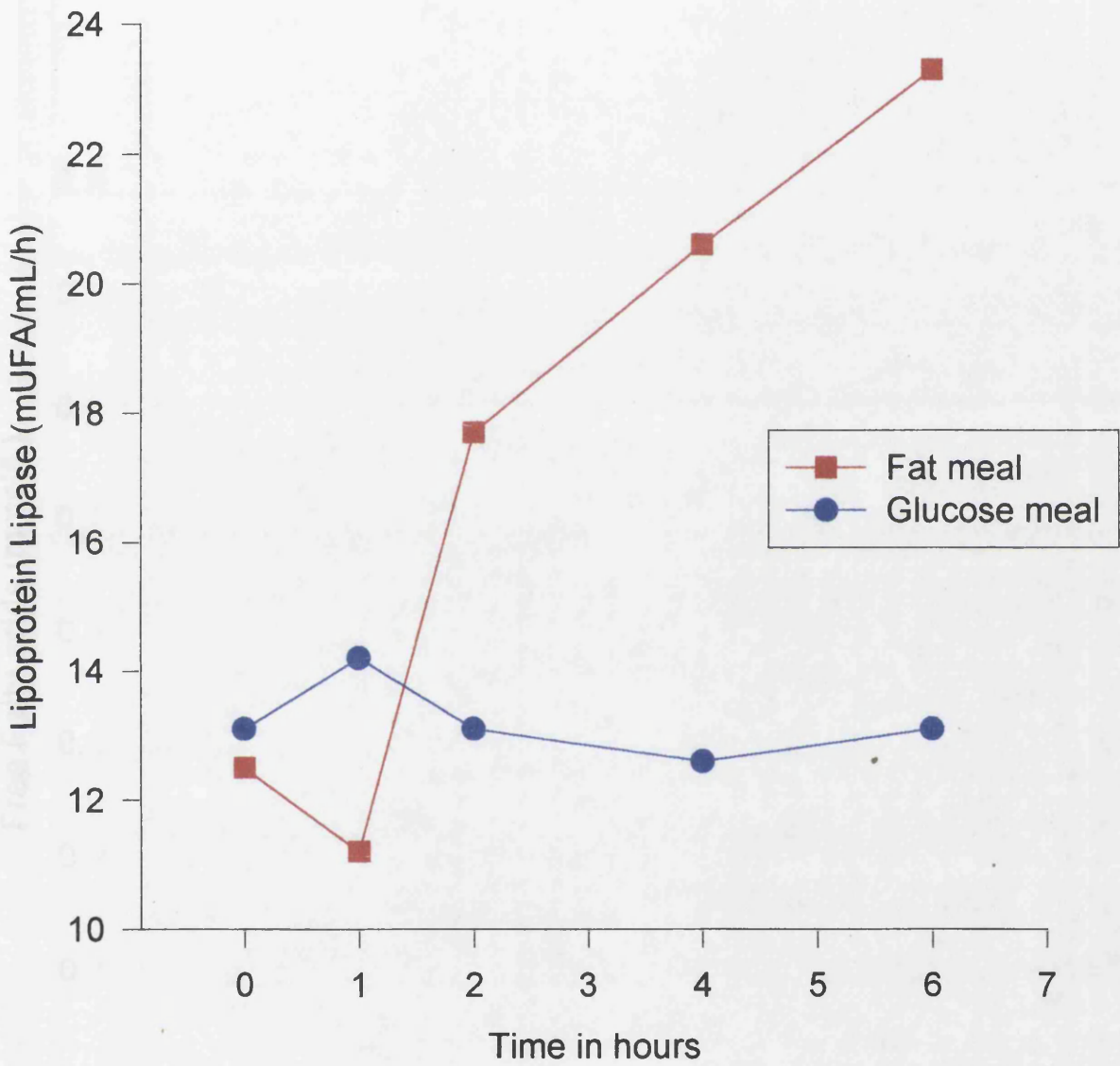


Fig 20: Comparing FFA response between meals

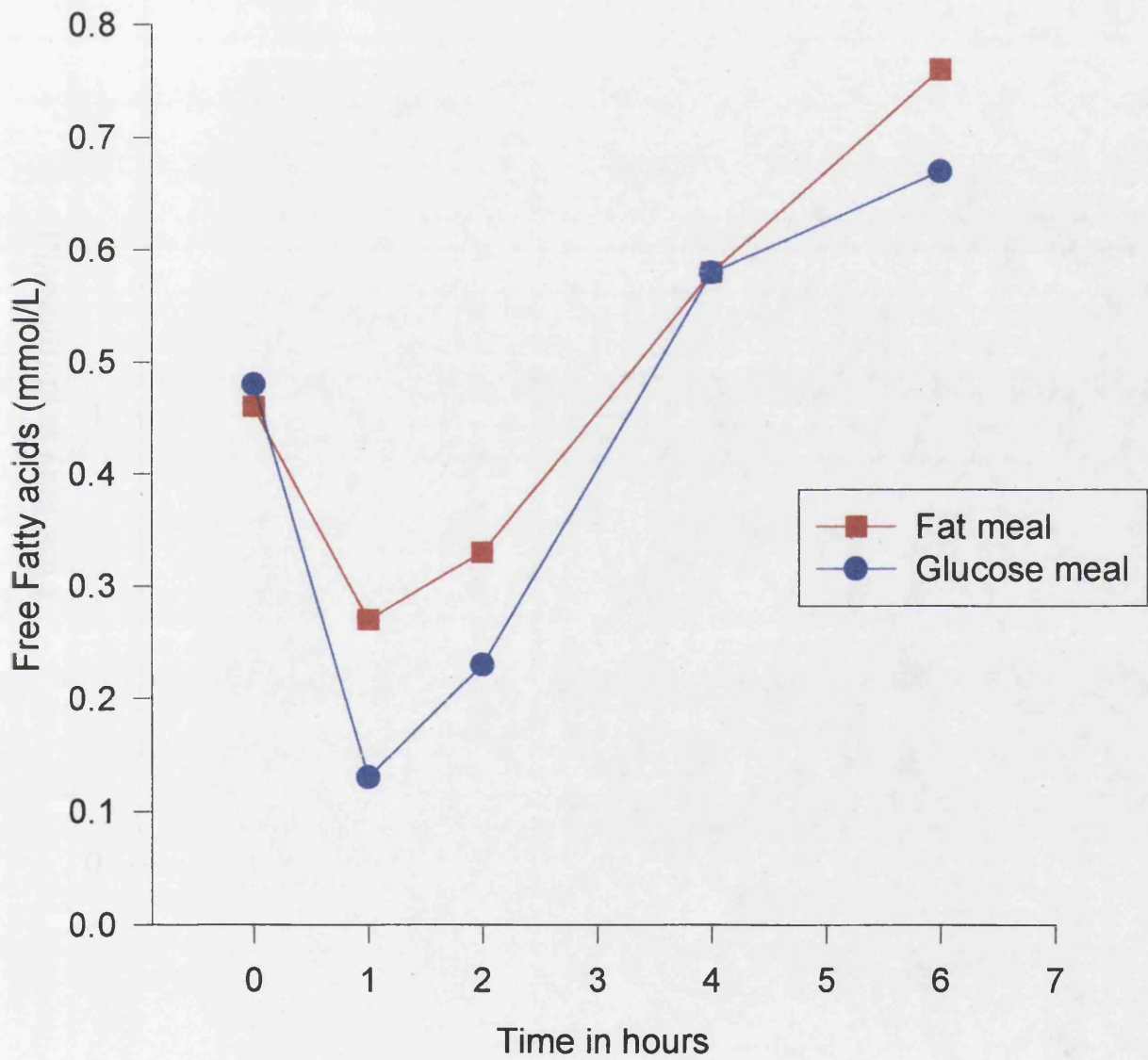


Fig 21: FFA response during fat meal challenge

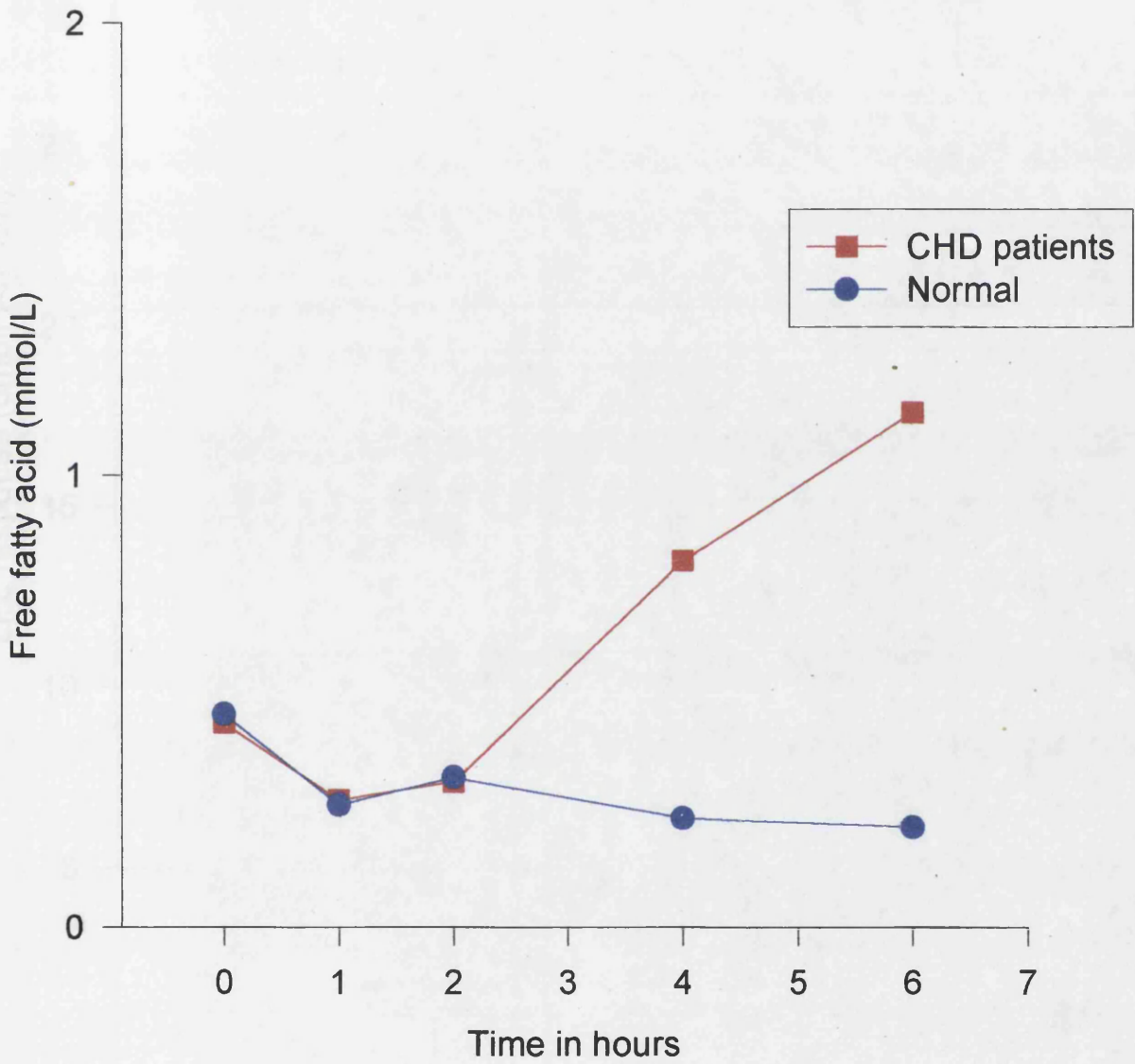


Fig 22: LPL response during fat meal

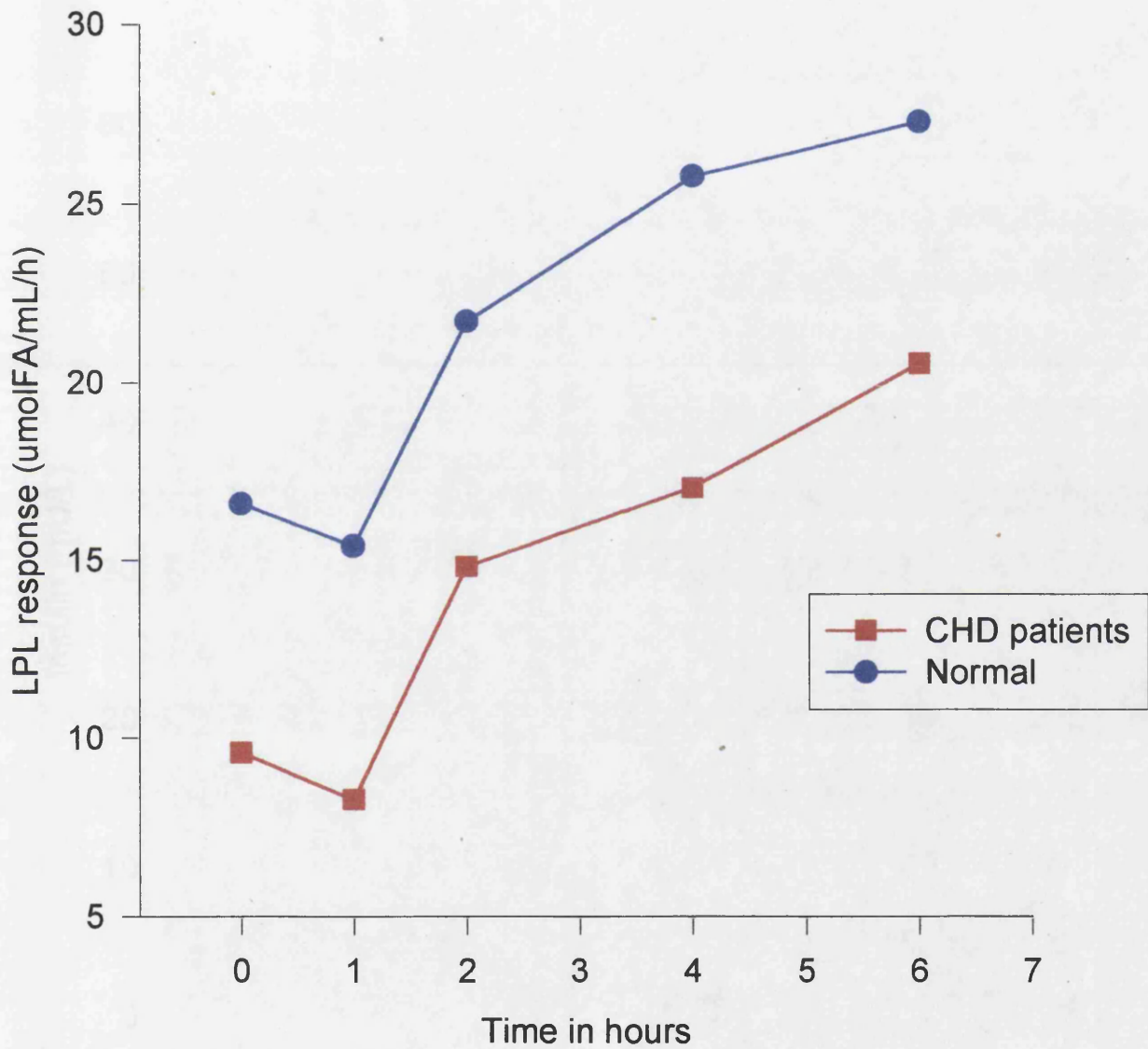


Fig 23: Insulin response during glucose meal

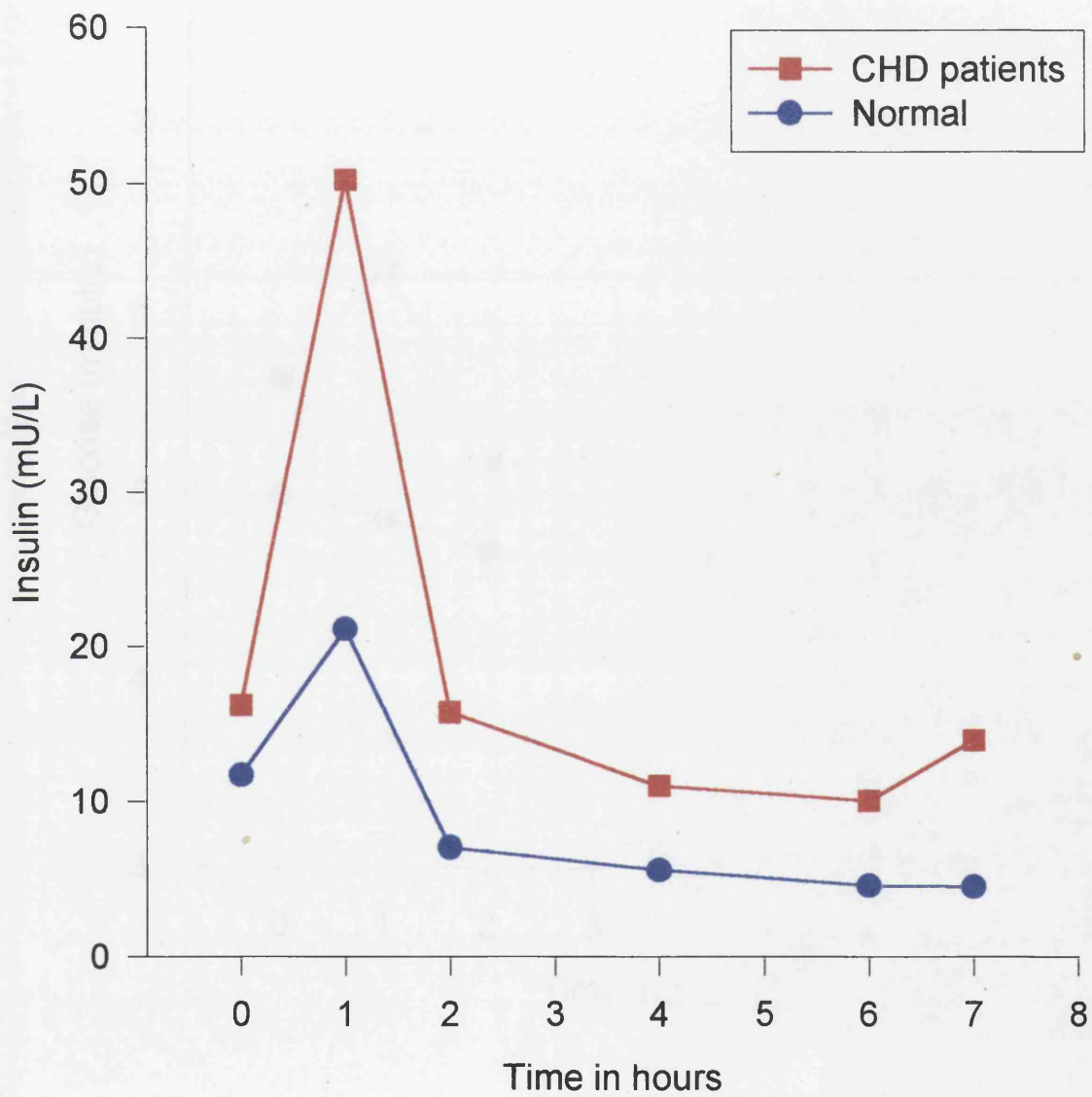


Fig 24: Glucose response during glucose meal

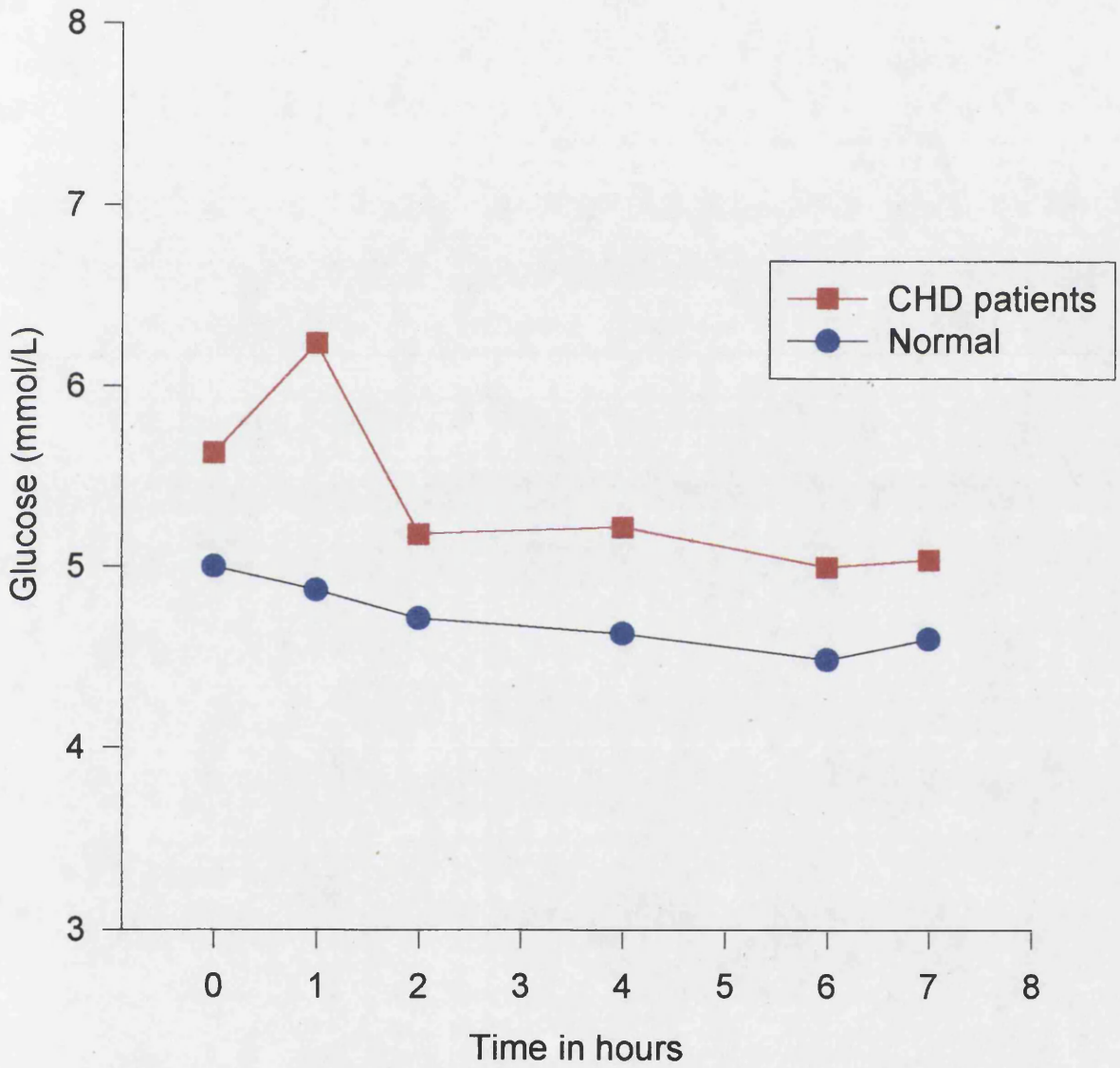
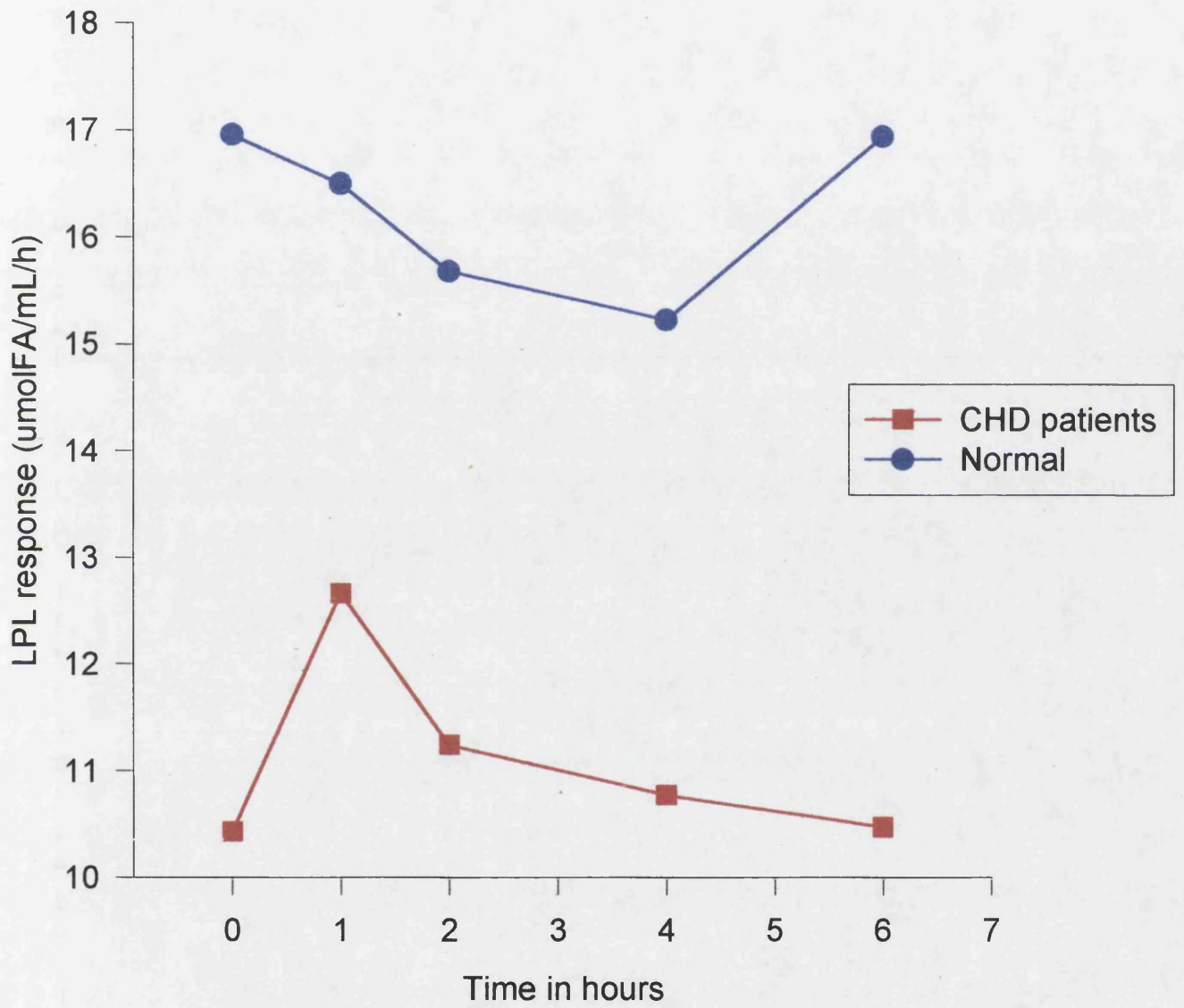


Fig 25: LPL response during glucose meal



Chapter 7 Risk Factors in Normal Males in Singapore

By knowledge the rooms are filled with all precious and pleasant riches.

Proverbs 24:4

7.1 Introduction

Singapore is a small island state which has a population of almost 2.7 million, consisting of 3 major ethnic groups i.e. Chinese, Malay and Indians. There are also many other groups represented, from Eurasians, Portuguese, Japanese, Thai, Sri Lankans, to various groups of European and American descent. Singapore is a city state and hence there is effectively no division into urban and non-urbanised areas. The majority live in high rise apartments and enjoy a good standard of living as well as medical care. It is unique in the epidemiological sense because the various ethnic groups live in a similar social economic milieu, unlike some countries where ethnic minorities may not enjoy the same privileges of life. The last population census showed that there are 77.6% Chinese, 14.2% Malay and 7.1% Indians with the other ethnic groups making up only 1.1% of the population. Although the standard of living is similar amongst the different races, yet it is interesting because many of the races have preserved their cultural heritage as well as dietary and eating habits. Hence it would not be surprising to find restaurants and other food outlet catering to the many differing tastes of both the local population and the increasingly large number of tourists from within the regions and other areas. The favourite pastime for many Singaporeans is dining and shopping. Obesity is an increasing problem, especially amongst the younger children because of the endless feasting and sedentary lifestyle. Many blame the problem on 'westernisation' of our diet, but that is being too simplistic. It is probably a combination of lifestyle changes and eating habits. Unfortunately, one of the prices we pay for such a lifestyle of good food and sedentary pleasures is increasing atherosclerosis, coronary heart disease and diabetes mellitus.

The prevalence of diseases amongst the various ethnic groups differ quite significantly.²³⁶ For example, Indians have the highest prevalence for diabetes mellitus followed by the Malays. Chinese have higher incidence of gut and colonic carcinomas than the other races. Even amongst the Chinese in Singapore, there are variation in disease patterns amongst the various dialect groups. The Cantonese (whose ancestors come from Canton, in China) have a much higher incidence of nasopharyngeal carcinoma whilst the Hokkiens (who have migrated from the province of Fujian, also in China) have higher prevalence of colonic carcinoma. This situation presents a fascination to the epidemiologist because it allows them to study the impact of both environmental and genetic factors on disease manifestations. Different ethnic groups living under similar conditions and yet having different susceptibility to diseases.

The last National Health Survey done in Singapore in 1992 (unpublished data),²³⁷ was a randomised survey involving more than 5000 males and females. This survey showed that the prevalence of diabetes had risen from 4.7% in 1984 to 8.6% in 1992 across all age groups (18-69 years of age). This same survey also showed that the mean cholesterol levels had fallen from 5.8 mmol/L to 5.3 mmol/L over the same period of time. This was largely through the efforts of intensive health education by the Health authorities. Unfortunately, this lowering of total cholesterol has not translated into a lowered

incidence of coronary heart disease. Two possible reasons were offered for this observation. Firstly, there could be a lag phase between the lowering of cholesterol and the benefits in terms of lowering of CHD risk. Furthermore, despite the lowered levels, this level of cholesterol in Singapore still represents one of the highest in the region as many of our neighbouring countries have levels well below 5.0 mmol/L. Secondly, whilst the risk from cholesterol may be decreasing, the risk of premature atherosclerosis is increasing from the high prevalence of diabetes mellitus, the majority of whom are type II diabetics with insulin resistance. Singapore now has the dubious honour of having one of the highest incidence of coronary heart disease amongst the East Asian countries (excluding the Indian Subcontinent). The National Health survey also revealed many other interesting facts, amongst which was the small number who exercised regularly (only 14%). Although we would like to believe that the prevalence of obesity has remained the same as in 1984 (4.3% in 1984 vs 4.4% in 1992), the truth is that this survey only measured body mass index as a marker of obesity but did not capture other indicators of central obesity such as waist to hip ratio or waist circumference, which many now believed to be a more important risk factor than just body mass index. Hence it was with this background in mind that a study was designed to examine coronary risk factors in a group of young, male Singaporeans without any history of coronary heart disease or diabetes mellitus.

7.2 Aims and objectives

The primary aim of the study was to determine the prevalence of the atherogenic lipoprotein phenotype and insulin resistance in a group of normal, healthy Singaporean males aged between 30 and 45 years old, with total cholesterol below 6.0 mmol/L. A secondary aim was to determine the relationship between the atherogenic lipoprotein phenotype and insulin resistance and whether the relationship between the two were similar to that demonstrated in the Scottish population. The age group of 30 to 45 years was chosen because it represented a young cohort most of whom should not have established CHD. The total cholesterol levels of 6.0 mmol/L was chosen as the entry criteria to exclude familial hypercholesterolaemia as well as the polygenic form of hypercholesterolaemia. With the high prevalence of diabetes mellitus and coronary heart disease in mind, it was felt important to determine what were the risk of coronary heart disease in a group of young male apart from high total or LDL cholesterol. This would also allow us to compare the group with an age matched group from the Scottish cohort. The working hypothesis was that the high prevalence of diabetes mellitus and/or insulin resistance would predispose to the triad of high plasma triglyceride, low HDL cholesterol and a predominance of dense, LDL particles.

7.3 Subject recruitment

Subjects were recruited from various sources, including hospital staff and their friends. Advertisements were also placed in the local newsletters at various worksites, so as to ensure a selection of males that were representative of the Singaporean males in this age group. Those who had no previous history of hypercholesterolaemia or hypertension, but were found at entry into study with total cholesterol of more than 6.0 mmol/L or had blood pressure of greater than 160/95 mmHg were excluded. Diabetes mellitus were excluded by sampling the fasting blood sugar, insulin and glycosylated haemoglobin and in borderline cases an oral glucose tolerance test was conducted. Consent was obtained from all and the study was approved by the hospital ethical committee, as well as the Medical Research Council of the Ministry of Health, Singapore. This study was also sponsored by a grant from the National Medical Research Council of Singapore. Results were made known to volunteers through the mail and advice were given to all who participated, regarding risk factors. Those with high risks were advised to seek medical consultation, and all volunteers were given the option to speak to the principal investigator to clarify any doubts that may arise from the results.

7.4 Profile of the Young, Male Singaporean

A total of 141 young, male Singaporeans without any previous history of hypertension, diabetes mellitus or coronary heart disease or dyslipidaemia were finally recruited. There were divided into various ethnic groups with 74.5% Chinese, 9.9% Malays, 9.2% Indians and 5.7% others and these were all Eurasians. The ethnic mix in the study sample were fairly similar to the population distribution of ethnic groups with the exception of the Eurasians, who were over-sampled for purposes of statistical evaluation. Amongst this group of males, there were 29% smokers and 71% non-smokers and this was again fairly similar to the National Health survey which showed that between 18 to 20% of the population smoked. The mean age of this group was 38 years, the body mass index (BMI) 23.9 kg/m² and waist to hip ratio(WHR) 0.87. In the last National Health survey (1992), obesity was defined as BMI of greater than 30 kg/m² whilst a BMI of between 25 and 30 kg/m² was considered overweight. Based on this classification, 2% of the study subjects were obese and 21% were overweight. This was again similar to the National survey with 5% and 21% respectively. The average systolic and diastolic blood pressures were well within normal limits. The volunteers came from diverse social backgrounds, from manual labourers to professionals, as well as differing educational levels. Care was taken to ensure there was no over representation from any particular social class.

Table 31: Anthropometry and Blood pressure

Variable	Mean±SD
Age in years	38±5.0
Body Mass index (kg/m ²)	23.9±2.7
Waist to hip ratio	0.87±0.05
Systolic blood pressure (mmHg)	121±11
Diastolic blood pressure (mmHg)	80±9

7.5 Lipid Profile and Insulin resistance

The mean cholesterol of the study sample was 5.3 mmol/L whilst the mean HDL cholesterol was 1.13 mmol/L. These values were consistent with the results of our National Health survey done in 1992, where the mean cholesterol and HDL cholesterol levels were 5.3 and 1.1 mmol/L respectively. Since the lipid levels between the study sample and the population means were similar, it was fair to assume that the study sample was representative of the normal, young, male Singaporeans. It was surprising that the mean plasma triglyceride levels were higher than expected, although the level of 1.87 mmol/L were still within the normal reference range. Unfortunately, the National Health survey did not report triglyceride levels and does not provide for comparison of the study sample with the population means.

LDL-I levels were lower than that observed in the Scottish population whilst the LDL-III levels were higher. The mean LDL-III concentration of the study sample was 97.5 mg lipoprotein/dL plasma, was very close to the level of 100 mg/dL, at which increased risk of atherogenesis is believed to occur. It was even more remarkable that amongst this group of normal males, 54% had LDL-III levels above 100 mg lipoproteins/dL plasma. The mean fasting glucose of 5.15 mmol/L and fasting insulin of 10.11 mU/L were unremarkable. However, the insulin resistance (IR) as calculated from the Homeostasis Model assessment appeared to be high, with mean levels of 2.38. This may not be surprising considering the high prevalence of diabetes mellitus in the Singapore population. In the Singapore study, three fasting samples of glucose and insulin were taken over a 15 minute period and the average of the three readings were used in the calculation of insulin resistance. This may be a better reflection of insulin resistance than either glucose or insulin alone.

Table 32: Lipid profile and markers of insulin resistance

Variable	Mean±sem
Total cholesterol(mmol/L)	5.3±0.1
Plasma triglyceride (mmol/L)	1.87±0.09
HDL cholesterol (mmol/L)	1.13±0.02
LDL cholesterol (mmol/L)	3.5±0.11
Apo A (g/L)	1.42±0.02
Apo B (g/L)	1.23±0.02
Lp (a) (mg/dL)	17.3±1.45
LDL-I (mg/dl)	32.0±3.0
LDL-II (mg/dL)	126.1±5.9
LDL-III (mg/dL)	97.9±7.4
Fasting glucose (mmol/L)	5.15±0.05
Fasting insulin (mU/L)	10.11±0.92
IR	2.38±0.25

HDL; high density lipoprotein, LDL; low density lipoprotein, IR; Insulin resistance

7.6 Factors predicting plasma lipids

In univariate analysis, plasma triglyceride, WHR, waist, IR and age exhibited significant correlation with total cholesterol. In general linear model, only triglyceride and waist to hip ratio remained significant predictors of cholesterol together accounting for almost 17% of the variability. For plasma triglyceride, there were significant correlations with total cholesterol, HDL cholesterol, BMI, WHR and waist. Using the general linear model involving all these factors, only cholesterol and HDL remained significant predictors, and together accounted for 23% of variability in plasma triglyceride levels. As for HDL, there were significant correlations with triglyceride, BMI, WHR and waist. In the general linear model, only plasma triglyceride remained as a significant predictor of HDL cholesterol levels. Apart from the significant univariate correlation with total cholesterol, it was surprising that IR did not show any correlation with the other lipid levels.

Table 33: Predictors of Lipids

Variable	Cholesterol		Triglyceride		HDL	
	Uni r ²	GLM r ²	Uni r ²	GLM r ²	Uni r ²	GLM r ²
Chol	-	-	13.4*	9.6*	0.2	2.2
TG	13.4*	11.3*	-	-	28.9*	15.5*
HDL	2.2	0.3	28.9*	13.7*	-	-
BMI	1.0	0.3	3.9‡	0.1	19.6*	0.9
WHR	26.2*	5.4*	6.2†	1.0	5.4†	0.1
Waist	59.6*	0.1	5.7*	1.1	14.4*	0.7
IR	3.2‡	0.02	2.1	0.4	1.1	0.1
Age	8.0†	0.3	2.2	0.7	0	0.1

BMI, body mass index; WHR, waist/hip ratio; HDL-C, high density lipoprotein-cholesterol; Chol, cholesterol; TG, triglyceride; IR, Insulin resistance

* P<0.001, † P<0.01, ‡ P<0.05 refer to the significance of the association r² (correlation coefficient squared) determined by linear regression in univariate analysis or by a multivariate analysis of variance General Linear Model including all of the tabled parameters.

7.7 Predictors of LDL Subfractions

The factors predicting LDL subfractions (Table 34 and 35) were next explored to determine if the relationship with the various lipids and anthropometric factors were similar to that observed in the Scottish population. Plasma triglyceride had a strong, negative correlation with LDL-I subfractions whilst HDL had a strong positive correlation. The other factor with significant negative, univariate correlation with LDL-I was BMI. In multiple regression using the general linear model, only plasma triglyceride and total cholesterol remained as significant predictors of LDL-I. Together, these accounted for 23% of the variability in LDL-I levels. There were significant correlations of triglyceride, cholesterol, HDL cholesterol, BMI and WHR with LDL-III levels. In the multiple regression using the general linear model, only three factors remained significant and these were triglyceride, total cholesterol and insulin resistance. Together they accounted for almost 17% of the variability of LDL-III.

In detailed analysis of the relationship of the LDL subfractions with plasma triglyceride, it was interesting to note that there were differences in this relationship between the Scottish population and the Singapore population. LDL-I levels showed a significant negative relationship with plasma triglyceride across the normal ranges of triglyceride levels (Fig 26). This was similar to that seen in the Scottish population and the fall off in LDL-I was similar in both population. The mean LDL-I fell from about 50 mg/dL at plasma triglyceride of 0.5 mmol/L to 20 mg/dL at a plasma triglyceride of 2.3 mmol/L, similar to that seen in the Scottish population, where the mean LDL-I fell from 100 mg/dL to 40 mg/dL over the same triglyceride range. However, it was obvious that over the same plasma triglyceride range, the Singapore males had lower LDL-I. The difference could be due to gender, since the Scottish study included both males and females, and females have higher levels of LDL-I. In a sub-analysis of the Scottish study, the mean LDL-I levels of age-matched Scottish males was 60 mg/dL versus 32 mg/dL in the Singaporean males. When the relationship of LDL-II with plasma triglyceride were examined, there were distinct differences. There was a strong negative relationship with plasma triglyceride demonstrated across the range of normal triglyceride levels, $r^2=24.8\%$, $p<0.001$. Unlike the group of Scottish males which showed a bimodal relationship, the Singapore males showed only a negative relationship with plasma triglyceride (Fig 27). In the Scottish population, there was a positive association of LDL-II with plasma triglyceride when the latter was less than 1.3 mmol/L and a negative relationship at higher plasma triglyceride levels. The relationship of LDL-III with plasma triglyceride showed a strong positive correlation across the range of plasma triglyceride levels, similar to that demonstrated in the Scottish study (Fig 28). The relationship of LDL-III with plasma triglyceride at level below 1.5 mmol/L had a regression equation of $LDL-III = 1.8 + 41.1TG$, and $r^2= 12.6$, $p<0.01$, whilst at triglyceride levels above 1.5 mmol/L, the relationship was represented by the equation $LDL-III = 41.6 + 4.4TG$, with $r^2 =18.4\%$ and $p<0.001$. A comparison of the slopes of the two equation using pairwise slopes and non parametric testing (Mann Whitney), showed significant difference in gradient of slopes ($p<0.001$).

Table 34: Predictors of LDL-I

Variable	Univariate: r^2 (%)	GLM: r^2 (%)
Triglyceride	25.9 (-)*	16.9*
Total cholesterol	0	5.9†
HDL cholesterol	20.2(+)*	1.4
Body mass index	5.0(-)‡	0.1
Waist to hip ratio	0.4(-)	0.02
Insulin resistance	0.6(-)	0.02

Table 35: Predictors of LDL-III

Variable	Univariate: r^2 (%)	GLM: r^2 (%)
Triglyceride	51.2(+)*	14.0*
Total cholesterol	25.4(+)*	1.2†
HDL cholesterol	21.9(-)*	1.2
Body mass index	7.8(+) [†]	0.3
Waist to hip ratio	10.0(+)*	0.2
Insulin resistance	0.1(+)	1.5‡

* $P < 0.001$, † $P < 0.01$, ‡ $P < 0.05$ refer to the significance of the association r^2 (correlation coefficient squared) determined by linear regression in univariate analysis or by a multivariate analysis of variance General Linear Model including all of the tabled parameters. The sign refer to the direction of the relationship.

7.8 Differences between the Ethnic groups

Differences in Anthropometry

The different ethnic groups were well matched in terms of the various parameters of anthropometry. None of the parameters used as indicators of obesity showed any significant differences between the ethnic groups. However, it would appear that Indians and Malays tended to be more obese than the Chinese or Others, who were predominantly Eurasians. The Indians were also more centrally obese than the other races. These results were again consistent with our National Health survey (1992), which showed that the Indians had the highest proportion of obese persons followed by the Malays. This is further support that the sample of males in this study is representative of the population of Singapore.

Table 36: Ethnic differences in anthropometry

Variable (mean±sem)	Chinese	Malays	Indians	Others
BMI(kg/m ²)	23.5±0.3	25.1±0.6	25.4±0.5	23.8±0.7
WHR	0.87±0.00	0.85±0.01	0.88±0.01	0.87±0.02
Waist(cm)	83.2±0.8	84.3±2.1	88.7±2.0	82.7±3.6

Differences in Plasma Lipids and LDL Subfractions

The different ethnic groups showed differences in their plasma lipids and LDL subfractions (Table 37 and 38). The mean cholesterol levels were highest in the Malays followed by the Indians, the Chinese and Others in descending order. The differences however, did not achieve statistical significance. The plasma triglyceride however, showed significant ethnic differences. Malays had the highest levels of triglyceride followed by Chinese and then the Indians. The Other races had the lowest level of triglyceride and there were significant differences between them and all the other ethnic groups. The levels of triglyceride found in the Other ethnic groups were similar to those in the Scottish male population. This was interesting as all the subjects in this group were Eurasians and were of mixed descent with the majority having some European ancestry. The HDL cholesterol demonstrated the reverse pattern, with the Malays having the lowest mean levels whilst the Chinese and Others had the highest levels. The differences in HDL cholesterol concentrations between the highest group, the Chinese, and the lowest group, the Malays, were statistically significant.

The LDL subfraction distribution were next explored and here again, there were significant ethnic differences. The Chinese males had the highest plasma concentrations of buoyant LDL-I subfractions whilst the Indians had the lowest levels. However, none of the differences achieved statistical significance. The plasma concentrations of LDL-I in the Chinese and the Others were similar. The levels of LDL-II did not pattern those seen in plasma lipids or LDL-I concentrations. LDL-II plasma concentrations were highest in the Others, followed by the Malays, Chinese and Indians. The significance of this is unclear. Concentrations of LDL-III were highest in the Malays. The Indians had the next highest mean concentrations and it was interesting to note that both the Malays and Indians had levels above 100 mg lipoproteins/dL plasma. The Chinese had concentration of LDL-III which were lower than the Indians although they had higher plasma triglyceride levels. The Others had the lowest mean concentrations of LDL-III, and again it was interesting to note that the levels in this group of normal males were similar to that demonstrated in the Scottish males. The difference between the highest group, the Malays, and the lowest group, the Others, achieved statistical significance.

Table 37: Ethnic differences in plasma lipids

Variable (mean±sem)	Chinese	Malays	Indians	Others
Chol(mmol/L)	5.28±0.1	5.44±0.2	5.35±0.3	5.18±0.3
TG(mmol/L)	1.92±0.1 ^{c†}	2.04±0.2 ^{d†}	1.67±0.2 ^{e‡}	1.17±0.2
HDL(mmol/L)	1.16±0.02 ^{a†}	0.98±0.04	1.05±0.14	1.16±0.08

a: difference between Chinese and Malays, b: difference between Chinese and Indians, c: difference between Chinese and Others, d: difference between Malays and Others, e: difference between Indians and others

*p<0.001, †p<0.01, ‡p<0.05, refers to the significance of difference between the ethnic groups by student's unpaired t-test

Table 38: Ethnic differences in LDL Subfractions

Variable (mean±sem)	Chinese	Malays	Indians	Others
LDL-I(mg/dL)	34.2±3.7	25.0±8.0	20.9±5.7	32.4±7.2
LDL-II(mg/dL)	123.4±7.1	134.7±1.9	115.4±11.0	156.3±19.0
LDL-III(mg/dL)	96.3±8.7	124.8±24.0 ^{d†}	100.5±25.0	64.3±16.0

a: difference between Chinese and Malays, b: difference between Chinese and Indians, c: difference between Chinese and Others, d: difference between Malays and Others, e: difference between Indians and others

*p<0.001, †p<0.01, ‡p<0.05, refers to the significance of difference between the ethnic groups by student's unpaired t-test

Differences in Insulin Resistance

In Singapore, there is a high prevalence of diabetes mellitus, particularly non-insulin dependent diabetes mellitus. However, we also know that the prevalence of diabetes mellitus amongst the different ethnic groups differ significantly, and hence it would be interesting to explore the ethnic difference in insulin resistance amongst the study groups. When the average fasting insulin were examined, the Malays and Indians had the highest levels followed by the Chinese. The Others had levels which were significantly lower when compared to the other 3 ethnic groups. The average fasting glucose levels did not demonstrate significant difference amongst the ethnic groups but the differences were similar to that noted in the fasting insulin. The IR, as calculated by the HOMA model, showed that Indians again had the highest level of insulin resistance, followed by the Malays and then the Chinese. The difference in IR between the Others and the 3 ethnic groups were statistically significant. However, there were no statistical difference in IR between the Chinese, Indians and Malays, although the trends were noted. The results showed that the Indians had the highest prevalence of insulin resistance followed by the Malays. The Others had the lowest prevalence of insulin resistance. Results of this study was again consistent with the National Health survey (1992), where the prevalence of diabetes mellitus was highest in the Indians and then the Malays, with the Chinese a close third. It was suggestive that the prevalence of insulin resistance could be high in the Asian population because of genetic influence and hence the difference between the Others, with their European ancestry and the other 3 ethnic groups of Asian descent.

The mean IR for the whole study group was 2.38. The group was then divided into 2 groups with one above the mean and the other group below the mean IR (Table 40). It became obvious that the group with high IR had significantly higher plasma triglyceride, lower HDL cholesterol, and higher levels of LDL-III. The insulin resistant group were also more obese as indicated by the higher BMI, as well as being more centrally obese, reflected by the WHR. This group with insulin resistant thus had the classical features of insulin resistance, represented by the more atherogenic lipid and lipoprotein profile as well as central obesity. This is further support of the close relationship between the insulin resistance syndrome and the atherogenic lipoprotein phenotype.

Table 39: Ethnic differences in Insulin Resistance

Variable (mean±sem)	Chinese	Malays	Indians	Others
Insulin(mU/L)	10.2±1.2 ^{c‡}	10.7±1.3 ^{d‡}	10.6±1.7 ^{e‡}	6.8±0.9
Glucose(mmol/L)	5.14±0.05	5.17±0.12	5.38±0.41	4.89±0.11
IR	2.41±0.3 ^{c‡}	2.48±0.3 ^{d‡}	2.60±0.5 ^{e‡}	1.48±0.2

a: difference between Chinese and Malays, b: difference between Chinese and Indians, c: difference between Chinese and Others, d: difference between Malays and Others, e: difference between Indians and others

*p<0.001, †p<0.01, ‡p<0.05, refers to the significance of difference between the ethnic groups by student's unpaired t-test. IR: Insulin resistance as derived by Homeostasis Model assessment.

Table 40: Difference between the Insulin Resistant and Non Insulin Resistant

Variable	Non-insulin resistant (mean±sem)	Insulin resistant (mean±sem)
Cholesterol(mmol/L)	5.22±0.1	5.38±0.1
Triglyceride(mmol/L)	1.66±0.12	2.23±0.16 [†]
HDL (mmol/L)	1.18±0.03	1.07±0.04 [‡]
LDL-I(mg/dL)	34.2±3.4	27.6±5.7
LDL-II(mg/dL)	136.9±6.9	105.7±11.0 [‡]
LDL-III(mg/dL)	84.5±8.0	123.3±14.0 [‡]
WHR	0.86±0.01	0.89±0.01 [†]
BMI(kg/m ²)	23.0±0.3	25.4±0.4*

*p<0.001, †p<0.01, ‡p<0.05, refers to the significance of difference between the insulin resistant and non insulin resistant groups by student's unpaired t-test

WHR; Waist to hip ratio; BMI; body mass index, LDL; low density lipoprotein

7.9 Discussion

The study was carried out in a group of young, male, Singaporean aged between 30 to 45 years old. The characteristics of this sampled group suggest that they were representative of the males in Singapore. The distribution of the various ethnic groups, the proportion of smokers as well as proportion of obese individuals were similar to the general population. The mean cholesterol and HDL cholesterol levels were also similar to the population means. As the other ethnic groups (apart from the Chinese, Malays and Indians) represented only 1% of the population, these were over-sampled in our study so as to enable ethnic differences to be studied. It was thus reasonable to assume that the sampled group was representative of the young male population in Singapore. The ethnic differences in BMI were similar to that found in the National Health survey, with Indian males being more obese and particularly that of central obesity. The problems of obesity amongst the Indians and Malays is strongly linked to their dietary habits. A typical Indian diet or Malay diet, in Singapore would consist of rice with generous servings of deep fried fish or meat cooked in coconut milk based curry. Often the rice is not steamed but also fried or cooked in oil with herbal flavourings. This is unlike the Chinese diet which consists mainly of steamed rice and much lesser amounts of curry and deep fried foods.

Hence it is not surprising to find a greater proportion of obese individuals amongst the Indians and Malays. However, the difference between the Chinese and the other races may not persist, as many Chinese are now abandoning their traditional carbohydrate based diet in preference for more meat and oil based diet. This is reflected in the growing problem of obesity amongst schoolchildren in Singapore.

The plasma lipids in the study subjects were again similar to the population means. In our subjects, the mean cholesterol level was 5.3 mmol/L, consistent with the findings of the National survey. Although this level of total cholesterol is similar to that found amongst Scottish male population, it is higher than in the United States and represents one of the highest levels in this region. The HDL cholesterol is again similar to that in the Scottish cohort but what was surprising was the high levels of fasting plasma triglyceride. The mean levels in our study was 1.87 mmol/L whilst the Scottish cohort had a mean plasma triglyceride level of only 1.2 mmol/L. The high levels of triglyceride may be an indication of the carbohydrate-based and oil-based diet or it could be an indication of underlying insulin resistance. When the lipid levels were analysed by ethnic groups, there were again differences. The Malays had the most atherogenic profile as the mean cholesterol and triglyceride levels were highest, whilst the HDL cholesterol levels were the lowest. The Indians had similar lipid profile and were also subjected to much higher coronary risk compared to the Chinese and Others. The adverse coronary profile in the Malays and Indians were further aggravated by the fact that they were generally more obese and particularly central obesity. What was particularly interesting was the very significant difference in lipid profile between the Eurasians (classified as others) and the other 3 major ethnic groups. This was despite the fact that the Eurasians have diet which are equally rich in oil and carbohydrate. Many Eurasians have Indian ancestry (apart from the European ancestry) and also have diets similar to the Indian community, and it was thus surprising to find that they had levels which were significantly lower than that seen in the Indians. However, it can be argued that because of the small sample size and the over-representation of the Eurasians in this study, the difference could simply be a sampling error. However, the differences in the lipoprotein subfraction distribution lends support to the fact that this difference may be real and not simply a sampling error.

Data in table 34 and 35 showed the various predictors of LDL-I and LDL-III respectively. The plasma triglyceride, HDL cholesterol and BMI all showed significant univariate correlation with LDL-I concentration. In multivariate analysis, only plasma triglyceride and cholesterol remained significant predictors and together predicted 23% of variability in LDL-I concentrations. In LDL-III, the factors which had significant univariate correlation were plasma triglyceride, cholesterol, WHR, BMI as well as waist circumference. In the multivariate analysis, the plasma triglyceride, cholesterol as well as IR remained as significant predictors of the variability in LDL-III. Together, they accounted for about 17% of the variability of LDL-III. There were differences between the different ethnic groups. The Indians and the Malays had higher plasma concentrations of LDL-III and lower levels of the buoyant LDL-I. It was interesting to note that in this group of 'normal' males, the Indians and Malay had mean concentration of LDL-III which exceeded 100 mg/dL. Although the Chinese males had lower LDL-III concentrations, than either the Malay or Indians, the mean concentration of LDL-III was

very close to 100 mg/dL. There were no significant difference in LDL-III concentrations amongst the 3 major ethnic group. In contrast, the Eurasians had mean LDL-III concentrations which were significantly lower than the other 3 ethnic groups. In fact, this group had plasma concentrations of LDL-III which were similar to that in the Scottish male cohorts.

The relationship of the LDL subfractions with plasma triglyceride across the normal range were explored. As the plasma triglyceride increases, the concentration of LDL-I fell as demonstrated by Figure 26. This relationship of plasma triglyceride with LDL-I is similar to that observed in the Scottish population except that the gradient of slope was much steeper. In the relationship of plasma triglyceride with LDL-II, there were significant difference from the Scottish cohort. Unlike the bimodal response seen in Scottish males, the Singapore males showed a strong negative correlation with plasma triglyceride (fig 27). Finally, the LDL-III relationship with plasma triglyceride were noted to be similar to that seen in the Scottish cohort, demonstrating a sharper increase in LDL-III levels when triglyceride exceeded 1.5 mmol/L. The difference in LDL subfraction relationship with plasma triglyceride between the Scottish cohort and the Singapore cohort is presently unclear but may be a reflection of the higher prevalence of insulin resistance and/or atherogenic lipoprotein phenotype in our population.

As mentioned in the preceding paragraphs, most of those classified under others in our population survey would be Eurasians and in this present study, all were Eurasians. The Eurasians are of mixed descent but many have European ancestry together with some Indian ancestry. There appears to be a clear distinction between those who are predominantly of Asian origin ie. Chinese, Malays and Indians, and those who have some European descent. It can be seen in the differences in plasma triglyceride, HDL cholesterol and LDL subfractions between the Eurasians and the 3 ethnic groups. Austin et al had previously suggested that the atherogenic lipoprotein phenotype occurs in about 30% of the population,¹³ but if this sampled group were representative of the general population, then the prevalence of the ALP may be much higher in Singapore. This study found that 54% of the males in this study had LDL-III above 100 mg lipoprotein/dL plasma, if all ethnic groups were considered together.

The markers of insulin resistance in this Singapore study were similar to those used in the Scottish cohort, with the exception that three fasting samples of glucose and insulin were taken to minimise the limitations of the use of HOMA model, as mentioned previously in chapter 4. There are no normal values for fasting insulin in our population but the mean fasting insulin in this group of males was noted to be higher than the Scottish cohort. Likewise, the IR, calculated from the HOMA model appeared to be higher than the Scottish cohort. This is not surprising, since insulin resistance is closely associated with diabetes mellitus, especially type II non-insulin dependent diabetes mellitus. The ethnic groups again showed differences with regards to the fasting insulin and IR. Indians had the highest levels of IR, followed by the Malays and then the Chinese. Likewise, the fasting insulin was highest in both Malays and Indians with the Chinese a close third. What is interesting is the clear distinction between these three ethnic groups and the Eurasians. If the presence of diabetes mellitus and insulin resistance has a strong genetic component, then it would not be surprising to find the high levels of insulin resistance

amongst individuals of Asian origin. This pattern of insulin resistance is similar to the prevalence of diabetes mellitus in Singapore, which is highest amongst the Indians (12.8%), followed by Malays (9.3%) and then the Chinese (8.0%).

The results in table 40 summarises the characteristics of the insulin resistant individual. Such were more likely to have high plasma triglyceride, low HDL cholesterol. They would also have lower levels of buoyant LDL-I and LDL-II whilst having significantly higher concentration of dense, LDL-III. They are also more likely to be obese, especially central obesity. The Singapore study also confirms the studies done in Scotland showing the close relationship between the insulin resistance syndrome and the atherogenic lipoprotein phenotype. It also demonstrated significant ethnic difference in lipid and lipoprotein subfraction profiles in population living under similar social environment. The ethnic difference may be the result of a combination of dietary and genetic influence.

Fig 26: LDL-I concentration vs Triglyceride

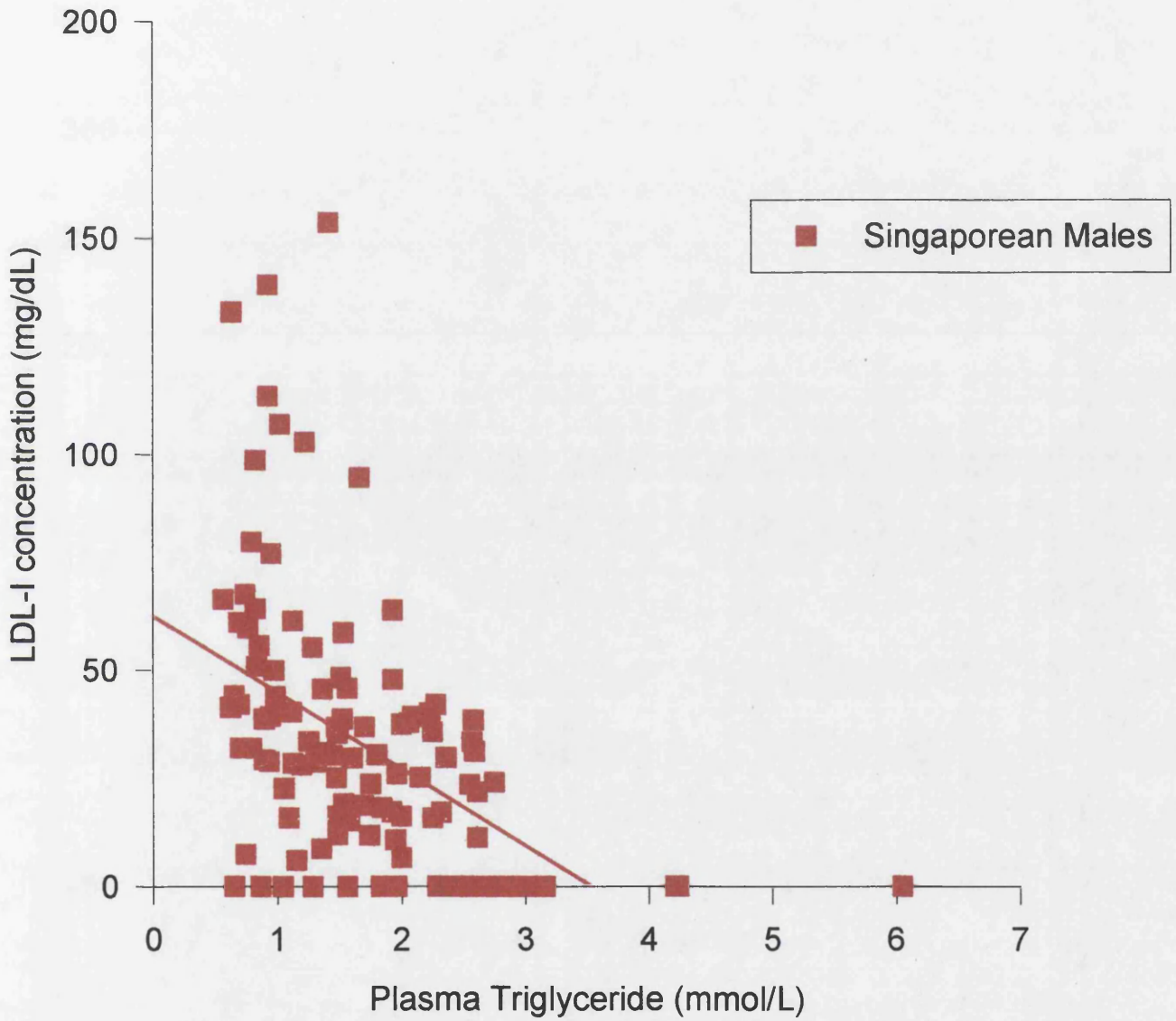


Fig 27: LDL-II concentration vs Triglyceride

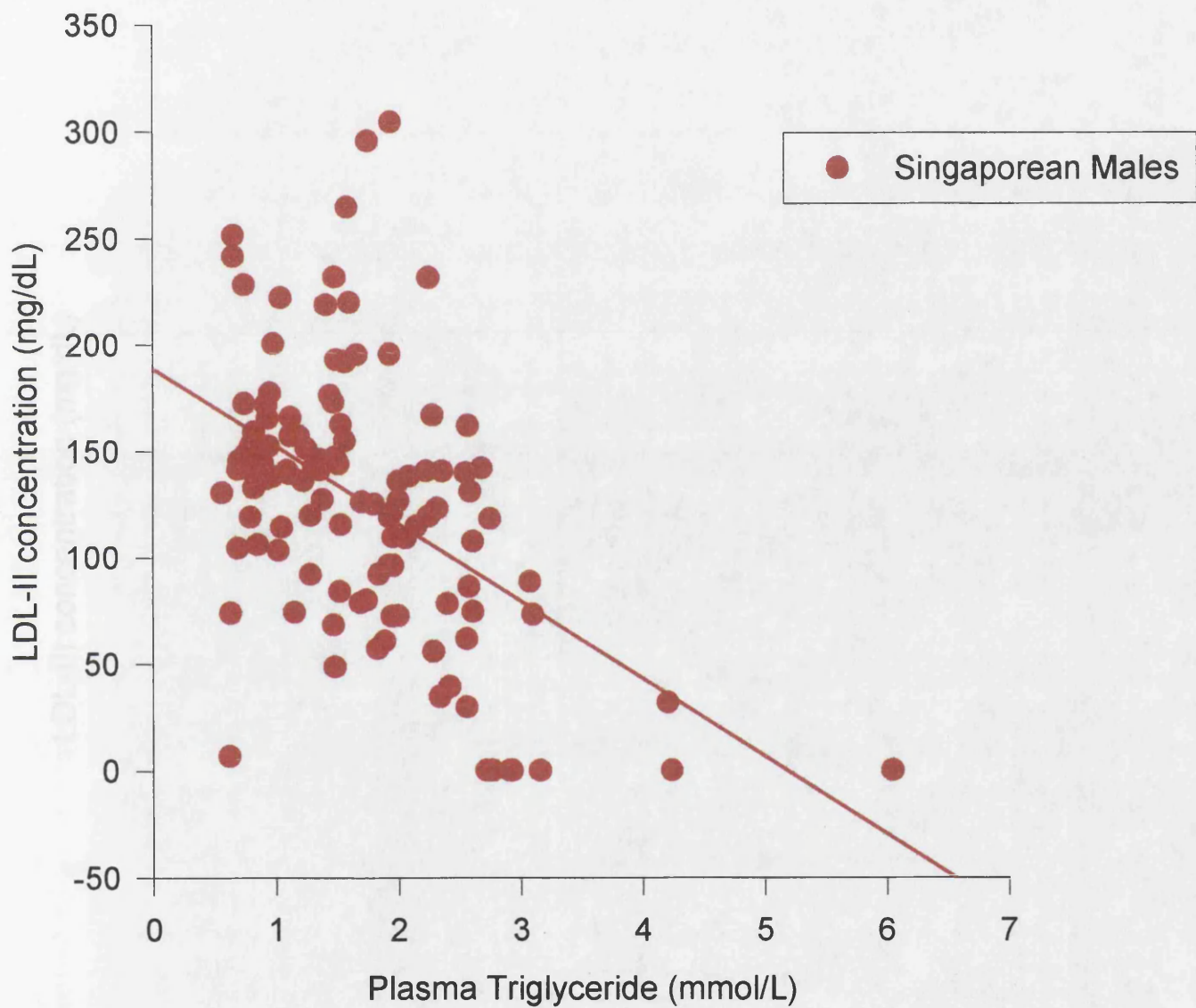
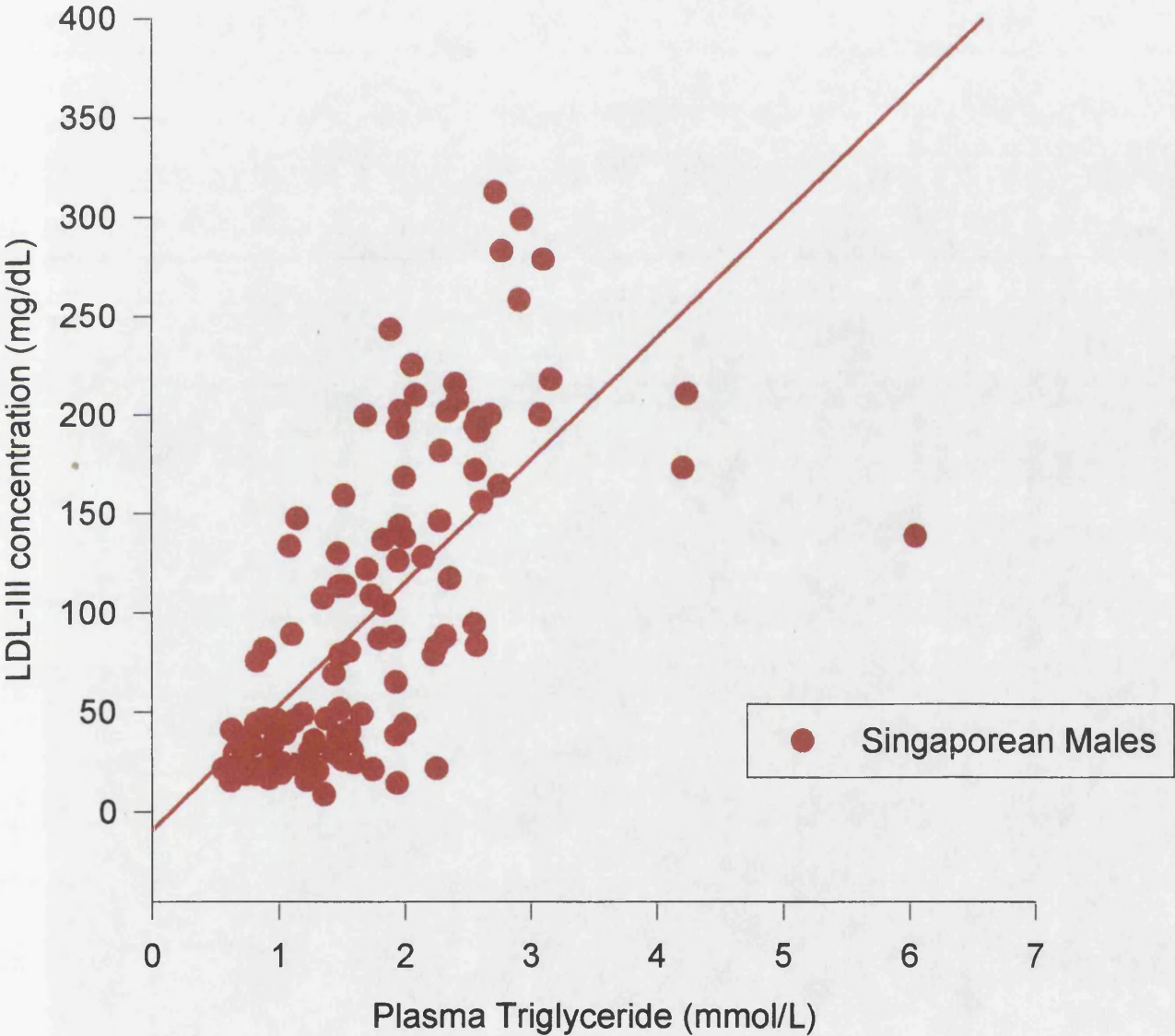


Fig 28: LDL-III concentration vs Triglyceride



Chapter 8 Conclusions

Give instruction to a wise man, and he will still be wiser; Teach a just man, and he will increase in learning.
Proverbs 9:9

8.1 Introduction

The aggregation of coronary artery disease (CAD) in families had been reported by several authors but the mechanisms of familial transmission of CAD are not fully understood. Inherited CHD is most apparent in families of young patients.²³⁸ Studies in twins²³⁹ have also suggested that the genetic component is stronger in early-onset than in late onset CAD. Hence associations and mechanisms for familial transmission are probably more developed in prematurely developed CAD. For example the recent European Atherosclerosis Study (EARS)²⁴⁰ showed that male offsprings of patient with premature CAD manifested some of the classical risk factors linked to CHD. Hence the study (chapter 5) carried out in Scotland sought to address the issue of environmental versus genetic factors in the manifestation of coronary risk factors in families with patients having premature atherosclerosis. The exclusion of probands with total cholesterol of more than 7.0 mmol/L was designed to exclude families with familial hypercholesterolaemia. There is no doubt that cholesterol, especially LDL cholesterol plays a major role in the atherosclerotic process but the family studies' objectives were to assess the impact of other CAD risks apart from hypercholesterolaemia.

The link between the insulin resistance syndrome (IRS) and the atherogenic lipoprotein phenotype (ALP) had been alluded to by Reaven and Krauss and this link was explored further throughout this thesis. Both conditions increases susceptibility to premature atherosclerosis and it was obvious that the links between the two needed further study. This was particularly important in Singapore, where 8.6% of the resident population suffer from diabetes mellitus and we also have one of the highest incidence of myocardial infarction rates in the region. Our last National Health survey on more than 5000 males and females, showed that the prevalence of diabetes mellitus had almost doubled over the last 10 years. Although the mean cholesterol levels had fallen from 5.8 mmol/L to 5.3 mmol/L, we still rank as one of the highest in the region. The Singapore myocardial infarct registry had also shown that the incidence rates for acute myocardial infarction had remained at about 76 per 100,000 Singaporeans aged 20 to 64 years from the mid eighties to the present time. This was alarming because the reduction in cholesterol had not translated into a reduction in CAD and I suspect it was partly because of the dramatic rise in diabetes mellitus and its accompanying dyslipidaemia. It was thus appropriate to study a group of normal Singapore males to determine the prevalence of insulin resistance and ALP in such a cohort. The prevalence of diabetes in Singapore is about four times that in Scotland and this would certainly have an impact on both insulin resistance and the ALP. Hitherto, we have been unable to diagnose the atherogenic lipoprotein phenotype in Singapore because of limitations of laboratory support in determining LDL subfractions. With the setup and transfer of technology from Glasgow to Singapore, we were able to fractionate the LDL by the density gradient ultracentrifugation and this allowed us to profile the ALP and document the prevalence of such profile in our local population. Of particular interest was the comparison between matched population of Scottish males and Singapore males as discussed further in this chapter. If the prevalence of the ALP is

higher in a group of Singapore males as compared to the Scottish cohort, it would lend further support to the hypothesis of a link between the insulin resistance syndrome and the atherogenic lipoprotein phenotype.

This thesis also explored the link between insulin resistance and the triglyceride metabolic capacity through the oral fat meal challenge. The majority of a person's time is spent in the post-prandial period, during which time the vessel walls are exposed to post-prandial lipoproteins which have been suggested to be particularly atherogenic.²⁴¹ The ability to handle triglyceride would be a strong determinant of the HDL cholesterol levels and the individual with IRS would often have low HDL cholesterol and high triglyceride, suggesting impaired triglyceride metabolic capacity.

8.2 Genetic Factors versus Lifestyle Factors

The statistical analysis in family studies posed a great challenge as there were no easy methods available. It was also difficult to tease apart the impact of genetic factors, environmental and lifestyle factors as they often occur together in families. Even studies on obesity in families have suggested that many members of obese families have similar eating habits and that could account for the obesity rather than genetic factors. From the study on families in chapter 5, it would appear that there are elements of both and it is likely that a combination of genetic factors and environmental influence determine susceptibility to premature atherosclerosis. Males from families with premature atherosclerosis were more likely to carry the ALP than the females, hence suggesting influence of gender on manifestations of ALP. Austin et al suggested that the ALP is not manifested until adult life but we found that males below the age of 30 years in such families manifested the ALP. This highlights the importance of genetic influence on the appearance of ALP probably moderated by the plasma triglyceride as discussed in chapter 3.

Garrison et al²⁴² suggested that environment was an important determinant of familial lipid relationships. They noted that the correlation of lipid levels in dizygotic twin pairs²⁴³ was higher than the correlation which they observed in non-twin sibling pairs. This was attributed to the greater sharing of environment by members of dizygotic twin pairs than by members of non-twin sibling pairs. However arguments against this observation have suggested that methodological and/or seasonal factors could have explained the quantitative differences between the twin pair and sibling pair correlation. Others have examined lipid relationships of families residing in kibbutzim and the families showed little evidence of total cholesterol familial aggregation.²⁴⁴ Sosenko et al had shown that there were greater correlation of total cholesterol and HDL cholesterol levels between child and mother than between child and father, and suggested that this was due to a greater sharing of environment between child and mother than between child and father.²⁴⁵ Obviously, predisposition to premature atherosclerosis is both a combination of inherited risk factors as well as environmental factors.

I sought to develop a risk scoring based on a combination of both lipid and lipoprotein associated factors as well as environmental influence, in particular smoking habits, to profile the families at risk. The data showed that family members of patients with

premature atherosclerosis were more likely to manifest at least 2 or more risk factors whilst those without family history of premature atherosclerosis had 1 or no risk factors. The use of this risk scoring appeared to distinguish between family members at risk and those who did not have family history of premature atherosclerosis. There was an aggregation of CAD risk which were determined by genetic as well as environmental influence, in such families with premature atherosclerosis. The social characteristics which distinguished families with premature atherosclerosis from the control group was the smoking habit. Family members were more likely to be smokers and were also more likely to be heavier smokers. Perhaps, it represents a learned habit, as children learn from their parents at a young age the smoking habits. Children brought up in families where the parents smoke, will accept smoking as part of the norms of life. Such social habits may be appropriately considered as 'inherited' social traits. Taken together, the results are compatible with the hypothesis that occurrence of CAD requires an interaction of both unfavourable genetic and environmental influences. Thus in families who do not have familial hypercholesterolaemia but have premature atherosclerosis, a clustering of risk factors are likely. These factors include higher plasma triglyceride, low HDL cholesterol, higher VLDL₁ subfractions and LDL-III particles, some of which have a genetic element. The presence of insulin resistance is also closely associated and it is likely that the risk factors act synergistically, contributing to the overall risks for premature atherosclerosis.

8.3 Modification of Risk Factors in Families with CHD

In the counselling of families with premature atherosclerosis, it became apparent that it was not possible to separate the genetic factors from the environmental influence. There are risk factors such as the ALP and insulin resistance, which are inherited but these are also modified by environmental influence. Furthermore, many family members have similar dietary patterns and in counselling at risk individuals about dietary change, I have found that it is ineffective to counsel them apart from the rest of the family. Pressures from within the family during meal times made it impossible for such individuals to adhere to any form of dietary plans. It was far more effective to counsel family units rather than individuals, particularly when we are dealing with high risk families. When the family changes its dietary habits, the patients are subjected to less temptations. Furthermore, family members can no longer have a detached attitude because they were able to see for themselves the risks that runs in their family, and this encourages all to change. This was particularly effective if each one had a set of results regarding his or her own coronary risks. It was also prudent to develop in the children at a young age, healthy eating habits rather than try to change their habits when it had been established. As mentioned previously, the smoking habits of such families have stood out as important socio-economic factors. The smoking habit is a learned behaviour and may even be considered an "inherited" trait. Again if patients are to succeed in quitting the smoking habit, the pressures from smokers within the family must be addressed. The benefit of cessation of smoking habits within the family unit cannot be understated. The support from family members would go a long way in helping "at risk" individual change their habits and lifestyle.

8.4 The Role of Plasma Triglyceride on Lipoprotein metabolism

There has been a lack of consensus on the role of triglyceride as an independent risk factor for CAD. The Paris Prospective study²⁴⁶ concluded that when interaction of cholesterol and triglyceride are considered, triglycerides are an independent risk factor. The Framingham study²⁴⁷ concluded that elevated triglyceride levels were a highly significant independent risk factor for CAD in women. Most agree that the lack of consensus may be related to flaws in the currently available statistical approaches to analysing triglyceride levels. The identification of subclasses of HDL and LDL particles, may lead to identification of more precise markers for possible underlying mechanisms related to risk of cardiovascular disease. In hypertriglyceridaemic subjects, the structure of LDL and HDL are altered by processes of lipid exchange (cholesteryl ester for triglyceride) and lipolysis. The resultant LDL and HDL are small, dense with the former becoming more atherogenic and the latter lower in concentration and less cardioprotective. Furthermore, there could be reduced clearance of VLDL particles, resulting in products of incomplete delipidization ie. VLDL remnants, which are potentially atherogenic. The results shown in chapter 3, Table 8 further supports the impact of hypertriglyceridaemia on lipoprotein metabolism, especially on the subfraction distribution. The group with triglyceride above 1.5 mmol/L had significantly higher levels of VLDL₁ and lower levels HDL₂. The decreased amounts of HDL₂, a particle which remains in the circulation for a longer period and is more efficient as acceptor of tissue cholesterol, means that the group with higher plasma triglyceride had a higher atherogenic potential. Similarly, the presence of a greater amount of LDL-III, which is more susceptible to oxidation and uptake by macrophages, translates into a greater atherogenic potential.

The risk of CAD in hypertriglyceridaemia may also depend on the presence of other risk factors such as hypertension, obesity, diabetes and cigarette smoking. It is known that subjects with isolated hypertriglyceridaemia have only limited increase in CAD risk. In contrast, the risks of CAD is considerably increased if the HDL cholesterol content is reduced and presumably replaced in part by triglyceride. Thus when hypertriglyceridaemia occurs in the presence of high cholesterol in familial combined hyperlipidaemia, the risk is particularly high.

The thesis also explored the significance of the alimentary challenge in predicting an individual's triglyceride metabolic capacity. It had been argued that the fasting plasma triglyceride may not be truly reflective of the totality triglyceride metabolism. Studies have shown that patients with CAD have an exaggerated triglyceride response after an oral fat meal challenge and the hypertriglyceridaemia remains for a longer period of time such that there is greatest distinction between normals and CAD patients at the 6th to 12th hour post meal. The ability to handle the fat meal is dependent on the lipoprotein lipase activity, and this had been shown to be subjected to hormonal influence such as insulin. The study on CAD proven men showed that pre-heparin LPL activity were decreased when compared to age-matched controls, and this may be related to the exaggerated triglyceride response post meal. The CAD patients were also insulin resistant and exhibited a hyperinsulinaemic response both to the standardised fat meal as well as to the modified glucose meal. The free fatty acid levels in CAD patients were higher than the

normals during the fat meal and yet these had a lower preheparin LPL level, suggesting that other mechanisms apart from the free fatty acid, may be responsible for the rise in LPL during a fat meal challenge. A resistance to the hormonal influence of insulin on LPL activity in CAD patients may be one possible mechanism for the observed difference in LPL levels. However, the significance of impaired preheparin LPL activity is unclear at the present moment as there are no correlation with postheparin LPL activity nor to the adipose and skeletal muscle LPL activity.

8.5 Comparing the Singapore males with the Scottish males

As the results of the study in Singapore were completed, it became increasingly apparent that there were significant differences between the Scottish male population and the Singapore males. These differences are summarised in table 41. A total of 141 Singaporean males and 65 Scottish males matched for age, were compared for differences in the lipids, lipoproteins and carbohydrate metabolism. Bearing in mind that the mean cholesterol levels for the Scottish population is about 6.2 mmol/L, it was not surprising that the cholesterol levels in the Scottish cohorts were significantly higher than those in the Singapore counterparts. However, what was remarkable were the differences in the other lipid and lipoprotein parameters. The Singapore males had significantly higher levels of plasma triglyceride whilst manifesting significantly lower levels of HDL cholesterol. There were also significant differences in the LDL subfraction patterns. The Scottish cohorts had significantly higher levels of buoyant LDL-I, as well as LDL-II whilst the Singapore males had significantly higher levels of the atherogenic LDL-III. There were also ethnic differences in the concentrations of LDL-III as those of Asian descent had levels of LDL-III close to or greater than 100 mg lipoprotein/ dL plasma, whilst the group with some European ancestry i.e. the Eurasians, had levels which were similar to that seen in the Scottish cohort. Taken together, the higher plasma triglyceride, lower HDL cholesterol as well as higher levels of LDL-III, it was obvious that the Singapore males manifested the atherogenic lipoprotein phenotype with greater frequency than its Scottish counterpart. More than half of the Singapore subjects had concentrations of LDL-III above 100 mg lipoprotein/ dL plasma.

The differences between the Singapore males and the Scottish males could be attributed to genetic influence, since both the insulin resistance and ALP are thought to be inherited, perhaps modified by other environmental factors. Given the high prevalence of diabetes mellitus in Singapore, it is reasonable to assume that there is a high gene pool of insulin resistance and hence the equally high prevalence of the atherogenic lipoprotein phenotype. Others may argue that the Singaporean males and the Scottish males have very different lifestyle and dietary habits and that alone may account for the difference, especially when the mean plasma triglyceride levels are high in the Singapore cohort. However, the ethnic differences in the Singapore study would lend further support to strength of the genetic influence, as the Eurasians, have similar lifestyle and dietary patterns to the typical Singaporean male but they have lipid and lipoprotein levels that were dramatically different. The data suggest that Eurasians in Singapore have lipid, LDL subfraction patterns and carbohydrate metabolism more akin to the Scottish population than the other ethnic groups in Singapore.

The lack of any significant fall in CAD incidences in Singapore over the past 10 years may in part be attributed in part to the rise in diabetes mellitus, insulin resistance and perhaps the atherogenic lipoprotein phenotype. In the past, the traditional Asian diet and poverty may have prevented those of Asian descent from suffering the manifestations of diabetes mellitus, insulin resistance and the atherogenic lipoprotein phenotype. Today, with increasing affluence and changing lifestyle and dietary patterns, such protection is no longer in place in a population with high genetic susceptibility.

Table 41: Differences between the Singapore and Scottish males

Variable	Singapore males (mean±sem)	Scottish males (mean±sem)	p
Cholesterol(mmol/L)	5.30±0.1	5.64±0.1	<0.05
Triglyceride(mmol/L)	1.87±0.1	1.32±0.1	<0.001
HDL-Chol(mmol/L)	1.13±0.02	1.21±0.03	<0.05
LDL-I(mg/dL)	31.9±3.0	67.2±4.6	<0.001
LDL-II(mg/dL)	126.1±5.9	187.5±8.4	<0.001
LDL-III(mg/dL)	97.9±7.4	69.6±8.5	<0.01
Fasting insulin(mU/L)	10.1±0.9	8.6±0.7	ns
Fasting glucose(mmol/L)	5.15±0.05	5.15±0.08	ns
Insulin resistance	2.38±0.3	1.96±0.2	ns

p value refers to the significance of difference between groups as determined by student's unpaired t-test

8.7 The Metabolic Dyslipidaemic Syndrome

Many patients with coronary artery disease have more than one metabolic risk factor. This is not unexpected because obesity, hypertension, glucose intolerance and dyslipidaemia are each very prevalent. However, such clustering of risk factors is greater than would be expected by chance.²⁴⁸ This has led to the hypothesis that these disorders may be pathophysiologically related.

The term insulin resistance had been originally and generally used to indicate impaired insulin action on glucose metabolism in skeletal muscle, adipose tissue, and the liver. However, the term is now more widely used to indicate abnormal insulin activation of other pathways such as antilipolysis, lipoprotein lipase activity, and hepatic lipoprotein metabolism.²⁴⁹ It is believed that insulin resistance increases the risk of CHD in non-diabetic subjects partly from the concomitant dyslipidaemic state. Prevalence data suggest that up to 25% of the non-diabetic population may be characterised by a state of insulin resistance.¹³ However, we do not know whether this prevalence holds true in population with high prevalence of diabetes mellitus such as Singapore and Hong Kong. In a small study conducted previously, we have found that up to 40% of non-diabetic, non obese, young hypertensive males in Singapore had insulin resistance.²⁷ The presence of the dense, LDL phenotype is associated with hypertriglyceridaemia, low HDL cholesterol as well as insulin resistance.⁷⁴ Thus an insulin resistant state appears to be a central component of the dyslipidaemic condition associated with hyperinsulinaemia. Although genetic factors are thought to play a significant role in insulin resistance and NIDDM, no

deleterious mutations of candidate genes have yet been found to explain the disease. It must be borne in mind that the association between variations in insulin sensitivity and plasma lipoprotein levels is only moderately high, suggesting that other factors, such as the variation in genes involved in lipoprotein metabolism, may modulate the magnitude of the dyslipidaemic state associated with insulin resistance.²⁵⁰

There are no consensus as to whether the changes in lipid metabolism are secondary to insulin resistance or vice versa. Prospective study have shown that insulin resistance precedes other changes and supports the hypothesis that insulin resistance is the underlying factor.²⁵¹ Others have argued that the primary change is in fat metabolism, and the increase in fat oxidation leads to insulin resistance in obesity and eventually to the high incidence of non-insulin dependent diabetes mellitus.²⁵²

In conclusion, the data reviewed in this thesis highlights the close relationship between insulin resistance and the atherogenic lipoprotein phenotype. Of particular interest was the significant difference between the Singapore cohort and the Scottish cohort, suggesting that genetic influence may play a significant role in the manifestations of insulin resistance and the dense LDL particles. It was further supported by the ethnic differences between those of Asian descent and those with some European ancestry, who despite living in similar social environment yet had differing profiles. Insulin resistance is a major risk factor in the development of a group of complications that increases risk for coronary heart disease. It is likely that insulin resistance and the atherogenic lipoprotein phenotype are part of a closely linked metabolic and cardiovascular syndrome. This concept has important clinical and therapeutic implications since effective treatment aimed solely at any given component of the syndrome may be completely offset by aggravation of other factors.

Appendix 1

Suppliers of Reagents, Equipment and Software

Abbot Laboratory

Rep of Ireland

Butterfly[®] -21 Venisystems[™]

Baker Instruments Ltd

Rusham Park, Whitehall Lane, Egham, Surrey, TW20 9NW, UK.

Encore Chemistry System

Centrifi Chem[®]

Beckman Instruments, Spinco Division

Beckman Instruments (UK) Ltd Analytical Sales and Service Operation

Progress Road, Sands Industrial Estate, High Wycombe, Bucks. HP12 4JL, UK.

L8-60M Ultracentrifuge

L8-70 Ultracentrifuge

Prep UV Scanner

AnF rotor

SW 40 rotor

Ti 60 rotor

Ultra-clear[™] centrifuge tubes

Ti 50.4 rotor

Polycarbonate centrifuge bottles and caps

Spinkote

Silicone Vacuum grease

Tube Slicer

Becton Dickinson

Dublin, Eire

Microlance[®]2 21G Needles

Syringes

BDH Laboratory Supplies

McQuilkin and Co., 21 Polmadie Avenue, Glasgow G5 0BB, UK

Triethylamine

Folin-Ciocalteu Reagent

Boehringer Mannheim GmbH

Boehringer Mannheim UK (Diagnostics and Biochemicals) Ltd

Bell Lane, Lewes, East Sussex BN7 1LG, UK.

ATP (MV 602)

Hitachi 717 automatic analyser

Kit No. 310328 (free cholesterol)

Kit No. 69184 (phospholipid)

Kit No. 704121 (cholesterol)

Kit No. 704113 (triglyceride)

CP Pharmaceuticals Ltd

Wrexham, UK

Hepsal[®] (10 units per mL)

Multiparin[®] (5000 units per ml)

Multiparin[®] (1000 units per mL)

Immunogenetics NV

Antwerp, Belgium

Innotest Lp(a)

Microsoft Corporation

1 Microsoft way, Redmond, WA, USA.
MS Word for Windows 3.1

Minitab Inc

3081 Enterprise Drive, State College, PA 16801-3008 USA.
PC Version of Minitab Release 9 for Windows

Paar Scientific Ltd

594 Kingston Road, Raynes Park, London SW 20 8DN, UK
Digital densitometer DMA 35

Technicon (Ireland) Ltd

Swords Co. Dublin, Eire.
Autoanalyser[®] II

Achema Pte Ltd

2 Soon Wing Road #03-11, Soon Wing Industrial Building, Singapore 1334
20-100uL single channel pipetter, 200-1000uL single channel pipettor
Stand for 6 Treff pipettors
Ismatech 8 channel peristaltic pump 2 colour coded stoppers Tygon tubings
Syringe infusion pumps

Beckmann Instruments Singapore Ptd Ltd

331 North Bridge Road, #07-01/02 Odeon Towers, Singapore 0718
Du650 Spectrophotometer and Colour monitor
External storage device
Single cell holder Flow cell and tubing kit
Fraction recovery system
SW 40 Ti rotor set 50.3 Ti rotor set
Cap for lipoprotein floatation
Ultra clear centrifuge tubes for SW 40Ti rotor
Ultra clear centrifuge tubes for 50.3 Ti
GS-15 Table top refrigerated centrifuge
F1010 Fixed angle rotor
3-5 ml Adapters 10 ml Adapters
Rotor cleaning kit

Laboratory Equipments Pte Ltd

153 Kampong Ampat, #02-04 Junjie Industrial Building, Singapore 1336
DMA 35 Digital Density Meter for liquids
Multisize tube racks
Test-tube peg racks

Schmidt Scientific Ptd Ltd

2 Jalan Kilang Barat, Singapore 0315
10 ml Vacutainer plain tubes 10 ml Vacutainer EDTA tubes
3 ml Vacutainer plain tubes 3 ml Vacutainer fluoride oxalate tubes

Quintech Scientific Pte Ltd

9 Chin Bee Avenue, Singapore 2261

1 ml Kimax Volumetric flask

Innovative BioTech Pte Ltd

77 Ayer Rajah Crescent, #03-04 Ayer Rajah Industrial Estate Singapore 0513

Sigma Maxidens

Sigma Diagnostics Protein Assay kit

Appendix 2

INCLUSION CRITERIA FOR CASES IN FAMILY STUDIES (Scotland)

1. Probands must be aged 55 and below
2. At least 3 months post CABG
3. Positive family history of siblings and or parents with documented history of premature ischaemic heart disease/ myocardial infarction(<55 years of age).
4. Members of family of proband must be accessible and willing to participate in the study.

Generation I Parents

Generation II Probands, siblings and spouses

Generation III Children of probands and probands' siblings

Adopted children and half sibs are excluded. All other members of family will be recruited

Exclusion Criteria:

1. Familial Hypercholesterolaemia
2. Diabetes Mellitus

INCLUSION CRITERIA FOR CONTROLS

1. Age under 55 years old
2. No family history of CHD in parents or siblings(<70 years of age)
3. No history of Diabetes mellitus, hypertension, hyperlipidaemia or CHD
4. At least 10 members of controls must be accessible and willing to participate in the study. All members of family will be recruited.

Volunteer Consent Form

I, _____, understand that Professor James Shepherd, Dr Chris Packard, Professor AR Lorimer, Professor Wheatley and Dr Chee-Eng Tan are undertaking investigations on coronary risk factors in families with and without premature coronary heart disease.

The studies involve the following:

1. Filling in a questionnaire on lifestyle assessment and medical history.
2. Taking 60 mL of fasted blood samples.
3. Taking a 75 gm glucose drink as part of an oral glucose tolerance test after (2), followed by half hourly blood sampling of 5 mL, for 2 hours.

I understand that my involvement in this study is entirely voluntary and that I may withdraw at any time.

Patient's signature _____ Date _____

Attending Physician's signature _____ Date _____

Volunteer Consent Form (Heparin Study)

I understand that a team of researchers under the supervision of Professor James Shepherd are investigating key factors which control blood fat levels in people.

The studies involve the following:

1. Taking a small initial blood sample for a full blood count.
2. Assessment by a Physician regarding my suitability to participate in the study.
3. Taking a larger fasting blood sample.
4. On the same occasion as (3) above, the administration of heparin followed by a small blood sample.

This nature of this study has been clearly explained to me by Dr CE Tan and my involvement in the study is entirely voluntary.

Patient's signature: _____

Date: _____

Attending Physician's
Signature: _____

Date: _____

Heparin Administration Medical Questionnaire

Volunteer - Name _____

Date of Birth _____

Dear Volunteer,

Thank you for participating in our study. You will be given an injection of heparin to enable us to measure the activity of key factors involved in fat metabolism. In order for us to be certain that it is safe to give you the heparin, would you please answer the questions and sign the sheet when you have finished. If you have any further questions or something is unclear, please ask.

1. Are you quite fit at the moment? Yes/No
2. Are you receiving any medical treatment? Yes/No
(including non-steroidal anti-inflammatory drugs)
If so, please specify: _____
3. Have you taken aspirin recently? Yes/No
4. As far as you know, are you sensitive to heparin? Yes/No
5. Do you have haemophilia or any other diagnosed bleeding disorder? Yes/No
6. Have you ever suffered from a stroke? Yes/No
7. Have you had rheumatic fever? Yes/No
8. Have you had a recent surgery? Yes/No
9. Have you had a recent head injury or trauma requiring hospital admission? Yes/No

Height:

Weight:

Blood pressure:

Signature: _____

Date: _____

LIFESTYLE ASSESSMENT QUESTIONNAIRE

Serial No:

Groups:

Family order:

PATIENT PARTICULARS

Date of Registration: _____

Forename: _____

Surname: _____

Address: _____

Tel: _____

Occupation: _____

Educational level: _____

Date of Birth: D/ M/ Y

Sex: 1=male, 2=female

Height(without shoes) . cm

Weight . kg

Blood pressure: To be taken seated from the right arm after 15 minutes rest and 5 minutes interval between readings.

	1st reading	2nd reading (nearest 2mmHg)
Systolic	<input type="checkbox"/> <input type="checkbox"/> <input type="checkbox"/> mmHg	<input type="checkbox"/> <input type="checkbox"/> <input type="checkbox"/> mmHg
Diastolic	<input type="checkbox"/> <input type="checkbox"/> <input type="checkbox"/> mmHg	<input type="checkbox"/> <input type="checkbox"/> <input type="checkbox"/> mmHg (Korotkov V)

Waist . cm (Waist is defined as the narrowest circumference below the ribs and above the umbilicus)

Hip . cm (Hip is defined as the broadest circumference between the superior border of iliac crest and the thigh)

Social History

Please fill up all columns and put in 0 if not applicable

Smoker 1=yes, 2=no

If current smoker cigarettes/day

If Ex smoker cigarettes/day

No of years of smoking

No of years since stopping smoking
(if applicable)

Alcohol consumption 1=yes,2=no

Alcohol intake in units per week
(NB: 1unit=half pint beer, 1glass wine or 1 tot spirit)

Level of Exercise

At work or in your leisure, how often are you physically active for a period of at least 20 minutes during which you become short of breath and perspire?

1=less than once per week, 2=once per week, 3=at least 2-3 times per week

Diet

Has your dietary pattern improved recently? 1=yes, 2=no
(in the last 6 months)

If yes, why?

1=relative recently diagnosed to have CHD, 2=Health education through the media,
3=Doctor's advice, 4=Personal motivation, 5=others

Medical History

Diabetes mellitus 1=yes,2=no

Age of onset of diabetes

No of years of Diabetes

Treatment of Diabetes _____

1=Diet only, 2=drug, 3=insulin, 4=drug+insulin, 5=others, please specify

Impaired glucose tolerance 1=yes, 2=no

(excluding Diabetes mellitus, ie. if you are already a diabetic, please fill this as 0)

Hypertension 1=yes, 2= no
(ie. diagnosed by GP, hospital, nurse practitioner or those already on treatment)

No of years of hypertension

No of years of treatment

Type of Treatment _____

1=Diuretics, 2=Beta blockers, 3=Calcium antagonist, 4=ACE inhibitors, 5=Diuretics + beta blockers, 6=no treatment, 7=others and specify

Coronary Heart Disease(CHD) 1=yes, 2=no
(CHD includes angina, myocardial infarction, ischaemic heart disease)

How was the diagnosis of CHD made?

1=Chest pain, angina, 2=ECG changes, 3=positive threadmill test, 4=coronary angiogram, 5=others, please specify

Duration of CHD (in years)

Dyslipidaemia 1=yes, 2=no

Duration of Dyslipidaemia (in years)

Type of Treatment _____

1=diet only, 2=resin binders, 3=fibrates, 4=statins, 5=olbetam, 6=combinations, please specify, 7=no treatment, 8=others, please specify

Other Medical History:

(You may fill more than one box)

1=Stroke, 2=Cancer, 3=kidney disease, 4=prostate disease, 5=respiratory disease, 6=liver disease, 7=neurological problems, 8=surgical procedures, please specify, 9=others, please specify

Any family history of coronary heart disease?

1=yes, 2=no

Appendix 6

Volunteer Consent Form: Visit 1 (fat meal study in Scotland)

I understand that a team of researchers under the supervision of Professor James Shepherd are investigating key factors which control blood fat levels in people.

The studies involve the following:

1. Taking a small initial blood sample for a full blood count.
2. Assessment by a Physician regarding my suitability to participate in the study
3. Taking another larger fasting blood sample.
4. On the same occasion as (3) above, the administration of heparin, which thins the blood, followed by a small blood sample.

This study has been clearly explained to me by Dr CE Tan and my involvement in the study is entirely voluntary.

Patient's signature: _____

Date: _____

Attending Physician's
Signature: _____

Date: _____

Volunteer Consent Form: Visit 2

I understand that a team of researchers under Professor James Shepherd are investigating the effects of dietary fat on key factors which control blood fat levels in people.

The studies involve the following:

1. Taking a small fasting blood sample.
2. Taking of a standardised fat meal
3. Taking of small blood samples at half hourly interval for 2 hours, and then 2 hourly interval up to the 6th hour.
4. Heparin will then be given at the 6th hour and a final blood sample 12 minutes later.

The nature of this study has been clearly explained to me and I understand that my involvement in this study is entirely voluntary.

Patient's signature: _____

Date: _____

Attending Physician's
signature: _____

Date: _____

Volunteer Consent Form: Visit 3

I understand that a team of researchers under Professor James Shepherd are investigating the effects of dietary glucose on key factors which control blood fat levels in people.

The studies involve the following:

1. Taking a small fasting blood sample.
2. Taking of a 22 g glucose load.
3. Taking of small blood samples at half hourly interval for 2 hours, and then 2 hourly interval up to the 6th hour.
4. Heparin will then be given at the 6th hour and a final blood sample 12 minutes later.

The nature of this study has been clearly explained to me by Dr CE Tan and I understand that my involvement in this study is entirely voluntary.

Patient's signature: _____

Date: _____

Attending Physician's
signature: _____

Date: _____

VISIT 1 (Alimentary Lipaemia study)**PATIENT PARTICULARS**

Date of Registration: _____

Forename: _____

Surname: _____

Address: _____

Tel: _____

Occupation: _____

Educational level: _____

Date of Birth: D/ M/ Y
 Sex: 1=male, 2=femaleHeight(without shoes) . cmWeight . kg

Blood pressure: To be taken seated from the right arm after 5 minutes rest and 5 minutes interval between readings.

	1st reading	2nd reading (nearest 2mmHg)
Systolic	<input type="checkbox"/> <input type="checkbox"/> <input type="checkbox"/> mmHg	<input type="checkbox"/> <input type="checkbox"/> <input type="checkbox"/> mmHg
Diastolic	<input type="checkbox"/> <input type="checkbox"/> <input type="checkbox"/> mmHg	<input type="checkbox"/> <input type="checkbox"/> <input type="checkbox"/> mmHg (Korotkov V)

Waist	<input type="checkbox"/> <input type="checkbox"/> <input type="checkbox"/> . <input type="checkbox"/> cm	(Waist is defined as the narrowest circumference below the ribs and above the umbilicus)
-------	--	--

Hip	<input type="checkbox"/> <input type="checkbox"/> <input type="checkbox"/> . <input type="checkbox"/> cm	(Hip is defined as the broadest circumference between the superior border of iliac crest and the thigh)
-----	--	--

Social History

Please fill up all columns and put in 0 if not applicable

Smoker 1=yes, 2=no

If current smoker cigarettes/day

If Ex smoker cigarettes/day

No of years of smoking

No of years since stopping smoking (if applicable)

Alcohol consumption 1=yes,2=no

Alcohol intake in units per week •
(NB: 1unit=half pint beer, 1glass wine or 1 tot spirit)

Level of Exercise

At work or in your leisure, how often are you physically active for a period of at least 20 minutes during which you become short of breath and perspire?

1=less than once per week, 2=once per week, 3=at least 2-3 times per week

Any family history of coronary heart disease? 1=yes, 2=no

CORONARY RISK FACTORS STUDY (IN SINGAPORE)

INCLUSION CRITERIA

Age: 30 to 45 years

Sex: Male

Normals must have HbA1C < 6.5%

Cholesterol must be less than 6.0 mmol/L

Date:

Category:

IC No: _____

PATIENT PARTICULARS

Name: _____

Address: _____

Tel: _____

Occupation: _____

Educational level: _____

Date of Birth:

	D/	M/	Y
<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>
<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>

Sex: 1=male, 2=femaleHeight(without shoes) . cmWeight . kg

Blood pressure: To be taken seated from the right arm after 5 minutes rest and 5 minutes interval between readings.

	1st reading	2nd reading (nearest 2mmHg)
Systolic	<input type="checkbox"/> <input type="checkbox"/> <input type="checkbox"/> mmHg	<input type="checkbox"/> <input type="checkbox"/> <input type="checkbox"/> mmHg

	1st reading	2nd reading (nearest 2mmHg)
Diastolic	<input type="checkbox"/> <input type="checkbox"/> <input type="checkbox"/> mmHg	<input type="checkbox"/> <input type="checkbox"/> <input type="checkbox"/> mmHg (Korotkov V)

Waist	<input type="checkbox"/> <input type="checkbox"/> <input type="checkbox"/> . <input type="checkbox"/> cm	(Waist is defined as the narrowest circumference below the ribs and above the umbilicus)
-------	--	--

Hip	<input type="checkbox"/> <input type="checkbox"/> <input type="checkbox"/> . <input type="checkbox"/> cm	(Hip is defined as the broadest circumference between the superior border of iliac crest and the thigh)
-----	--	---

Social History

Please fill up all columns and put in 0 if not applicable

Smoker 1=yes, 2=no

If current smoker cigarettes/day

If Ex smoker cigarettes/day

No of years of smoking

No of years since stopping smoking
(if applicable)

Alcohol consumption 1=yes, 2=no

Alcohol intake in units per week
(NB: 1 unit=half pint beer, 1 glass wine or 1 tot spirit)

Level of Exercise

At work or in your leisure, how often are you physically active for a period of at least 20 minutes during which you become short of breath and perspire?

1=less than once per week, 2=once per week, 3=at least 2-3 times per week

Any family history of coronary heart disease? 1=yes, 2=no

Medications: _____

Consent

I, _____, understand that Dr CE Tan and his team of doctors are conducting a study on risk factors for heart disease in patients and this requires taking of 3 samples of blood at 5 minutes interval after a 10 hour fast. I also understand that my consent and participation in this study is entirely voluntary and that I may withdraw from the study at any time.

Patient's Signature and Date

Physician's Signature and date

Glossary

CHD:	Coronary Heart disease
CAD:	Coronary artery disease
NIDDM:	Non insulin dependent diabetes mellitus
IDDM:	Insulin dependent diabetes mellitus
IR:	Insulin resistance
IRS:	Insulin resistance syndrome
ALP:	Atherogenic lipoprotein phenotype
HDL:	High density lipoprotein
VLDL:	Very low density lipoprotein
LDL:	Low density lipoprotein
HL:	Hepatic lipase
LPL:	Lipoprotein lipase
AUC:	Area under curve
sem:	Standard error of mean
SD:	Standard deviation
BMI:	Body mass index
WHR:	Waist to hip ratio

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sensitivity to insulin of healthy men. *Clin Sci* 1935; 2: 67
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