The Atherogenic Lipoprotein Phenotype in Renal Disease

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SUMMARY

Nephrotic range proteinuria and chronic renal failure are associated with both qualitative and quantitative changes in lipoproteins and increased cardiovascular risk. LDL exhibits heterogeneity with increased small dense LDL (LDLIII) associated with increased risk of coronary heart disease (CHD). In normal populations, production of small dense LDL is physiologically linked to mild hypertriglyceridaemia and low HDL, a combination that has been labelled 'the atherogenic lipoprotein phenotype'. This thesis aimed to explore abnormalities in the metabolism of triglyceride-rich lipoproteins in patients with chronic renal dysfunction, with particular reference to changes in LDL subfraction distribution and remnant lipoprotein particles. The main population investigated were patients with nephrotic range proteinuria secondary to primary glomerular disease, however this work was extended to examine changes in lipoproteins in patients with proteinuria secondary to diabetic nephropathy in type 2 diabetes, and also patients with end-stage renal failure. Our hypothesis was that in patients with proteinuria, hypertriglyceridaemia occurs as a result of impaired catabolism of VLDL. The consequence is the generation of small dense LDL (LDLIII) and remnant lipoproteins, both of which are pathogenic agents which may contribute to accelerated vascular disease and to the rate of progression of renal failure.

Quantitative analysis of both LDLIII and remnant lipoproteins was performed in 27 patients with nephrotic range proteinuria and well preserved renal function. This revealed a marked increased in plasma LDLIII concentration, to levels that are noted to be atherogenic in normal populations. This increase primarily due to a shift in particle size towards smaller denser particles. Remnant lipoproteins (RLP) were

similarly increased in proteinuric patients. The excess of both of these atherogenic particles was independently related to the increase in plasma triglyceride prevalent in this population, an increase that previously been demonstrated to result in part, from delayed clearance of the $VLDL_1$ subfraction of very-low density lipoprotein (VLDL).

The 27 patients studied were found to have normal in vitro lipase activity, however marked abnormalities in the lipid and apolipoprotein content of the $VLDL_1$ particles was observed. Despite apparently normal plasma concentration of both apolipoprotein (apo) CII and apoCIII, the patients with proteinuria possessed $VLDL_1$ particles that were deficient in both of these apolipoproteins. This deficiency was accompanied by a relative deficiency in both plasma and $VLDL_1$ apoE. $VLDL_1$ particles were also found to be smaller than those in the control group with a higher surface ratio of free cholesterol: phospholipid. The presence of smaller, free cholesterol enriched, apolipoprotein deficient particles could easily account for the observed delay in clearance of $VLDL_1$ observed in this population.

12 patients with >1.5g/24hrs of urinary albumin, hypercholesterolaemia and plasma triglyceride >1.5mmol/l were studied in a randomised crossover trial comparing the effects of a statin (cerivastatin) and a fibrate (fenofibrate) on the concentration of LDLIII and remnant lipoproteins (RLP). Fenofibrate produced a significant reduction in both LDLIII and RLP. Cerivastatin reduced LDLIII but not RLP, moreover the reduction in LDLIII was greater on fenofibrate than following cerivastatin. The two treatments also differed in the manner in which plasma LDLIII was reduced, thus the reduction in LDLIII following fenofibrate correlated with plasma triglyceride reduction whilst the LDLIII reduction on cerivastatin was associated with LDL-C

reduction. Therefore fenofibrate seemed to reduce LDLIII by removing the excess triglyceride essential for formation of small dense LDL, with cerivastatin reducing LDLIII by decreasing the total amount of LDL present.

The association between dyslipidaemia and progression of chronic renal failure was studied in 30 patients with primary glomerular disease and >1.0g/24hrs of urinary albumin. Mean urinary albumin was found to have the strongest correlation with the rate of progression of renal failure, however non HDL-C was also independently related to the rate of progression. From amongst the spectrum of lipoproteins covered by non HDL-C, the only lipoprotein parameter that correlated with the rate of progression was VLDL$_2$. No relationship was seen between the rate of progression of renal failure and the concentration of small dense LDL.

The influence of albuminuria on LDLIII concentration was examined further by studying patients with type 2 diabetes who had either diabetic nephropathy, microalbuminuria or normal urinary albumin excretion (diabetic controls). All three groups were compared to non-diabetic control patients. All groups were matched for age and sex, with the diabetic groups also matched for glycaemic control. The patients with diabetic nephropathy were found to have excess LDLIII compared with all three other groups, whilst the microalbuminuric and diabetic control patients possessed excess LDLIII compared with normal controls. These increases in LDLIII were found despite each group having a similar plasma LDL concentration. Once more, plasma triglyceride was the most important determinant of LDLIII concentration, however an independent effect of having diabetes was present and a clear relationship was seen between plasma LDLIII concentration and urinary albumin. As the groups were well

matched, it is likely that in the patients with diabetic nephropathy, the dyslipidaemic environment in which excess LDLIII was formed resulted from the excess urinary albumin.

The final study assessed the influence of end-stage renal failure and renal replacement therapy on LDL subfractions. Excess LDLIII was present in the patients with renal failure, with plasma triglyceride and hepatic lipase activity being independent determinants of LDLIII. It is noteworthy that hepatic lipase activity was decreased compared with controls. In normal populations, a plasma triglyceride >1.5mmol/l and hepatic lipase activity >15umol/FA/ml/hr is required to form atherogenic levels of LDLIII. As a result of the reduced lipase activity in uraemic patients, the level of plasma triglyceride required to form atherogenic levels of LDLIII was noted to be increased to approximately 2mmol/l.

In conclusion, the importance of the impaired metabolism of triglyceride-rich lipoproteins in patients with chronic renal disease has been demonstrated in this work. The consequence is formation of excess atherogenic particles, that are not detected by routine measurement of cholesterol and LDL-C but will clearly contribute to the excess coronary artery disease present in patients with either proteinuria, diabetic nephropathy or end-stage renal failure. A clear relationship was also demonstrated between dyslipidaemia and progression of renal failure. This clearly requires further investigation. Lipid lowering therapy is successful in reducing the plasma concentration of these particles, however due to the extent of the dyslipidaemia, aggressive treatment is likely to be required to achieve optimal results.

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Author's Declaration

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

Christopher Deighan, October 1999

Dedication

To my loving wife Tricia and children Hannah and Michael

List Of Abbreviations

Apo	Apolipoprotein
BMI	Body mass index
BP	Blood pressure
CAD	Coronary artery disease
CETP	Cholesteryl ester transfer protein
CHD	Coronary heart disease
CrCl	Creatinine Clearance
d	Density
Da	Daltons
DBP	Diastolic blood pressure
HDL	High density lipoprotein
HL	Hepatic Lipase
HMG CoA	3-hydroxy,3-methyl glutaryl coenzyme A
IDL	Intermediate density lipoprotein
IQR	Inter-quartile range
LCAT	Lecithin cholesterol acyl transferase
LDL	Low density lipoprotein
Lp(a)	Lipoprotein (a)
LpL	Lipoprotein lipase
RLP-C	Remnant lipoprotein - cholesterol
RLP-TG	Remnant lipoprotein - triglyceride
SD	Standard deviation
Sf	Svedberg flotation units
VLDL	Very low density lipoprotein

CHAPTER 1 - INTRODUCTION

1.1. MACROVASCULAR DISEASE IN PATIENTS WITH CHRONIC RENAL DISEASE

Across a wide range of pathological processes, the development of chronic renal disease is associated with a marked increase in cardiovascular morbidity and mortality. The presence of excess urinary protein is recognised to be an independent risk factor for cardiovascular disease [1, 2] with 2 fold increase in the relative risk of cardiovascular disease in normal populations found to have dipstick positive proteinuria. [3]. In patients with chronic renal failure, there is an increased incidence of cardiovascular disease, accounting for more than 50% of deaths amongst patients on renal replacement therapy [4]. Moreover, despite the reduction in mortality from cardiovascular disease in the general population [5], the proportion of patients receiving renal replacement therapy who die of cardiovascular disease has remained constant [4]. Finally in both insulin dependent and non-insulin dependent diabetes, the development of diabetic nephropathy with persistent proteinuria is also associated with increased cardiovascular mortality [2, 6].

When discussing cardiovascular disease in the context of underlying renal dysfunction, it is important to distinguish between: (1) atherosclerotic disease which is manifested primarily as myocardial ischaemia secondary to coronary artery disease, (2) congestive cardiac failure which may result from myocardial ischaemia, but may also be secondary to fluid overload, uraemia or hypertension, (3) left ventricular hypertrophy, which also results from fluid overload and hypertension but is also a

consequence of chronic anaemia and (4) cerebrovascular disease, which may be secondary to atherosclerosis, but more frequently results from hypertension.

If the cardiovascular risk assessment is restricted to morbidity and mortality from coronary heart disease however, the increased risk remains prevalent across the spectrum of renal disease. In the MRFIT study, the relative risk of death from coronary heart disease was increased 2 fold in patients with persistent 1+ positive proteinuria on dipstick urinalysis [3], increasing to 2.5 times with an increase in the proteinuria to 2+ [3]. The relative risk continues to rise as proteinuria increases and studies in patients with nephrotic range proteinuria demonstrate a 3 fold increase in the risk of coronary death and a 5.5 times increase in the risk of myocardial infarction [7]. In the case of the MRFIT study, the increased risk was independent of age, initial cholesterol, diastolic blood pressure, smoking, antihypertensive use, change in renal function and race [3], whilst in the study by Ordonez [7] the risk from nephrotic range proteinuria was independent of both hypertension and smoking. In patients with chronic but not end-stage renal failure, the incidence of myocardial infarction is 2.5-3 times higher in male patients than in the general male population [8], whilst those on renal replacement therapy in the United States have a prevalence of coronary artery disease of 40% compared with the prevalence in the general population of 5% in those aged 45-64 and 12% in those over 65 years [9]. Age specific death rates from myocardial ischaemia and infarction in patients on renal replacement therapy in the UK follow along similar lines and continue to be 7-10 times higher, even after excluding patients with diabetes mellitus [4]. In patients with diabetes mellitus, the impact of renal disease on morbidity and mortality from coronary heart disease is even more profound. Coronary artery disease is the major determinant of the excess

mortality in patients with insulin dependent diabetes mellitus (IDDM) and persistent proteinuria [10], with an 8 fold increase in the incidence of coronary artery disease compared with matched insulin dependent diabetic patients without proteinuria [11]. In NIDDM, although microalbuminuria is strongly associated with coronary artery disease, it is unclear whether this is simply a reflection of underlying vascular damage rather than a risk factor for the future development of coronary artery disease [12]. However, the development of heavy proteinuria in NIDDM has been shown to be associated with increased atherosclerosis [13]. The prognosis in diabetics with end-stage renal failure is even worse. Mortality from ischaemic heart disease is a further 2-3 times higher in diabetics compared to non-diabetic patients on renal replacement therapy [4]. A further illustration of the extent of coronary artery disease in this population can be seen by comparing the mortality from myocardial infarction in diabetics from the MRFIT study (10 per 1000 patient/years) and diabetics on dialysis (65 per 1000 patient/years) [14]. These two data sets clearly demonstrate the excess coronary risk of having both NIDDM and end-stage renal failure.

Therefore, it is clear that not only is cardiovascular disease extremely prevalent across the wide spectrum of chronic renal disease, but coronary heart disease and atherosclerotic disease in general are major contributors to morbidity and mortality in this population. Indeed data from the United States Renal Data System has shown that overall 1 year mortality following the diagnosis of a first myocardial infarction in patients with end-stage renal failure is 59%, twice the mortality following acute myocardial infarction in the general population [15].

1.2. RISK FACTORS FOR THE DEVELOPMENT OF CORONARY HEART DISEASE

In normal adult populations, factors associated with increased risk of coronary heart disease (CHD) are well recognised. They include hypertension, hyperlipidaemia, cigarette smoking, diabetes and family history. More recently additional risk factors have been identified e.g. fibrinogen, homocysteinaemia, lipoprotein (a) and C reactive protein (CRP). It is now clearly established that intervention to attenuate the effect of a number of these risk factors reduces morbidity and mortality from coronary heart disease.

Raised levels of total cholesterol, increased plasma triglyceride, and reduced HDL-C are established risk factors for coronary heart disease. These will be discussed in more detail later in this chapter. Coronary heart disease (CHD) is the most common cardiovascular disease that is influenced by hypertension. Increases in both systolic (SBP) and diastolic blood pressure (DBP) are major contributors to death from CHD [16]. From the large MRFIT sample size it is calculated that the optimal level for SBP is 120mmHg and for DBP 80mmHg, with a 1.5 fold increase in the risk of CHD death for an increase in the SBP of 20mmHg and 2.5 fold increase for a 40mmHg increment. Antihypertensive treatment reduces CHD by about 14% [17], with the benefit extended to those aged >60 years, where it has been demonstrated that reduction in systolic hypertension results in a 27% decrease in non-fatal MI or CHD death [18]. It is interesting to note that the optimal blood pressure calculated from the MRFIT cohort is not dissimilar to the figure obtained from randomised controlled studies, which suggests that the relationship between on-treatment blood pressure and stroke flattens out below a blood pressure of 135/85 [19].

Smoking multiplies the effect of other coronary risk factors, with the adverse effect seen in both men and women. This increased risk is particularly profound in women who smoke whilst taking oral contraceptives [20]. The absolute risk imparted by cigarette smoking on coronary heart disease, is greater than the absolute risk of chronic obstructive airways disease, pulmonary neoplasms or pulmonary heart disease [21]. There is a 3-4 fold increase in the relative risk of acute myocardial infarction [22], with the risk increasing with the duration of smoking [21] and the number of cigarettes smoked [22]. However the risk decreases quickly with cessation of smoking, even in long term smokers [23].

Diabetes increases the risk of CHD 2-4 fold [24]. This increased risk is related to the increased prevalence of both dyslipidaemia and hypertension [25], but is also related to poor glycaemic control [25]. Recent evidence has suggested that overall cardiovascular mortality is increased with mild asymptomatic hyperglycaemia [26], but whether hyperglycaemia itself contributes to CHD or simply results in worsening of associated risk factors, remains controversial. In insulin dependent or diabetes mellitus (IDDM), the development of premature CHD is closely linked to nephropathy and hypertension [27]. In non-insulin dependent diabetes mellitus (NIDDM), lipid lowering reduces CHD death and non-fatal MI [28], and tight blood pressure control reduces macrovascular disease including stroke and CHD [29]. Intensive glycaemic control has been shown to decrease microvascular disease, however it has not been shown to reduce macrovascular disease [30]. Obesity is associated with several cardiovascular risk factors, including hypertension [31], dyslipidaemia [32] and glucose intolerance [33]. Waist to hip ratio (an index of central obesity) rather than

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body mass index (BMI) has been shown to predict fatal MI. Central obesity is part of the insulin resistance syndrome, which is associated with increased risk of CHD, however data from the Framingham cohort suggests that effect of obesity as a risk factor for CHD and coronary death, is also independent of conventional risk factors including glucose intolerance, cholesterol, SBP, smoking and left ventricular hypertrophy [34].

A family history of CHD is recognised to be a strong independent risk factor for CHD [35]. This may be mediated by genetic influence on other risk factors e.g. obesity, hypertension, dyslipidaemia or diabetes [36]. However there may also be an independent influence e.g. the deletion allele of the insertion deletion polymorphism of the angiotensin converting enzyme has been shown to be a risk factor in NIDDM patients who are at low risk of CHD due to the presence of a favourable lipid profile [37]. Hormonal status clearly exerts a profound influence with premenopausal women protected against CHD to an extent not accounted for by differences in blood pressure, total cholesterol and cigarette smoking.

Epidemiological studies have demonstrated that a sedentary lifestyle is associated with increased risk of both morbidity and mortality from CHD [38], moreover adults who undertake moderate physical exercise, have a lower mortality rate from CHD than less active subjects [39]. The beneficial effects of physical exercise on CHD result from improvements in blood pressure, lipids, weight and insulin resistance, however exercise also increases cardiac output and tissue oxygen extraction from the blood [40] with an independent and inverse correlation seen between physical fitness and cardiovascular mortality [41].

More recently clotting factors, homocysteine and inflammatory markers have been shown to correlate with CHD. Factors affecting both coagulation and fibrinolysis are important, with fibrinogen, Factor VII, Von Willebrand factor (VWF), tissue plasminogen activator and plasminogen activator inhibitor activity-1 (PAI-1) all being implicated. A number of prospective studies have identified an independent association between increased plasma fibrinogen and increased morbidity and mortality from CHD [42-44], and it has been suggested that a component of the risk of cigarette smoking is mediated via increased fibrinogen [42]. In hypercholesterolaemic patients, a high fibrinogen level increases coronary artery risk, whilst a low fibrinogen level identifies patients at low risk despite having hypercholesterolaemia [44]. Possible mechanisms by which fibrinogen increases coronary artery risk include facilitating the development of the atherosclerotic plaque, increasing plasma viscosity, increasing the ability of platelets to aggregate and increasing the amount of fibrin contained in thrombi [45].

VWF and tissue plasminogen activator have both been shown to be independent predictors of acute coronary events [44]. Factor VII concentration is increased in patients with coronary artery events [42,44], however this has not been found to be an independent risk factor [46]. Increased PAI-1 has been demonstrated to be an independent risk factor, particularly for secondary cardiac events [47]. Hyperhomocysteinaemia is associated with a 2 fold increase in coronary artery disease after correction for conventional risk factors [48]. Moreover, in the Physicians Health Study, over 5 years, a 3-4 fold increase in the risk of myocardial infarction was demonstrated in patients with increased homocysteine levels [49].

Evidence of inflammation after MI is associated with increased risk of recurrent coronary events [50]. Small increases in C reactive protein (CRP) are predictive of the development of acute myocardial infarction [51] and CRP is an independent risk factor for death following unstable angina or a non-Q wave MI [52]. Meta-analysis has demonstrated that an increase in CRP of 1.4mg/dl is associated with a 1.7 fold increase in the relative risk of coronary events [53]. Overall, increases in CRP are strongly associated with acute coronary events but are not determinants of chronic stable coronary disease [54].

1.3. NORMAL LIPID AND LIPOPROTEIN METABOLISM

(1) Lipids and Lipoproteins

The main lipids in plasma are cholesterol, cholesterol esters, triglycerides and phospholipids. They are found in the circulation complexed to apolipoproteins as lipoprotein particles. Cholesterol is required for the formation of cell membranes, steroid hormones and vitamin D, whilst triglycerides provide a major source of energy for the body. Cholesteryl ester and triglycerides are both hydrophobic neutral lipids, are insoluble in water, and thus are located in the core of lipoprotein particles. The amphipathic lipids, phospholipid and free cholesterol, form a surface monolayer in which proteins (apolipopoproteins) are interspersed.

Lipoproteins can be separated and classified by a number of different methods. Density gradient ultracentrifugation in a salt solution separates particles by their density and flotation characteristics, electrophoretic mobility on a gel separates

particles by the charge on the apolipoprotein content of the particles, and gel chromatography separates particles by their size. Separation into density classes is the method in most widespread use, however in recent years it has become evident that even within each density range, lipoproteins exhibit marked heterogeneity and can be separated into subgroups with distinct structures and functions [55,56].

The physical properties and apolipoprotein compositions of the various lipoproteins are given in table 1.1. Table 1.2 lists the main plasma apolipoproteins, the particles on which they are found, and their main functions. The largest and lightest lipoproteins are chylomicrons. These particles are most abundant post-prandially and are virtually absent in the fasting state. They are formed in intestinal epithelial cells and their main core lipid content is triglyceride, derived from dietary fat, along with lesser amounts of phospholipid, free cholesterol, cholesterol esters and protein. The main apolipoprotein (apo) is apoB48 however they also contain apo A-I, A-IV, ApoC and ApoE (table 1.2).

Very-low density lipoproteins (VLDL) are very similar to chylomicrons, and are the main triglyceride carrying lipoprotein in the fasting state. The main differences between VLDL and chylomicrons are the site of synthesis, the source of triglyceride being transported and the apolipoprotein B moiety. VLDL particles are synthesised in hepatocytes and their main function is the transport of endogenously synthesised triglyceride. VLDL contains apoB-100, apoCII, apoCIII and apoE, which are the apolipoproteins essential for the synthesis and clearance of the particles (fig 1.1).

ApoB is common to both chylomicrons and VLDL. It is essential for the assembly of both triglyceride rich lipoproteins, forming the structure around which both chylomicrons and VLDL are assembled. ApoB-100 and apoB-48 arise from an identical gene. ApoB-100, a 4563 amino acid protein, is synthesised in the liver and contains in its C terminal domain, the ligand region for the LDL receptor (table 1.2). ApoB-48 is the N-terminal 2152 amino acids of apoB-100, is synthesised in the intestine and does not bind the LDL receptor.

It was previously thought that intermediate-density lipoproteins (IDL) were simply intermediate particles in the process of forming low-density lipoprotein (LDL) from VLDL, however it is now evident that IDL itself has important properties and may contribute to atherogenesis [57]. IDL contains apoB-100 and apoE with a reduced triglyceride content and an increased cholesteryl ester content compared with VLDL. LDL is the main cholesterol carrying lipoprotein in plasma, accounting for more than 70% of circulating cholesterol. It contains very little triglyceride and the only apolipoprotein in LDL is apoB-100, accounting for approximately 25% of the particle mass (fig 1.2). Excess LDL is associated with atherogenesis, with humans possessing much greater concentrations of LDL compared with other mammals.

The smallest and most dense lipoprotein is high-density lipoprotein (HDL). The main function of HDL is to transport cholesterol from peripheral tissues to the liver, thus the main lipid found in HDL is cholesteryl ester. The major apolipoproteins in HDL are apo A-I and apoA-II, however HDL also possesses lesser amounts of ApoC and ApoE. Despite being the most abundant lipoprotein particles, HDL only carries 20%

of the circulating cholesterol. An important inverse relationship is seen between HDL and coronary artery disease risk.

(II) ApoB Containing Lipoproteins and Endogenous Lipoprotein Transport

In humans, there are two main pathways for the transportation of lipids in the plasma. The endogenous pathway involves transport of lipids from the liver to peripheral tissues using apoB-100 containing lipoproteins, and then the mechanism of reverse cholesterol transport uses HDL to return cholesterol to the liver. The exogenous pathway involves transport of dietary fat, mainly in the form of triglycerides, in chylomicrons to adipose tissue and skeletal muscle and will be discussed briefly later in this chapter.

Fig 1.3 illustrates an outline of endogenous lipoprotein metabolism. VLDL particles are assembled in hepatocytes, with each particle having a triglyceride rich core and containing one apoB moiety along with a number of apolipoprotein C and E molecules. The relative importance of apoB production and the availability of triglyceride in regulating production of VLDL remains to be clarified. What is clear is that not only is apoB essential for the normal production of VLDL, as patients with abetalipoproteinaemia do not produce VLDL [58], but that in the presence of excess triglycerides, larger VLDL particles are produced [59]. Following secretion from the liver into the bloodstream, VLDL particles acquire apoC and apoE from HDL. They are then transported to peripheral tissues where they undergo lipolysis via the endothelial bound enzyme, lipoprotein lipase. The apoC moieties on VLDL are cofactors for this process. ApoCII is necessary to activate lipoprotein lipase, whilst apoCIII inhibits the enzyme. Lipolysis of the triglyceride core of the particle shrinks

the VLDL particle and results in the formation of either smaller VLDL remnant particles or IDL. The VLDL remnant particles have 2 main fates, they can either undergo further lipolysis to IDL via either lipoprotein lipase or hepatic lipase, or they can be cleared directly from the bloodstream via hepatic receptors, which recognise apoE on the VLDL surface.

In addition to hydrolysis of triglyceride rich lipoproteins, a large amount of exchange of lipids and apolipoproteins takes place between VLDL and HDL. This process is facilitated by cholesteryl ester transfer protein (CETP). Cholesteryl ester is transferred from HDL to VLDL in exchange for triglyceride which transfers in the opposite direction. Thus not only do VLDL particles lose triglyceride via lipolysis, they are further triglyceride depleted whilst being cholesteryl ester enriched under the influence of CETP. IDL is formed, which contains apolipoproteins B and E, and a core which has a reduced triglyceride content and greater quantities of cholesteryl ester. Further lipolysis of the triglyceride in IDL is mediated via hepatic lipase, the triglyceride content is further reduced, apoE is lost and IDL is transformed into LDL.

LDL can be regarded as the end-product of VLDL metabolism. Some direct hepatic secretion of LDL occurs but the majority of LDL particles are formed from VLDL. LDL clearance occurs in both the liver and peripheral tissues, however in humans, the liver is responsible for over 70% of LDL catabolism. Clearance of LDL particles can take place via receptor mediated or receptor independent pathways. The LDL receptor recognises apoB in the LDL particle, the particle is internalised and cholesterol is delivered to the cell. Between 50 and 70% of LDL in normolipaemic subjects is metabolised this way. Not only is the LDL receptor responsible for binding LDL

particles, but along with the enzyme HMGCoA reductase, it plays an integral part in maintaining normal cholesterol homeostasis. If intracellular cholesterol stores are replete, the number of LDL receptors is reduced and HMGCoA reductase activity also decreases. This results in less cholesterol entering the cells via the LDL receptor and reduced de novo production of cholesterol by the cell. On the other hand when cholesterol is needed, upregulation of LDL receptors takes place and activity of the HMGCoA reductase enzyme increases. This increases the amount of cholesterol entering the cell and also increases de-novo production of cholesterol.

The alternative route by which LDL is removed from the circulation is by non-receptor mediated routes or the so called 'scavenger receptor'. This method involves phagocytosis of LDL by macrophages and is uncontrolled. LDL penetrates the endothelium and accumulates both in the extracellular space and in the smooth muscle cells of the arterial intima. LDL is often modified by oxidation pre-phagocytosis and the end product is the development of fatty streaks and ultimately atherosclerotic plaques.

(III) The Exogenous Lipoprotein Transport Pathway

The exogenous lipid transport pathway involves transport of dietary lipid (mainly triglyceride) in chylomicrons from the intestine to the liver. Chylomicrons are synthesised in the villi of the small intestine in response to the absorption of dietary lipid. Chylomicron synthesis is dependent upon production of apolipoprotein B-48. Particles are secreted from intestinal mucosal cells into the lymphatics or the portal circulation. The remainder of chylomicron metabolism follows along very similar lines to that of VLDL. Particles acquire apoC and apoE in the circulation, are

hydrolysed by lipoprotein lipase (which in fact preferentially binds to chylomicrons compared with VLDL), and surface components are transferred to HDL. Chylomicron remnants are taken up by the liver via receptor mediated mechanisms, with the LDL receptor related protein (LRP) recognising apoE on the surface of the chylomicron remnants.

(IV) HDL

HDL is synthesised in the liver as disc like particles known as 'nascent HDL'. These contain phospholipid, free cholesterol and apoA1. HDL takes part in the process of reverse cholesterol transport (fig 1.3). This involves transporting cholesteryl esters from peripheral tissues to the liver, either directly by HDL or indirectly by transfer of cholesteryl ester to VLDL (and ultimately LDL) via CETP. Cholesterol that is transferred from peripheral tissues to HDL is converted to cholesteryl ester by the action of lecithin cholesterol acyl transferase (LCAT). Exchange of cholesteryl ester and triglyceride takes place between HDL and VLDL, with the triglyceride rich HDL particle that is formed, rapidly hydrolysed by hepatic lipase. As a result of this process, it can be seen that a low HDL-C is physiologically linked to increased VLDL, as the rate limiting step for transfer of cholesteryl ester from HDL to VLDL is the availability of VLDL to accept the transferred cholesteryl ester. This explains why such a close inverse correlation is consistently found between plasma triglyceride and HDL-C.

(V) Lp(a) metabolism

Lipoprotein (a) is lipoprotein that consists of an LDL particle that is covalently linked to an apo(a) molecule by a disulphide bond with apoB. This particle was first

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discovered in 1963 [60], when it was considered to be a variant of LDL. Apo(a) is a glycosylated protein that resembles plasminogen. Apo(a) is extremely polymorphic, varying in size from 300-800kDa [61]. Plasma concentration of Lp(a) is under genetic control and is heavily influenced by the size of the apo(a), which is inversely related to plasma level of Lp(a) [61]. Lp(a) and the apo(a) protein are synthesised in the liver with plasma levels regulated by the rate of production rather than clearance of the particle. Less is known about mechanisms by which Lp(a) is cleared from the circulation. The kidney is thought to contribute, with Lp(a) concentration increased in renal failure, however the major sites of Lp(a) catabolism remain to be determined. The normal physiological role of Lp(a) is still not understood, however it is evident that the protein sequence of the apo(a) shows marked homology with plasminogen, and it has been suggested that Lp(a) may represent a bridge between the fields of atherosclerosis and thrombosis [62].

Table 1.1: Properties of Plasma Lipoproteins

Lipoprotein	Density (g/ml)	Flotation Coefficient (Sf)	Size (nm)	Molecular Weight	Major Neutral Lipid
Chylomicrons	<0.94	>400	75-1200	$50\text{-}1000 \times 10^6$	Triglyceride
VLDL	<1.006	20-400	30-80	$10\text{-}80 \times 10^6$	Triglyceride
IDL	1.006-1.019	12-20	25-35	$5\text{-}10 \times 10^6$	Cholesteryl Ester
LDL	1.019-1.063	0-12	18-25	$2\text{-}3 \times 10^6$	Cholesteryl Ester
HDL	1.063-1.21	0-9*	5-12	$65\text{-}386 \times 10^6$	Cholesteryl Ester

* at solvent density 1.20kg/l

Adapted with permission from Ref #162

Table 1.2: The Major Apolipoproteins in Plasma

Apolipoprotein	Particle	Function	Molecular weight
A-I	HDL	Ligand for HDL Receptor, Activator of LCAT	28,000
A-II	HDL	Ligand for HDL Receptor, Cofactor for LCAT	17,500
A-IV	HDL	Possibly involved as cofactor for LCAT	46,000
B-48	Chylomicrons	Forms Structure of Chylomicron	264,000
B-100	VLDL, IDL & LDL	Forms Structure of VLDL, IDL & LDL. Ligand for LDL receptor	512,000
CII	VLDL, Chylomicrons	Activates Lipoprotein Lipase	9000
CIII	VLDL, Chylomicrons	Inhibits Lipoprotein Lipase	9000
E	VLDL, IDL, Chylomicrons, Lipoprotein Remnants	Ligand for LDL Receptor, LDL-RP & VLDL Receptor	34,000

LDL-RP - LDL Receptor Related Protein,   LCAT - Lecithin:cholesterol acyltransferase

Fig 1.1: VERY LOW DENSITY LIPOPROTEIN

apo B-100

Apo CIII

Triglyceride

Unesterified Cholesterol

Phospholipid

Apo CII

Apo E

Cholesterol Ester

DIAMETER : 400-700 Å

Fig 1.2: LOW DENSITY LIPOPROTEIN

apo B-100

Triglyceride

Cholesterol Ester

Unesterified Cholesterol

Phospholipid

DIAMETER : 225-275 Å
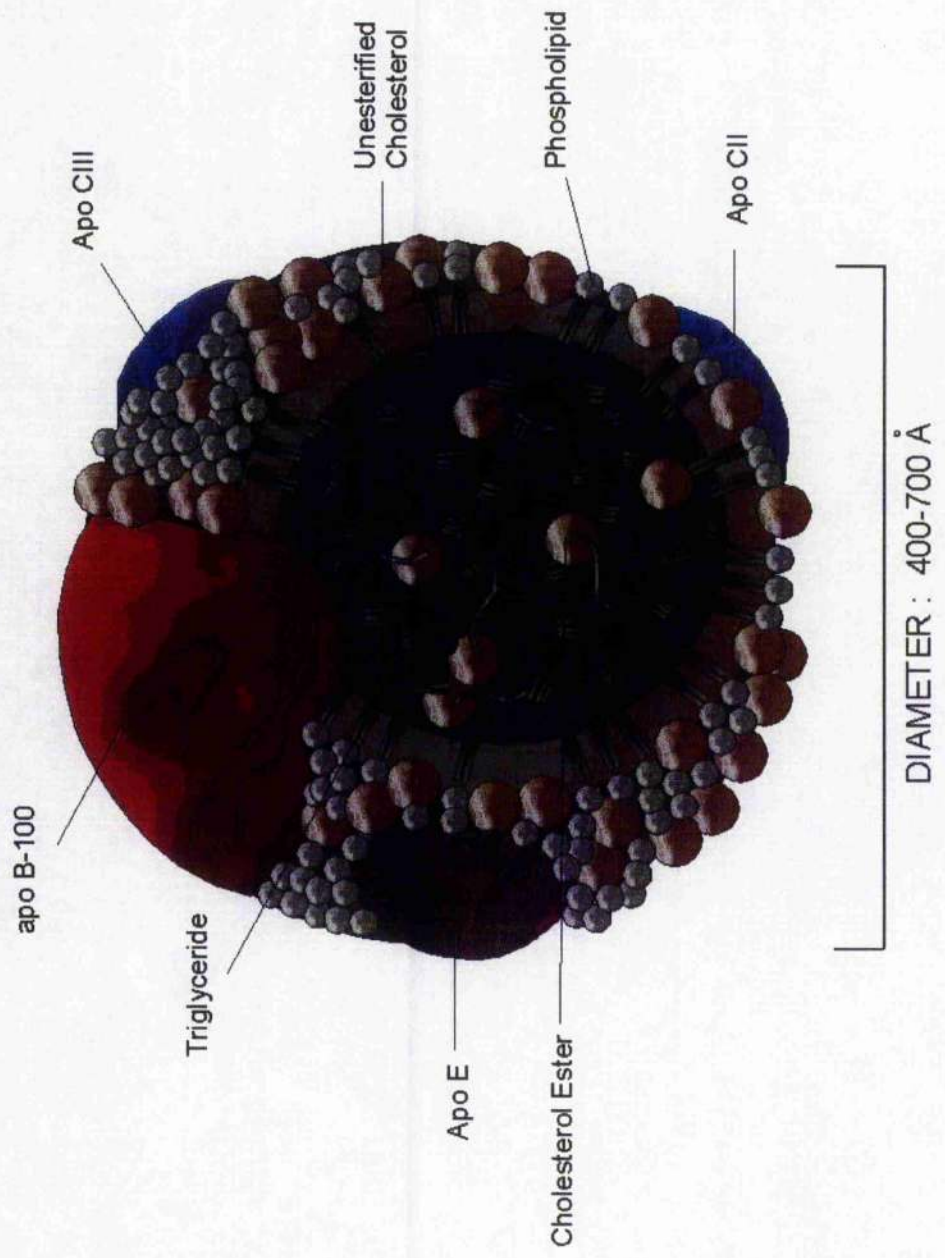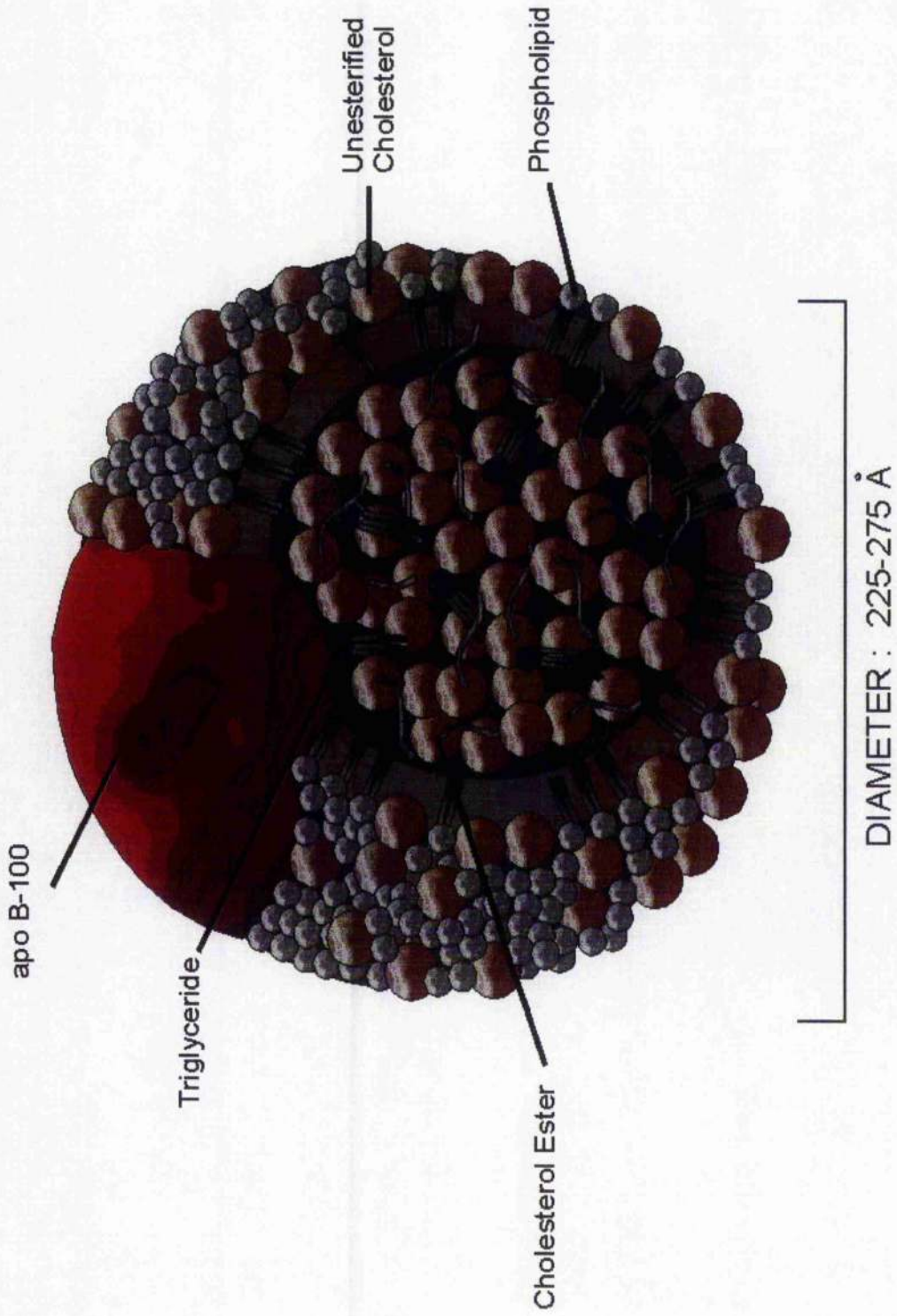
Fig 1.3: ENDOGENOUS LIPID METABOLISM

Enzymes & Receptors
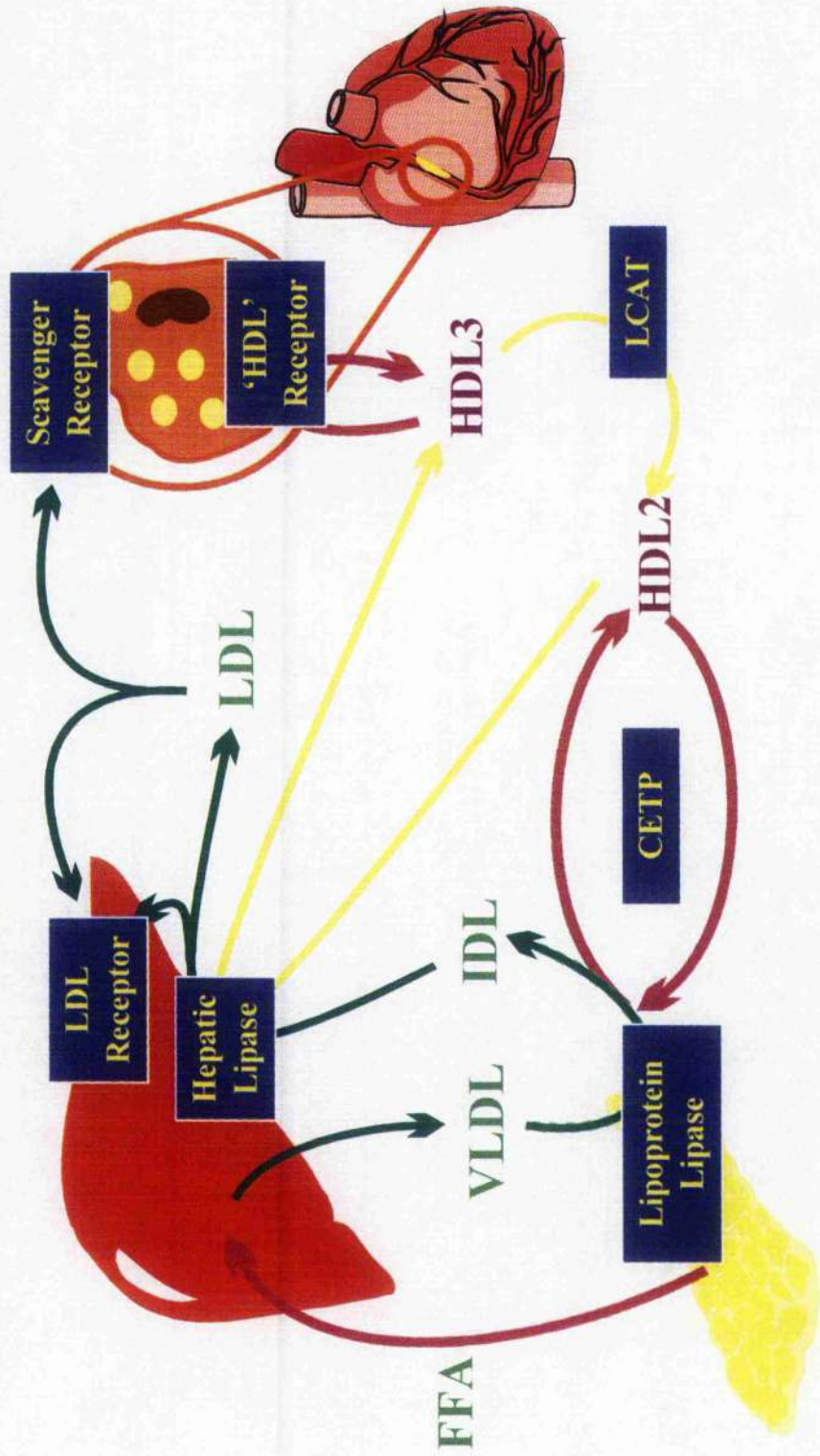
1.4. LIPIDS, LIPOPROTEINS AND CORONARY HEART DISEASE

The influence of dyslipidaemia on CHD has been recognised for some time, with a clear relationship between CHD risk and increasing plasma total cholesterol [63]. A gradient of increased risk of death from coronary heart disease has been shown for total cholesterol levels of >4.7mmol/l, with a 2-3 fold increase for a total cholesterol of 5-6.3mmol/l and a 5 fold increase for a cholesterol >6.3mmol/l [16]. Furthermore, it is clearly established that reducing cholesterol with HMGCoA reductase inhibitors ('statins') reduces morbidity and mortality from CHD in patients with hypercholesterolaemia, both in the context of primary prevention and secondary prevention [64,65]. Moreover for secondary prevention, this benefit extends to patients with total cholesterol levels that would not be regarded as high [66,67]. It has also recently been demonstrated that a similar risk reduction can be obtained using statins as primary prevention in a population with a normal cholesterol and below average HDL-C levels [68].

The relationship between coronary artery disease and raised plasma triglycerides has not been defined as clearly as the risk of hypercholesterolaemia, nevertheless, it is now clear that hypertriglyceridaemia is an independent risk factor for CHD. The role of triglycerides has been clouded by the physiological link between raised triglycerides and a low HDL-C, thus confounding attempts at multivariate analysis when both of these closely linked variables are inserted. Both the PROCAM study [69] and the Helsinki Heart Study [70] have found marked increases in cardiac events in patients with plasma triglyceride >200mg/dl (2.3mmol/l) and a meta-analysis of 17 studies has demonstrated that increased plasma triglyceride is a risk factor for coronary artery disease, even after adjusting for HDL-C [71]. Data from the Helsinki

Heart Study [72] has suggested that lowering plasma triglyceride leads to a reduction in coronary events. In this prospective randomised controlled study, fibrates (Gemfibrozil), whose main action is to increase lipolysis of triglyceride rich lipoproteins, reduced coronary events, with the benefit seen almost exclusively in the subgroup with raised triglycerides and a low HDL.

An inverse relationship exists between the plasma concentration of HDL-C and CHD [73,74], and it has been estimated that a 1mg/dl (0.025mmol/l) decrease in HDL-C increases the risk of coronary artery disease by 2% in men and 3% in women [75]. The cardioprotective role of HDL-C is thought partly to be related to its role in reverse cholesterol transport, as it is known that lipid rich macrophages can have cholesterol removed by HDL [76]. Other potential cardioprotective mechanisms include stabilisation of prostacyclin in the vessel wall [77] or enhancement of endothelial repair [78]. However given the physiological link between low HDL-C and excess triglyceride rich lipoproteins, and the atherogenic effect of these triglyceride rich lipoproteins, it is now felt that part of the cardioprotective effect of raised HDL-C is simply related to it being a marker for efficient lipolysis of triglyceride rich lipoproteins, with the resulting low plasma concentrations of these lipoproteins. It is suggested that, in comparison to HDL-C, the greater variability of plasma triglycerides on repeated testing means that when both variables are inserted into a multivariate analysis, HDL-C rather than triglycerides is chosen as the independent variable with a consistent underestimate of the contribution of triglyceride to the disease state [79].

More recently remnant lipoproteins have been implicated in atherogenesis. These particles are formed from chylomicron or VLDL particles which have been partially

metabolised by lipoprotein lipase. These remnant-like lipoproteins (RLP) are apoE rich, exist across a wide range of densities, and are characteristically found in patients with hypertriglyceridaemia [80]. It is likely that this connection results from hypertriglyceridaemia and increased RLP having a common origin, i.e. inefficient or incomplete lipolysis of triglyceride rich lipoproteins. Increased RLP have been found in patients with NIDDM [81] and patients on haemodialysis [82]. RLP-cholesterol levels are increased in men with coronary artery disease [83]. In addition a high RLP-cholesterol is a predictor for coronary artery stenosis [84], sudden cardiac death [85], and myocardial infarction in vasospastic angina [86]

Most studies show higher concentrations of Lp(a) to be an independent risk factor for CHD [87, 88]. The precise mechanism by which Lp(a) confers its increased coronary risk remains to be elucidated however it has been shown that Lp(a) promotes proliferation of smooth muscle cells [89], is deposited in atherosclerotic plaques [90], and is thrombogenic, competing with plasminogen and decreasing plasminogen activation [91].

1.5. VASCULAR RISK FACTORS, ATHEROGENESIS AND PLAQUE STABILITY

The role that established cardiovascular risk factors play either in initiating atherosclerosis or precipitating acute coronary events can be seen by examining their impact on either the development or the stability of the atherosclerotic plaque. Factors contributing to atherosclerotic plaque development include accumulation of oxidised LDL in the vascular wall, endothelial dysfunction, activation of macrophages,

alteration of smooth muscle cells, platelet activation and pro-coagulant states [92]. A typical atherosclerotic plaque (fig 1.4) has a lipid rich core (derived from oxidised LDL which has been endocytosed by macrophages to form foam cells), macrophages (often in the form of foam cells) which surround the lipid core, and a fibrous cap (consisting mainly of collagen but also containing smooth muscle cells) [93]. The majority of acute coronary events occur in unstable plaques which rupture, resulting in thrombus formation and coronary artery occlusion. Unstable plaques are characterised by having large, highly thrombogenic, lipid rich cores, thin fibrous caps with few smooth muscle cells or collagen fibres, large numbers of inflammatory cells (including macrophages and lymphocytes) within and surrounding the cap (fig 1.4), and areas of endothelial denudation [93-95]. In contrast, stable plaques have a thick fibrous cap with large numbers of smooth muscle cells and collagen fibres, and a smaller lipid core with few inflammatory cells [93-95]. Vascular risk factors can help trigger coronary events by effecting plaque stability, by contributing to plaque rupture via changes in blood flow or endothelial function or by aiding propagation of thrombus.

Fig 1.5 summarises the effect of vascular risk factors on plaque stability, rupture and thrombus propagation. LDL is an essential component in atherosclerotic plaque development. Plasma cholesterol concentration is closely related to the concentration of LDL in the aortic intima [96], cholesterol enrichment of an atherosclerotic plaque increases plaque instability, and the beneficial effects of cholesterol lowering is related to a reduction in the lipid-rich core of plaques [97]. Patients with hypertriglyceridaemia develop smaller denser LDL particles which penetrate endothelium more easily and are more susceptible to oxidation, both essential process

in the formation of atherosclerotic plaques [55,56]. Lp(a) is found in atherosclerotic plaques, increases local cholesterol deposition, competes with plasminogen and accumulates in thrombi [61]. Hypertension is associated with endothelial dysfunction, however evidence suggests that hypertension also plays a key role in triggering acute plaque disruption, causing shear stress on the arterial wall [94]. Concentrations of angiotensin II (a potent vasoconstrictor) are often raised in hypertensives, contributing to endothelial dysfunction and promoting atherogenesis by stimulating smooth muscle growth. [98]. The effect of cigarette smoking on coronary heart disease results from alteration in haemodynamics, vasomotor tone, platelet activation, increased fibrinogen and decreased oxygen carrying capacity of the blood [99,100]. Diabetes mellitus is frequently associated with dyslipidaemia and hypertension, however both insulin and the glycation of lipoproteins may also increase cholesteryl ester accumulation in macrophages and in the arterial wall [101], and both platelet activity and coagulation are enhanced in diabetes [94]. It is known that high homocysteine concentrations are associated with a prothrombotic state, homocysteine is also toxic to endothelium and reduces nitric oxide availability (thus impairing vasodilatation) [98], whilst increased levels of fibrinogen, factor VII, VWF and PAI-1 will all increase the propensity for thrombus to form on the vessel wall. Thus it is evident that the epidemiological associations between these recognised risk factors and either atherogenesis or acute coronary events can be explained by examining the contribution that the recognised risk factors make to plaque instability, rupture or to the propagation of thrombus.

Fig 1.4: CHARACTERISTICS OF UNSTABLE AND STABLE PLAQUES

Unstable

Inflammatory Cells

Thin Fibrous Cap

Few SMCs

Eroded Endothelium

Activated Macrophages

Cholesterol Enrichment

Stable

Lack of Inflammatory Cells

Thick Fibrous Cap

More SMCs

Intact Endothelium

Foam Cells
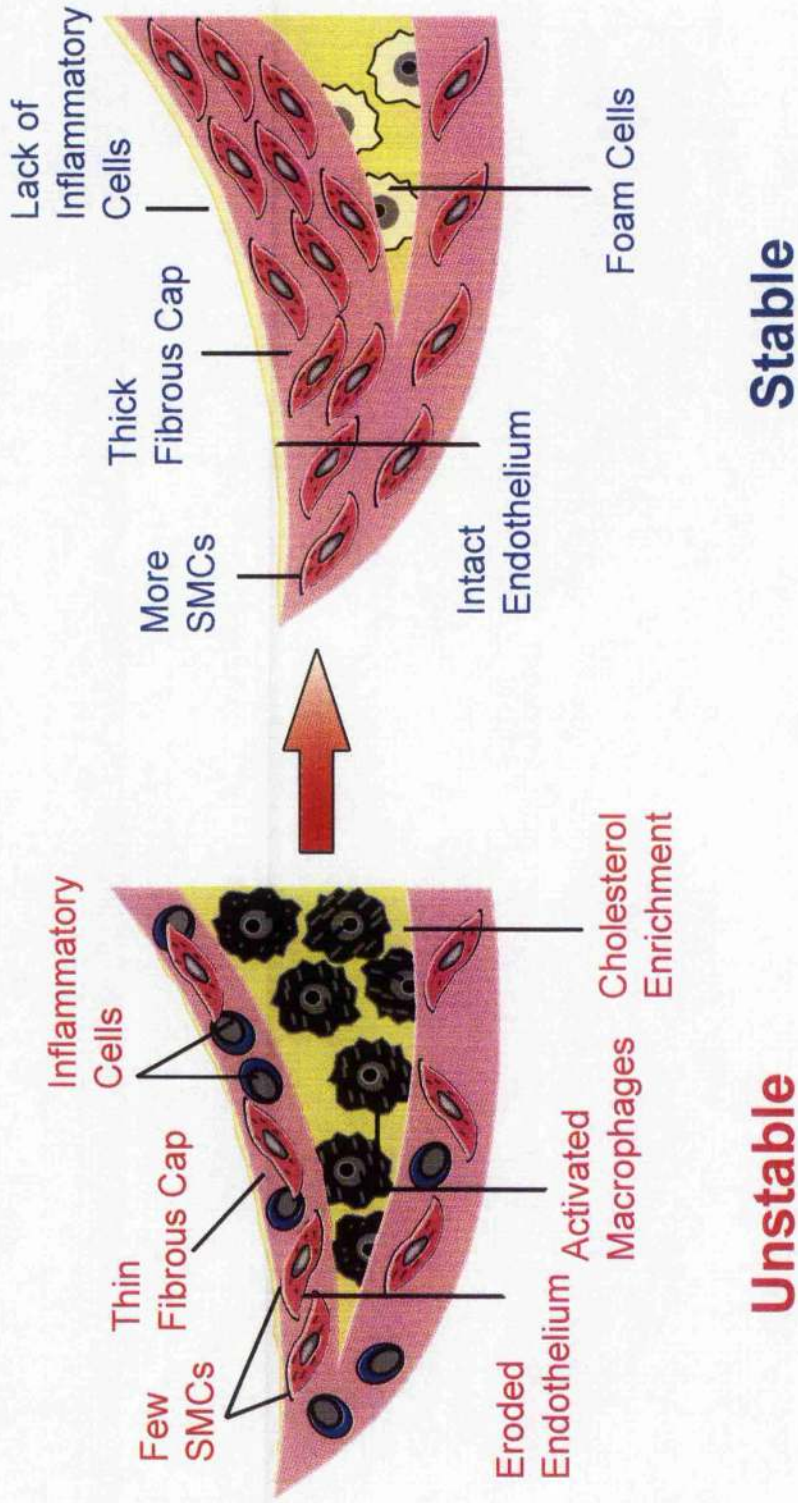
Fig 1.5: VASCULAR RISK FACTORS & PLAQUE STABILITY

1. Hypercholesterolaemia - Lipid Rich Core

2. Raised Triglycerides - Oxidised LDL

3. Hypertension - Endothelial Dysfunction, Shear Stress

4. Smoking - Endothelial Dysfunction, Platelet Activation, Increased Fibrinogen

5. Glucose Intolerance - Hypertension, Dyslipidaemia, Increased Thrombosis, Platelet Activation, Lipid Rich Macrophages.

6. Homocysteine - Pro-Thrombotic State, Endothelial Dysfunction .

7. Increased Fibrinogen, PAI-1, Factor VII, VWF - Increased Thrombosis

Lumen

Smooth Muscle Cells

Macrophages
Endothelium
Platelets
Lipid Core

1.6. DYSLIPIDAEMIA IN RENAL DISEASE

The prevalence, extent and origin of dyslipidaemia in chronic renal disease varies with the cause of the renal dysfunction, the level of renal impairment and the degree of proteinuria. One of the first reports documenting the connection between hyperlipidaemia and the nephrotic syndrome was published in 1917 [102], although the association between chronic renal disease and hyperlipidaemia has been recognised since the 19th century [103]. In nephrotic range proteinuria both quantitative and qualitative changes in lipids and lipoproteins exist. Total cholesterol and LDL cholesterol (LDL-C) are invariably raised [104,105]. Triglycerides may also be increased [106] and very low density lipoprotein cholesterol (VLDL-C) is raised [104,105], even in patients with proteinuria, normal serum albumin and normal renal function [107]. This dyslipidaemia results from a combination of increased production and reduced clearance of apoB containing lipoproteins. VLDL is overproduced, lipolysis of VLDL to IDL and LDL is delayed, and receptor mediated clearance of LDL reduced [104].

Despite extensive animal and human study, the underlying cause of the alterations in the production and clearance of lipoproteins in patients with both the nephrotic syndrome and nephrotic-range proteinuria remains to be clarified. It has been postulated that the increase in production of apoB containing lipoproteins simply reflects a general increase in hepatic protein synthesis consequent of having excess loss of plasma proteins. However not only is albumin synthesis not increased in the nephrotic syndrome [104] but metabolic studies have shown that changes in apoB synthesis do not match changes in albumin synthesis [108]. The explanation for the impaired clearance of VLDL also remains to be fully explained. Reported activity of

both lipoprotein and hepatic lipase enzymes varies [104,105,109]. It has been postulated that loss of cofactors (e.g. apoCII) in the urine might account for the impaired lipolysis [110], however this would not explain the finding of normal clearance of chylomicrons in the context of impaired clearance of VLDL in patients with the nephrotic syndrome [104].

The extent of dyslipidaemia in non-nephrotic patients with moderate chronic renal failure is less well defined. Increased plasma triglyceride and low HDL-C are the characteristic abnormalities in advanced renal failure however the level of renal function at which abnormal lipid levels become more prevalent is less clear and studies performed tend to have only small numbers of patients. Attman reported that triglycerides tended not to be increased when GFR was greater than 15-20ml/min [111]. However Grutzmacher suggested that triglycerides may be increased when GFR is less than 30ml/min [112] and Arnadottir reported increased triglycerides in 26 patients with non-nephrotic proteinuria and a GFR of 50ml/min [113]. In end-stage renal failure (ESRF) hypertriglyceridaemia and low HDL-C are typical [114,115], very-low density lipoprotein cholesterol (VLDL-C) is raised, however levels of total cholesterol and low-density lipoprotein cholesterol (LDL-C) are generally normal and may be low [114,115]. The primary metabolic defect is thought to be impaired catabolism of triglyceride rich lipoproteins (mostly VLDL) by the enzymes lipoprotein and hepatic lipase, and studies have found activity of lipoprotein and hepatic lipase to be low [114]. However the precise mechanism underlying the impaired lipolytic activity remains to be established [114,116,117]. The lipoprotein abnormalities also differ according to modality of renal replacement therapy with patients on peritoneal dialysis tending to have higher total cholesterol and LDL-C compared with those

treated by haemodialysis [118].

1.7. LIPIDS IN RENAL DISEASE: CARDIOVASCULAR AND CORONARY RISK

In contrast to the data linking dyslipidaemia to coronary artery disease, and despite the high prevalence of coronary artery disease in patients with chronic renal dysfunction, direct epidemiological evidence linking dyslipidaemia to coronary artery disease in this population remains limited. This is partly due to the relatively small numbers of patients with chronic renal disease compared to other organ dysfunction (e.g. diabetes mellitus) but is also due to the extensive comorbidity present in this population. This has clouded links between mortality from CHD and dyslipidaemia to the extent that renal physicians have been reluctant to embark on lipid lowering therapy due to the link between a low cholesterol and decreased survival [119], a link that is now recognised to result from excess comorbidity in the study population.

To our knowledge, at present, no epidemiological studies have examined the relationship between dyslipidaemia and either cardiovascular disease or coronary artery disease in patients with nephrotic range proteinuria. Clearly patients with nephrotic range proteinuria or dipstick positive proteinuria have increased coronary artery disease [3,7], however the extent to which dyslipidaemia contributes to this increased risk remains to be established. No prospective trials looking at cardiovascular end-points in patients with proteinuria that is not secondary to diabetic nephropathy, have been undertaken. However in patients with persistent proteinuria, it

is difficult to imagine that the significant dyslipidaemia resulting from persistent heavy proteinuria will not contribute to the development of atherosclerosis.

There is extensive evidence linking dyslipidaemia with cardiovascular and coronary artery disease in both IDDM and NIDDM, with raised total cholesterol, LDL-C and plasma triglycerides, along with a low HDL-C, all risk factors for coronary artery disease [120-129]. Moreover in insulin-dependent diabetics with proteinuria, a link between CHD and dyslipidaemia has been established. In patients with IDDM and persistent proteinuria, CHD is increased compared with those without proteinuria, moreover the risk of CHD is associated with high total cholesterol levels [10]. Similarly in NIDDM, Stephenson reported that proteinuria was associated with both raised cholesterol and excess cardiovascular disease (the vast majority of which were due to ischaemic heart disease), however the increased cardiovascular mortality was independent of the increased cholesterol [6]. In diabetics with end-stage renal failure, a low HDL-C has been found to be a risk factor for CHD in a study looking at 110 patients with insulin dependent diabetes [130], whilst data from 412 type 1 and type 2 diabetic patients on dialysis again found a low HDL-C to be an independent predictor of cardiac and non-cardiac mortality [131], although the same authors in a study of coronary angiography in 105 diabetics on renal replacement therapy failed to show a difference in lipid profiles between patients with and without CHD [132].

An increasing number of studies have identified dyslipidaemia as a risk factor for either cardiovascular disease or coronary artery disease in chronic renal failure. In a study looking at patients with chronic but not end-stage renal failure, Jungers found that patients with myocardial infarction had higher triglycerides, LDL-C,

apolipoprotein B and Lp(a), with a lower HDL-C [8]. In this study, HDL-C was an independent risk factor. A number of studies have highlighted the importance of triglycerides and HDL-C in patients on haemodialysis. In a study of 252 chronic haemodialysis patients, those with CHD had higher plasma triglyceride and lower HDL-C compared with those without CHD [133]. In a prospective study which followed 419 patients (1% diabetic) on chronic haemodialysis for a mean follow-up of 6 years, plasma triglyceride was found to be an independent risk factor for ischaemic cardiovascular disease [134]. In a recent cross sectional study looking at 607 haemodialysis patients (33% diabetic), independent predictors of coronary artery disease included a low HDL-C, increased plasma apoB and apo(a) phenotype [135]. The largest study looking at Lp(a) in end-stage renal failure prospectively followed up 129 patients for 4 years and found that high Lp(a) levels predicted atherosclerotic cardiovascular events [136].

Overall, although data remains limited, the risk of coronary artery disease posed by dyslipidaemia in chronic renal disease is beginning to emerge. On multivariate analysis, the most frequently quoted parameter contributing to increased risk is a low HDL-C. However most of these studies insert plasma triglyceride and HDL-C into the same multivariate equation and are therefore liable to the same underestimation of the importance of plasma triglyceride that has been explained by Austin [79]. The relevance and contribution of increased plasma triglyceride as compared with reduced HDL-C can be illustrated by performing more detailed lipoprotein analysis in order assess subfractions of the lipoprotein density classes.

1.8. HETEROGENEITY OF APOB CONTAINING LIPOPROTEINS AND THEIR ROLE IN ATHEROSCLEROSIS

Although the traditional classification of lipoproteins divides them according to their density into VLDL, IDL, LDL and HDL, within each density range these lipoproteins exhibit marked heterogeneity in terms of size, density and composition. The first studies of low-density lipoprotein heterogeneity in normal populations, divided LDL by size using gradient gel electrophoresis [137]. As a result it was found that LDL consisted of a discrete number of subfractions with particular sizes and densities. Patients who had LDL that was mostly large were labelled as having 'Pattern A', whilst those with small LDL had 'Pattern B' [55]. Alternatively, in order to quantify the amount of each LDL subfraction present, LDL can be separated by density gradient ultracentrifugation into three main subfractions (LDLI d=1.025-1.034g/ml, LDLII d=1.034-1.044 and LDLIII d=1.045-1.060) [138]. LDLI predominates in premenopausal females, the most common subfraction for males is LDLII (fig 1.6), whilst an increasing number of studies have demonstrated a link between CHD and either the small dense LDL phenotype (Pattern B) or an increase in plasma LDLIII concentration. In a case control study of survivors of myocardial infarction, 50% of cases had pattern B compared with 26% of controls [139]. Multivariate analysis demonstrated that plasma triglycerides and HDL-C were associated with formation of small dense LDL and contributed to the risk associated with the small dense subfraction pattern [139]. Thus as a result of this and other studies, the combination of excess small dense LDL, raised plasma triglycerides and low HDL-C have come to be known as the **ATHEROGENIC LIPOPROTEIN PHENOTYPE** [55,56,140]. The risk of excess small dense LDL has been further quantified in cross-sectional studies,

and an LDLIII level greater than 100mg/dl has been shown to confer a seven times increase in the risk of myocardial infarction [141].

Fig 1.6: TYPICAL LDL SUBFRACTION PROFILES

LDLI   II   III

Premenopausal Female

Male

CAD

Absorbance 280nm

1.025   1.034   1.044   1.060g/ml
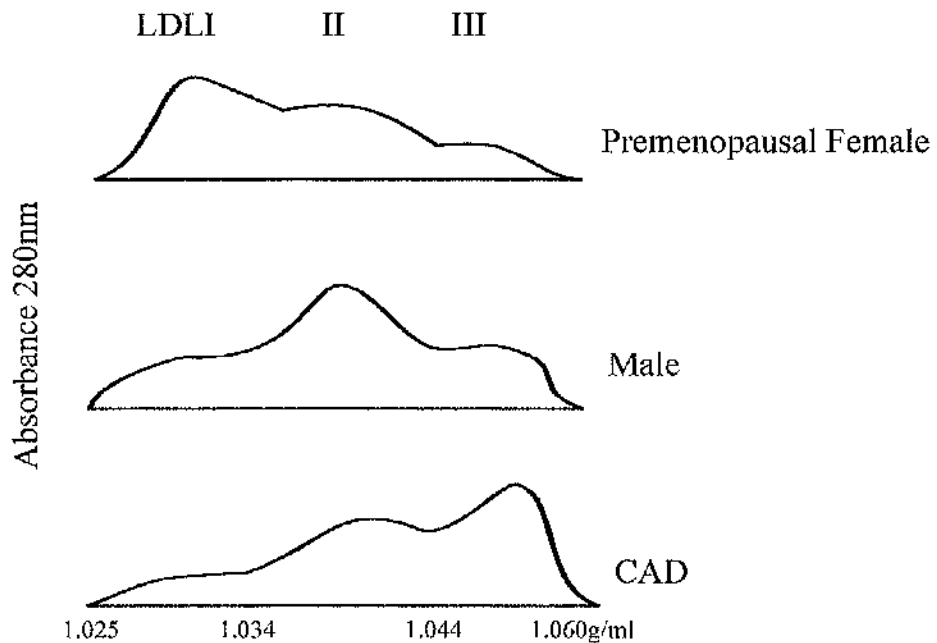
The atherogenic risk of small dense LDL has been further substantiated by 2 more recently published prospective studies. Data from the Physician's Health Study cohort has shown that cases of MI have a significantly smaller LDL diameter compared with controls [142], whilst Gardner in the same edition of the Journal of the American Medical Association demonstrated that in men and women, LDL size is smaller in patients with coronary artery disease compared with controls [143]. Both of these studies used non-fasting samples, and in the second study LDL size was found to be independent of other lipid parameters, despite the close links that have been demonstrated between LDL size and plasma triglyceride [144].

In addition to LDL, VLDL can also be divided into 2 subfractions. This is generally performed using cumulative-gradient ultracentrifugation, with VLDL subfractions being divided by their flotation coefficients. $VLDL_1$ (Sf 60-400) is larger, lighter and triglyceride rich, $VLDL_2$ (Sf 20-60) is smaller, denser and more enriched in cholesteryl ester. Production of $VLDL_1$ and $VLDL_2$ is regulated independently. $VLDL_1$ production is increased in patients with hypertriglyceridaemia, in patients with insulin resistance [145] and following carbohydrate loading [146]. As a result excess $VLDL_1$ is associated with raised triglycerides [144] and excess partially metabolised atherogenic lipoprotein remnants. $VLDL_2$ is overproduced in patients with raised total and LDL cholesterol [56]. $VLDL_1$ is slowly cleared from the circulation, $VLDL_2$ on the other hand is rapidly metabolised to LDL and increased $VLDL_2$ formation is the main cause of the excess LDL found in common types of hypercholesterolaemia [56]. The importance of excess $VLDL_1$ is further highlighted by looking at the physiological relationship between $VLDL_1$ and LDL subfractions, as formation of excess small dense LDL (LDLIII) requires an increase in plasma triglyceride (in the form of $VLDL_1$), along with adequate plasma activity of hepatic lipase and cholesteryl ester transfer protein (CETP) [56].

1.9. THE ATHEROGENIC LIPOPROTEIN PHENOTYPE IN RENAL DISEASE

(I) Background

It can therefore be seen that not only are small dense LDL, plasma triglycerides and a low HDL-C physiologically linked (the atherogenic lipoprotein phenotype) but small

dense LDL is associated with an increase in the risk of coronary artery disease. To assess whether patients with proteinuria possessed excess small dense LDL, we performed a pilot study in 12 patients with nephrotic range proteinuria and relatively well preserved renal function. The most important factor influencing LDL particle size is generally the plasma triglyceride level, therefore our initial hypothesis was that the excess of triglyceride rich lipoproteins found in this population would result in increased amounts of small dense LDL being present. We found that the patients studied had LDLIII levels twice those of the normal controls, and at a concentration known to be associated with increased atherogenic risk in normal populations (mean 135mg/dl). This excess of LDLIII resulted from a shift in particle size towards smaller denser particles, as the total LDL concentration did not differ between the two study populations [109]. In this pilot study, we also demonstrated that both $VLDL_1$ and $VLDL_2$ subfractions were increased in this population, confirming data previously published from our laboratory [104]. This study therefore suggested that in addition to hypercholesterolaemia, the presence of excess small dense LDL in patient with nephrotic-range proteinuria was an additional atherosclerotic risk factor.

(II) Triglyceride Rich Lipoproteins and Small Dense LDL

In normal populations, excess triglyceride rich lipoproteins are essential for the formation of atherogenic levels of small dense LDL. Current models of lipoprotein metabolism suggest that adequate plasma activity of hepatic lipase and cholesteryl ester transfer protein are also required. A further consequence of impaired clearance of triglyceride rich lipoproteins in normal populations is the presence of excess remnant lipoproteins. Remnants of VLDL have not been studied in this population, however given the impaired VLDL clearance in patients with proteinuria, it is likely

that these lipoproteins will be present in excess. Remnants may also contribute to the atherogenic risk attributed to the atherogenic lipoprotein phenotype.

(III) Dyslipidaemia and Progression

In addition to atherosclerotic disease, dyslipidaemia may contribute to progression of renal disease. There are pronounced pathogenetic similarities between atherosclerosis and glomerulosclerosis [147]. Proteinuria is the most important risk factor for progression of chronic renal failure and the associated dyslipidaemia may contribute to this progression [148]. Several reports have suggested a relationship between lipoprotein abnormalities and progression of renal disease [149-151]. Thus a cyclical decline may occur in which renal disease generates dyslipidaemia which in turn aggravates the renal disease. LDL is seen as the prime candidate promoting the pathological changes in both the arterial wall and the kidney.

(IV) Lipid Lowering Therapy and Proteinuria

HMG CoA reductase inhibitors (statins) and fibrates have been shown to be effective lipid lowering agents in proteinuric dyslipidaemia, although there has been some concern expressed about the use of fibrates in severe hypoalbuminaemia, due to the extent of their protein binding. Statins reduce total cholesterol and LDL-C by preventing intracellular cholesterol synthesis and as a result, upregulating the LDL receptor. This has the net result of increasing influx of LDL from plasma to the liver. It is thought that the main effect of fibrates is to suppress apolipoprotein CIII (apoCIII) synthesis and promote lipoprotein lipase activity [152]. Thus their action leads to a reduction in plasma triglyceride and triglyceride rich lipoproteins. In normal populations, statins reduce small dense LDL by lowering all 3 LDL subfractions in

concert [153]. Fibrates on the other hand, promote a shift in LDL size towards larger lighter particles, with or without a change in total LDL [154,155]. Statins have been shown to reduce cholesterol and LDL-C in patients with nephrotic dyslipidaemia [104]. This cholesterol lowering effect is accompanied by plasma triglyceride reduction and increased receptor mediated LDL clearance [156]. Small dense LDL formation is a consequence of mild increases in plasma triglyceride [56], and therefore the triglyceride lowering effect of statins may have a beneficial effect not only on LDLIII concentration but also on the relative proportions of each LDL subfraction and on remnant lipoproteins.

(V) NIDDM, Proteinuria and Dyslipidaemia

A strong relationship exists between vascular disease and both plasma triglyceride and LDL-cholesterol in NIDDM. Patients with NIDDM typically have smaller LDL and excess quantities of LDLIII [157,158], and therefore possess the atherogenic lipoprotein phenotype. It has recently been suggested that patients with diabetic nephropathy and macroalbuminuria possess LDL that is smaller than normal diabetic controls [159], however the extent to which small dense LDL is formed in diabetic nephropathy and macroalbuminuria secondary to NIDDM has not been quantified.

(VI) Small Dense LDL in ESRF

Proteinuria is the most consistent risk factors for progression of renal failure in glomerular disease. Qualitative analysis of LDL in end-stage renal failure (ESRF), [160,161] has been published suggesting an increase prevalence of small dense LDL in ESRF. Plasma triglyceride has been shown to be an independent risk factor for cardiovascular disease in ESRF, however the relationship between triglyceride rich

lipoproteins, lipase activity and small dense LDL in this population has not been explored.

Therefore, we planned to explore in more detail lipoprotein heterogeneity in chronic renal disease, with a particular focus on small dense LDL in patients with proteinuric renal disease. In addition to the focus on the atherogenic lipoprotein phenotype, we hoped to highlight the importance of detailed lipoprotein analysis in order to fully assess cardiovascular risk in a population with a high risk of coronary artery disease.

1.10. HYPOTHESIS

Our hypothesis is that in patients with proteinuria, hypertriglyceridaemia occurs through impaired catabolism of VLDL. The primary consequence is the generation of small dense LDL which is a pathogenic agent contributing both to rate of progression of renal failure and accelerated vascular disease. A further consequence of the impaired clearance of triglyceride rich lipoproteins is that excess atherogenic remnant lipoproteins may also be formed. These remnants may be associated with excess small dense LDL and contribute to the increased vascular risk attributed to small dense LDL.

The main population studied in this project were patients with excess proteinuria, generally secondary to primary glomerular disease, but also secondary to NIDDM. Given that proteinuria is consistently the most important factor influencing the renal prognosis in this population, a further study was performed to assess the effect of end-stage renal failure on the quantity and quality of LDL.

1.11. AIMS

The aims of this project are encapsulated in the following questions.

1. To what extent are LDLIII and remnant lipoproteins present in patients with nephrotic range proteinuria? What is the origin of these abnormal lipoproteins in patients with nephrotic range proteinuria? In particular we planned to investigate differences in plasma triglyceride, VLDL subfractions, activity of cholesterol ester transfer protein (CETP), hepatic and lipoprotein lipases whilst attempting to identify if there is any relationship between the plasma concentration of LDLIII, remnant lipoproteins and both renal function and proteinuria.

2. In normal populations, excess $VLDL_1$ is essential for the development of atherogenic levels of LDLIII. Are there specific abnormalities in the composition of $VLDL_1$, that may account for the delayed clearance of the lipoprotein in patients with nephrotic range proteinuria? Our laboratory has previously shown activity of lipoprotein lipase to be normal in this population, we therefore we planned to investigate plasma and $VLDL_1$ apolipoprotein CII, CIII and E levels, along with the lipid composition of $VLDL_1$ particles.

3. What are the effects of treatment with lipid lowering agents, on the lipoprotein subfraction profile and remnant lipoproteins in patients with proteinuria. The aim of this study therefore, was to compare the ability of a statin (cerivastatin) and a fibrate (fenofibrate) to lower levels of these atherogenic lipoproteins in patients with nephrotic range proteinuria.

4. Is there a relationship between rate of deterioration of renal function and dyslipidaemia? If there is, does this relationship involve subfractions of either LDL or VLDL ? Examination of this issue requires further investigation into the

relationships between lipoprotein subfraction profiles, level of renal function, and degree of proteinuria in patients with glomerular disease, heavy proteinuria and progressive renal failure.

5. Does the development of diabetic nephropathy in NIDDM result in changes in the atherogenic lipoprotein phenotype? Do any changes develop with the onset of microalbuminuria or frank proteinuria? This study will involve assessing small dense LDL formation across the spectrum of NIDDM and nephropathy, from normoalbuminuria to frank proteinuria, and comparison with non-diabetic controls.

6. What is the prevalence of small dense LDL in patients with end-stage renal failure, and how does the quantity and quality of LDL differ between patients who have not yet commenced dialysis, those on haemodialysis and those on peritoneal dialysis? This study will also involve assessing factors that determine LDLIII formation in end-stage renal failure and compare VLDL and LDL subfractions in patients on haemodialysis and peritoneal dialysis to see if this provides further information regarding the origin of the different lipid and lipoprotein profiles obtained in these 2 populations.

CHAPTER 2 - METHODS

All analyses of lipids and lipoproteins were performed in either the lipid research laboratory or the routine lipid laboratory in the Department of Pathological Biochemistry, Glasgow Royal Infirmary University NHS Trust.

2.1. B QUANTIFICATION OF LIPOPROTEINS

Plasma cholesterol, triglyceride, VLDL cholesterol, LDL cholesterol and HDL cholesterol were analysed by a modification of the standard Lipid Research's Clinics protocol [163]. 5ml of plasma is overlayered with d<1.006 solution and centrifuged at 35,000 rpm for 16 hours. The solution is split into an upper VLDL fraction and a lower fraction containing LDL and HDL. Heparin and Manganese Chloride is used to precipitate LDL in the lower fraction leaving HDL in solution. Cholesterol measurements are performed on the total plasma, the top VLDL fraction, the total bottom fraction, and the bottom fraction with the precipitate removed (following centrifugation). Thus LDL is a calculated measured value. i.e. Total Cholesterol - (VLDL cholesterol + HDL cholesterol).

2.2. VLDL, VLDL SUBFRACTIONS AND IDL

VLDL$_1$ (S$_f$ 60-400), VLDL$_2$ (S$_f$ 20-60) and IDL (S$_f$ 10-20) were isolated from fresh plasma by a modification of the cumulative-gradient ultracentrifugation procedure [164]. Stock solutions of density 1.006g/ml and 1.182g/ml were prepared using NaCl, NaBr and Na$_2$EDTA. These were used to make up 6 density solutions, which ranged from 1.0588 to 1.0988g/ml. The density of 2mls of plasma was adjusted to 1.118g/ml

by the addition of 0.341g NaCl. The density solutions were then carefully overlayered on the plasma as detailed in table 2.1. Ultracentrifugation of the sample was performed, with the speeds and times shown in table 2.2. At the end of each run, samples were carefully removed using a long-tipped glass pipette. $VLDL_1$ is removed in 1ml, with $VLDL_2$ and IDL removed in 0.5ml aliquots.

Table 2.1: Density Gradient for Separation of $VLDL_1$, $VLDL_2$ and IDL

Solution	Density (mg/l)	Volume (mls)	Position
6	1.0588	2	Top
5	1.0641	2	
4	1.0722	2	
3	1.0790	2	
2	1.0860	1	
1	1.0988	1	
Plasma	1.118 (adjusted)	2	
	1.182	0.5	Bottom

Adapted with permission from Ref #162

2.3. LOW DENSITY LIPOPROTEIN

LDL was isolated from fresh plasma by sequential ultracentrifugation [162]. The density of 2mls of plasma was increased to 1.109g/ml by the addition of a solution of density 1.182g/ml. The resulting solution was overlayered using 1.019g/ml salt solution and the sample underwent centrifugation overnight for 18hrs on a fixed angle rotor at 15°C, 35000rpm. The top 2mls (containing VLDL and IDL was removed),

further 1.182g/ml solution was added followed by overlayering with 1.063g/ml solution. Again, the sample was spun overnight at 15°C, 35000rpm and the top 1ml removed (LDL d 1.019-1.063 g/ml).

Table 2.2: Conditions for Cumulative Ultracentrifugation of VLDL₁, VLDL₂ and IDL

	Sf	Speed (rpm)	Time (hours:min)
VLDL₁	60-400	39,000	1:38
VLDL₂	20-60	18,500	15:41
IDL	12-20	39,000	2:35

Adapted with permission from Ref #162

2.4. CHOLESTEROL, TRIGLYCERIDE, PHOSPHOLIPID AND PROTEIN

The triglyceride, free cholesterol, cholesteryl ester, phospholipid and protein content of the VLDL₁, VLDL₂, IDL and LDL collected, were assayed and the lipoprotein concentrations were calculated as the sum of these components (concentration = protein + free cholesterol + esterified cholesterol + phospholipid). Protein was analysed by a modification of the method by Lowry et al. [165]. Cholesterol in plasma is present in esterified and free form. Free cholesterol is 22-30% of total cholesterol. Total cholesterol was measured by enzymatic hydrolysis of cholesterol esters to form free cholesterol, with subsequent oxidation to give hydrogen peroxide, which is quantitated by formation of a coloured product (quinineimine) at 505nm (Boehringer Mannheim, Germany, Kit No: 1491458). Free cholesterol was assayed using the same method but omitting the enzyme cholesterol esterase (which would otherwise react

with the cholesterol esters: Boehringer Mannheim, Germany Kit No: 310328).

Cholesterol ester was calculated as the total cholesterol - free cholesterol. Triglyceride

was assayed by enzymatic hydrolysis with subsequent enzymatic determination of

liberated glycerol by colorimetry at 505 nm (Boehringer Mannheim, Germany, Kit

No: 1730711). Phospholipid was determined by a enzymatic colorimetric assay

method measuring absorbance at 500nm (Boehringer Mannheim, Germany, Kit No:

691844), absorbance is proportional to the amount of phospholipids present. Note this

kit only measures phosphatidyl choline, the most abundant phospholipid, and so the

values obtained are estimates of total phospholipid content.

2.5. LDL SUBFRACTIONS

Three LDL subfractions were isolated from fresh plasma by nonequilibrium density

gradient ultracentrifugation as previously described [138]. Following centrifugation

for 24h at 40,000rpm and 23°C in a swinging bucket rotor, the contents of the

centrifuge tube were eluted by upward displacement and the presence of 3 fractions

(LDL I d 1.025-1.034 g/ml, LDL II d 1.034-1.044 g/ml and LDL III d 1.044-1.063

g/ml) were detected by continuous monitoring at 280nm. The individual subfraction

areas were quantified, corrected for previously calculated extinction coefficients and

expressed as percentage of total LDL. The lipoprotein mass of LDL (d 1.019-1.063

g/ml) was determined as above and used to generate individual subfraction

concentrations in mg lipoprotein per 100ml plasma.

2.6. LIPOPROTEIN AND HEPATIC LIPASE

Lipoprotein and hepatic lipase activities were measured in post-heparin plasma

incubated with a gum arabic-stabilized triglyceride emulsion containing glycerol tri(1-

^{14}C) oleate at a specific activity of 30μCi/mmol triglyceride fatty acids as substrate [166]. For the specific assay of LPL, plasma was preincubated with sodium dodecyl sulfate to inhibit HL, and pooled pre-heparin plasma was added to the incubation as a source of cofactor apoCII to activate the LPL enzyme. HL was assayed in the presence of 1.0 mol/l NaCl to inactivate LPL. Enzyme activities are expressed in μmol of fatty acids released per hour per ml of plasma.

2.7. CHOLESTERYL ESTER TRANSFER PROTEIN

Cholesteryl Ester Transfer Protein (CETP) was assayed using a CETP assay kit from Roar Biomedical Inc. HDL containing fluorescein labelled cholesteryl ester was incubated with plasma from the subjects studied. The fluorescent labelled lipid is contained in the core of the HDL particle. CETP mediated transfer of cholesteryl ester from HDL to VLDL was determined by the increase in fluorescence as the labelled lipid is moved from the core of the HDL particle to the acceptor VLDL particle. The assay was read in a fluorescence spectrometer. The activity is expressed as pmol cholesteryl ester transferred per ml of plasma.

2.8. REMNANT-LIKE PARTICLES

The cholesterol and triglyceride in remnant like particles (RLP-C, RLP-TG) were measured using a diagnostic reagent kit from Japan Immunoresearch Laboratories (JIMRO), Takasaki, Japan. Remnant lipoproteins were separated from other lipoproteins by an immunoaffinity gel containing monoclonal antibodies to human apoB100 and human apoA1 [167]. The cholesterol and triglyceride contents of the unbound fraction was measured using very sensitive enzymatic spectrophotometric assays.

9. APOLIPOPROTEINS

Apolipoproteins B, CII, CIII and E were analysed from EDTA plasma and the $VLDL_1$ fraction using kits purchased from Wako Pure Chemical Industries (Japan).

2.10. URINARY ALBUMIN

Urinary Albumin is measured by an automated (Hitachi 911) immunoturbidimetric method using a monoclonal antibody produced by SAPU.

2.11. STATISTICS

Statistical analyses were performed by using MINITAB 10X for Windows (Minitab Inc.), SPSS statistics package and Microsoft Excel 7 for Windows 95 (Microsoft Corp.). Statistical methods used in each study are outlined in the appropriate chapters. Factors that were not normally distributed were subjected to log transformation. Results are shown as means and standard deviations or as medians and interquartile ranges.

CHAPTER 3 -

The Atherogenic Lipoprotein Phenotype: Small Dense LDL and Lipoprotein Remnants in Nephrotic Range Proteinuria

3.1. INTRODUCTION

Normal populations require raised triglycerides (in the form of triglyceride rich $VLDL_1$) and adequate activity of hepatic lipase and cholesteryl ester transfer protein (CETP) to form atherogenic levels of LDLIII [56]. Excess lipoprotein remnants (RLP) are a characteristic finding in patients with hypertriglyceridaemia [80]. There is increasing evidence to suggests that excess lipoprotein remnants (RLP) are atherogenic [168] and a risk factor for coronary artery disease [83]. RLP have not previously been studied in patients with nephrotic range proteinuria however given the delayed clearance of triglyceride-rich lipoproteins found in this population, excess RLP are likely to be present and may contribute to the atherogenicity of nephrotic dyslipidaemia.

The aim of this study was to assess factors determining formation of both small dense LDL and remnant lipoproteins (RLP) in patients with nephrotic range proteinuria. We aimed to study triglyceride rich lipoproteins, lipoprotein lipase and hepatic lipase activity, and cholesteryl ester transfer protein. At the same time, this would allow us to confirm our previous finding of excess LDLIII in this population, clarify the influence of renal dysfunction, plasma and urinary albumin on LDLIII and RLP, and examine the relationship between lipoprotein remnants and the atherogenic lipoprotein phenotype in

proteinuric renal disease.

3.2. METHODS

Subjects

27 patients (22 males, 5 females) were recruited consecutively from the out-patient clinic of the Glasgow Royal Infirmary Renal Unit, according to the following inclusion criteria (a) urinary albumin excretion >2.0g/24hrs (b) serum creatinine <300umol/l. (c) primary glomerular disease. They were compared to 27 age and sex matched controls. Patients suffering from other diseases or on treatment that might influence their lipid profile were excluded, specifically patients with underlying liver disease, diabetes mellitus, amyloid, any neoplastic disorder, systemic lupus erythematosis or taking thiazide diuretics, fat soluble beta blockers, corticosteroids or other immunosuppressive agents. Treatment with other antihypertensives or diuretics was permitted. Patients receiving lipid lowering therapy had their lipid lowering medication stopped for a period of 4 weeks prior to inclusion in the study. All patients had a biopsy proven diagnosis. They were compared to 27 age and sex matched controls who were either relatives of patients attending the renal unit or laboratory staff members. All controls were free of acute illness with normal serum creatinine, albumin and liver function tests. Any patient or control with a bleeding diathesis or with a recent history of peptic ulceration was excluded. All patients and controls gave written consent before participating. The study was approved by the Ethics Committee of Glasgow Royal Infirmary. Patients and controls attended after an overnight fast. An intravenous cannula was inserted and samples were taken for the estimation of lipids and lipoproteins, LDL and VLDL

subfractions, remnant lipoproteins, cholesteryl ester transfer protein, serum creatinine and albumin. 70units/kg body weight of intravenous heparin was then given followed by further sampling 10 minutes later for hepatic and lipoprotein lipase measurement. All patients collected a 24 hour urine for analysis of urinary albumin excretion.

Statistics

Statistical analyses were performed by using MINITAB 10X for Windows (Minitab Inc.). Any factors that were not normally distributed were subjected to log transformation. These included: BMI, plasma TG, VLDL-C, HDL-C, LPL, HL, total VLDL and VLDL subfractions, total LDL and LDL subfractions and lipoprotein remnants. Results are shown as either median and interquartile range (IQR) or as mean and standard deviation. Samples were compared using the two-sample t-test. Simple regression analysis was performed to identify significant correlations. Stepwise regression was used for multivariate analysis.

3.3. RESULTS

Anthropometry, Diagnoses and Renal Function

The patients were well matched for age and body mass index (table 3.1). The mean creatinine was 139μmol/l (range 85-290). Creatinine clearance (CrCl) was greater than 50ml/min in 20 patients (calculated using the Cockcroft & Gault formula [169]), and greater than 70ml/min in 15 patients. Urinary albumin excretion was 3.9g/24hrs (range 2.0-11.4). Serum albumin was well preserved at 35.0±6.7g/l (6 patients had a serum albumin <32g/l). The diagnoses were membranous nephropathy (8 patients) IgA

nephropathy (7 patients), with 3 cases each of mesangiocapillary glomerulonephritis, minimal change, focal and segmental glomerulosclerosis, and chronic glomerulonephritis.

Table 3.1: Anthropometry, Lipids, Lipoproteins and Lipase activity: (Median+IQR)

	PATIENTS n=27	CONTROLS n=27	p
Age	48.0 (29.0-62.0)	45.3 (30.1-60.5)	nsd
Sex (M: F)	22 : 5	22 : 5	nsd
Body Mass Index	26.5 (25.1-30.2)	24.5 (22.8-28.6)	nsd
Creatinine (μmol/l)	120 (100-180)	100 (95-120)	<0.006
Cholesterol (mmol/l)	6.8 (5.6-7.5)	5.1 (4.6-5.8)	<0.0001
Triglyceride (mmol/l)	2.2 (1.6-3.2)	1.0 (0.8-1.3)	<0.0001
VLDL-C (mmol/l)	1.0 (0.5-1.3)	0.3 (0.2-0.4)	<0.0001
LDL-C (mmol/l)	4.2 (3.7-5.4)	3.4 (2.8-4.0)	<0.002
HDL-C (mmol/l)	1.0 (0.8-1.2)	1.2 (1.0-1.6)	<0.003
Lipoprotein Lipase (μmolFA/ml/hr)	3.7 (2.9-5.6)	4.0 (3.4-5.4)	nsd
Hepatic Lipase (μmolFA/ml/hr)	16.2 (10.1-24.3)	13.8 (9.6-16.1)	nsd

Lipids, Lipoproteins and Post-Heparin Lipase Activity

The results for basic lipids, lipoproteins and post-heparin lipase activity are shown in table 3.1. Cholesterol, plasma triglyceride and VLDL-C, were all higher in patients with

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proteinuria compared with controls (all $p<0.0001$), LDL-C was similarly raised ($p<0.002$) with HDL-C reduced in the patients ($p<0.003$). Comparing the two groups revealed no difference in activity of either lipoprotein or hepatic lipase. Urinary albumin correlated with plasma cholesterol ($r^2=22\%$ $p<0.02$), LDL-C ($r^2=15\%$ $p<0.05$) and negatively with HDL-C ($r^2=21\%$ $p<0.02$). Plasma albumin was also negatively correlated with cholesterol ($r^2=41\%$ $p<0.001$) and LDL-C ($r^2=41\%$ $p<0.001$). There was no observed relationship between either plasma creatinine or CrCl and any lipid parameter, within the range of the population studied.

VLDL Subfractions, IDL and LDL concentration

There was a 3-4 fold increase in total VLDL (table 3.2, $p<0.001$), due to an increase in the concentration of both $VLDL_1$ and $VLDL_2$ in proteinuric patients (both $p<0.001$). IDL mass in the patients was double that of the controls ($p<0.0001$), and total LDL mass ($p<0.02$) was also increased. Urinary albumin was weakly associated with increasing IDL mass ($r^2=17\%$ $p<0.04$) but not LDL mass. Low plasma albumin was associated with both IDL ($r^2=27\%$ $p<0.006$) and LDL ($r^2=19\%$ $p<0.03$). No relationship was observed between renal function and any of the lipoproteins.

LDL Subfractions

A marked shift in LDL subfraction profile was observed. Proteinuric patients had a reduced concentration of large light LDLI and LDLII with a large increase in LDLIII mass ($p<0.0001$, table 3.2). LDLIII mass was greater than 100mg/dl in 74% of the patients studied, compared with 4% of controls. Similarly, the percentage of LDL that was LDLIII was increased in the patients ($p<0.0001$), with a corresponding decrease in

the %LDLI (p<0.0001) and %LDLII (p<0.0003, table 3.2), indicating that the difference in LDL profile was independent of the LDL mass.

Table 3.2: VLDL subfractions, IDL and LDL subfractions: (Median + IQR)

	PATIENTS n=27	CONTROLS n=27	p
VLDL Total mg/dl	236 (172-364)	79 (52-113)	<0.0001
VLDL$_1$ mg/dl	156 (57-212)	43 (24-74)	<0.0001
VLDL$_2$ mg/dl	109 (75-140)	30 (21-43)	<0.0001
IDL mg/dl	80 (63-113)	44 (34-60)	<0.0001
LDL Total mass mg/dl	351 (298-440)	292 (252-349)	<0.02
LDLI mass mg/dl	36 (24-43)	69 (46-101)	<0.0005
LDLII mass mg/dl	124 (79-220)	178 (129-236)	<0.04
LDLIII mass mg/dl	182 (84-267)	31 (26-62)	<0.0001
%LDLI	9 (7-12)	19 (15-33)	<0.0001
%LDLII	36 (21-63)	63 (53-69)	<0.0003
%LDLIII	57 (22-72)	11 (8-19)	<0.0001

Effect of Renal Function and Plasma Albumin

The CrCl was >70ml/min in 15 of the 27 patients studied. These patients had a creatinine of 100 (90-120)µmol/l (median + IQR) and a CrCl of 90ml/min. Direct comparison of the two subgroups based on a CrCl greater than or less than 70, revealed no difference in urinary albumin (CrCl >70: 2.7 (2.1-4.9) vs CrCl <70: 2.9 (2.2-

6.1g/24hrs), plasma albumin (35±6 vs 35±7g/l) or any lipoprotein parameter (table 3.3). When the CrCl >70 subgroup was compared with the control population, the total LDL mass was not increased (probably as a result of inter-patient variability). All other results were similar to those achieved with the entire proteinuric group. The mass and percentage of LDLIII were both increased (both p<0.0005) with corresponding reductions in the mass and percentage of LDLI (both p<0.0005) and LDLII (mass p<0.03, percentage p<0.005). No correlation was observed between renal function (using either CrCl or reciprocal of creatinine), and the mass or percentage of LDLIII.

21 of the 27 patients studied had a plasma albumin >32g/l. If the patients with hypoalbuminaemia were omitted, the results remained similar to that for the whole study group, with the only that difference being that LDL mass was not significantly elevated (patients 347 (301-416) vs controls 292 (252-349) mg/dl). The proteinuric but normoalbuminaemic patients had a mean LDLIII mass of 174 (67-274)mg/dl, with 50% of the LDL in these patients in the form of LDLIII. No relationship was observed between plasma albumin and LDLIII mass although albumin was negatively correlated with LDLI (r^2=29% p<0.004) and LDLII mass (r^2=21% p<0.02). No relationship was observed between urinary albumin and LDL subfractions. The 12 patients with a creatinine clearance >70 and a normal plasma albumin had a mean LDLIII mass of 165 (35-286)mg/dl with 48% of the LDL present in the form of LDLIII.

Table 3.3: Lipids, Lipoproteins, VLDL & LDL subfractions according to renal function. (Median + IQR)

	Patients: Creatinine Clearance >70 (n=15)	Patients: Creatinine Clearance <70 (n=12)
Chol (mmol/l)	6.9 (5.5-7.4)	6.6 (5.7-8.4)
Trig (mmol/l)	2.3 (1.4-3.2)	2.6 (1.8-3.4)
VLDL-C (mmol/l)	1.0 (0.6-1.3)	0.9 (0.5-2.2)
LDL-C (mmol/l)	4.2 (3.2-5.3)	4.4 (3.8-5.9)
HDL-C (mmol/l)	1.0 (0.8-1.3)	0.9 (0.8-1.2)
VLDL Total (mg/dl)	236 (143-375)	227 (178-356)
VLDL$_1$ (mg/dl)	159 (66-191)	125 (79-148)
VLDL$_2$ (mg/dl)	109 (62-137)	103 (79-148)
IDL (mg/dl)	78 (63-113)	82 (64-114)
LDL Total (mg/dl)	329 (279-451)	368 (323-440)
LDL I mass (mg/dl)	33 (24-37)	41 (17-50)
LDL II mass (mg/dl)	115 (76-197)	135 (80-256)
LDL III mass (mg/dl)	195 (49-288)	149 (88-258)
%LDLI	9 (7-12)	11 (8-18)
%LDLII	36 (21-65)	37 (23-62)
%LDLIII	57 (17-72)	51 (23-70)

Cholesteryl Ester Transfer Protein (CETP) Activity & Lipoprotein Compositions

CETP activity was increased in the proteinuric patients (patients 59.4 ± 21.0 vs controls

45.8 ± 11.1 pmoles CE/ml plasma, p<0.005). In keeping with the excess CETP activity, both $VLDL_1$ and $VLDL_2$ were found to be cholesterol enriched and triglyceride deplete compared with controls, whilst LDL was cholesterol deplete and triglyceride enriched (table 3.4).

Table 3.4: Lipoprotein Compositions: (Mean ± SD)

	Patients (n=27)	Controls (n=27)	P
$VLDL_1$ % Cholesterol	10.7 ± 3.1	7.3 ± 2.2	<0.0001
$VLDL_1$ % Triglyceride	64.2 ± 4.1	67.1 ± 4.5	<0.0001
$VLDL_2$ % Cholesterol	24.2 ± 4.2	18.8 ± 3.9	<0.0001
$VLDL_2$ % Triglyceride	38.1 ± 5.5	42.3 ± 7.8	<0.03
IDL % Cholesterol	35.7 ± 4.2	36.3 ± 3.2	nsd
IDL % Triglyceride	17.0 ± 5.9	15.2 ± 3.4	nsd
LDL % Cholesterol	37.6 ± 3.6	39.6 ± 1.2	<0.02
LDL % Triglyceride	8.9 ± 3.4	6.7 ± 1.1	<0.004

Remnant Lipoproteins

Proteinuric patients had increased RLP-Cholesterol (RLP-C, Patients: 18.8 (10.9-26.8) vs Controls: 7.6 (5.9-8.8) p<0.0001) and RLP-Triglyceride (RLP-TG: 35.8 (11.8-54.7) vs 7.1 (4.3-10.0) p<0.0001, all mg/dl, median + IQR). No relationship was observed between RLP and urinary albumin, plasma albumin or renal function. Similar results were obtained for the patients with a CrCl >70 compared with those <70ml/min (RLP-C: 18.8 (8.0-25.3) vs 20.0 (12.1-41.9), RLP-TG: 35.8 (9.4-47.0) vs 32.1 (12.6-67.6), all

mg/dl). Proteinuric patients with normal serum albumin levels had significantly more remnant lipoproteins compared with controls (RLP-C 18.8mg/dl (10.3-25.8), RLP-TG 35.8 (11.6-54.2), both p<0.0001 vs controls).

Table 3.5: Factors associated with LDLIII and Remnant Lipoprotein concentration

	LDLIII mass (r^2%)	%LDLIII (r^2%)	RLP-C (r^2%)	RLP-TG (r^2%)
Triglyceride	45.7[a]	54.9[a]	85.2[a]	87.5[a]
VLDL$_1$ mass	29.7[b]	57.9[a]	68.8[a]	85.4[a]
VLDL$_2$ mass	39.1[a]	20.2[d]	27.6[b]	18.3[d]
LDL mass	24.3[c]	0.0	0.0	0.1
HDL-C	38.0 (-)[b]	32.9 (-)[b]	39.2[a]	45.0[a]
CETP	24.6[c]	20.2[d]	46.0[a]	39.3[a]
Lipoprotein Lipase	0.0	0.5	0.4	1.6
Hepatic Lipase	0.5	5.4	0.2	3.5

r^2% = coefficient of determination   [a] p<0.001, [b] p<0.005, [c] p<0.01, [d] p<0.05

Factors determining LDLIII and Lipoprotein Remnants in Proteinuric Patients

LDLIII mass was correlated with plasma triglycerides, CETP activity (both fig 3.1), VLDL$_1$, VLDL$_2$ and total LDL (table 3.5). An inverse relationship was seen with HDL-C. No relationship was observed between LDLIII mass and activity of lipoprotein or

hepatic lipase (fig 3.1). Multivariate analysis revealed plasma triglycerides and LDL mass to be independent predictors of LDLIII mass accounting for 62.2% of the variance (TG 45.7%, LDL mass 16.5%). The %LDLIII was related to plasma triglyceride and $VLDL_1$ with a weaker relationship with $VLDL_2$ and CETP activity. An inverse relationship was seen with HDL-C, with no correlation seen with total LDL or lipase activity. On multivariate analysis plasma triglyceride (r^2= 54.9%) was the only independent predictor of %LDLIII.

RLP-C and RLP-TG were closely correlated with plasma triglyceride, $VLDL_1$ concentration and plasma CETP activity (table 3.5, fig 3.2). An inverse correlation was noted with HDL-C. No relationship was observed between remnant lipoproteins and lipase activity or LDL. On multivariate analysis plasma triglyceride was the only predictor of RLP-C mass (r^2=85.2%), with plasma triglyceride, $VLDL_1$ mass and $VLDL_2$ mass independent factors determining RLP-TG level (total r^2=94.1%: triglyceride 87.5%, $VLDL_1$ 5.1%, $VLDL_2$ 1.5%). A clear relationship was observed between the mass and percentage of LDLIII and RLP. LDLIII mass was associated with both RLP-C (r^2=31.3% p<0.003, fig 3.3) and RLP-TG (r^2=33.6% p<0.003), with a closer relationship between %LDLIII and both RLP-C (r^2=46% P<0.001, fig 3.3) and RLP-TG (r^2=55.5% p<0.001).

Fig 3.1: Plasma Triglyceride (TG), CETP Activity and Hepatic Lipase Activity vs LDLIII mass in Proteinuric Patients

Triglyceride vs LDLIII mass: Patients
(log : log plot)

$r^2 = 45.7\%$
$p < 0.001$

y-axis: LDLIII mass (mg/dl)
x-axis: Plasma Triglyceride (mmol/l)

CETP vs LDLIII mass: Patients

$r^2 = 24.6\%$
$p < 0.01$

y-axis: LDLIII mass (mg/dl) (log plot)
x-axis: CETP Activity (pmolesCE/ml)

Hepatic Lipase vs LDLIII mass: Patients
(log:log plot)

$r^2 = 0.5\%$
$p = nsd$

y-axis: LDLIII mass (mg/dl)
x-axis: Hepatic Lipase (umolFA/ml/hr)
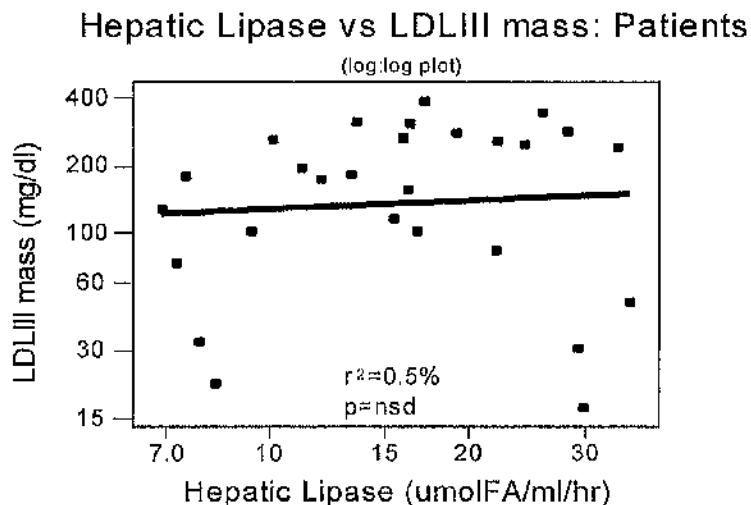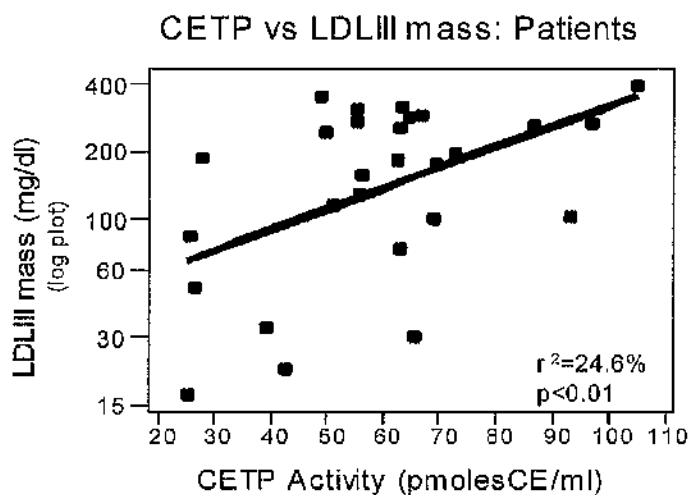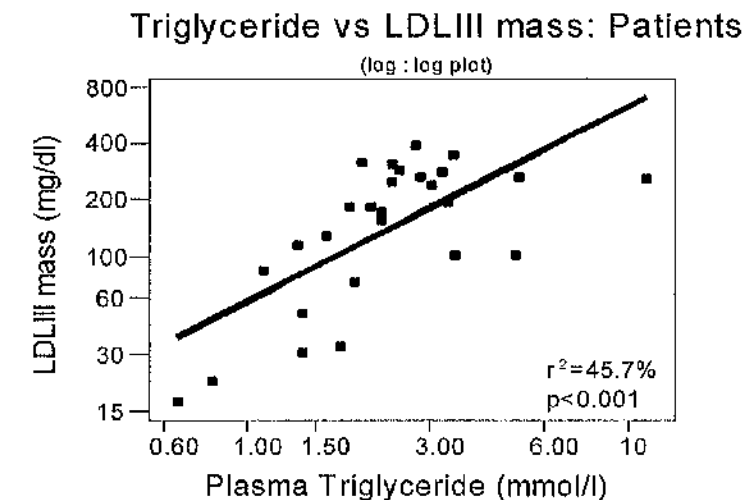
Fig 3.2: Plasma Triglyceride, VLDL₁ mass and CETP Activity vs Remnant

Lipoprotein Cholesterol (RLP-C) in Proteinuric Patients

Triglyceride vs RLP-C: Patients
(log : log plot)

r^2=85.2%
p<0.001

Plasma CETP vs RLP-C: Patients

r^2=46.0%
p<0.001

VLDL1 vs RLP-C: Patients
(log : log plot)

r^2=68.8%
p<0.001
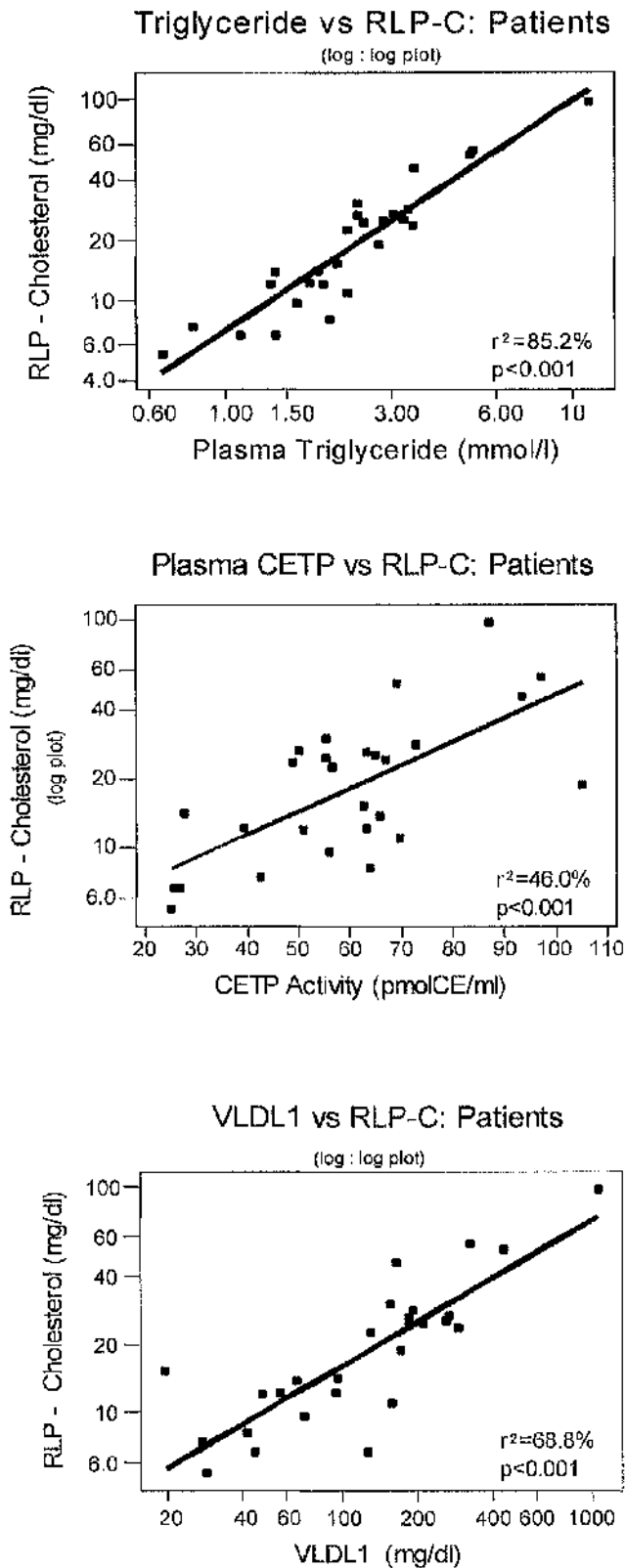
Fig 3.3: LDLIII mass and %LDLIII vs RLP-Cholesterol: Patients

LDLIII mass vs RLP-C: Patients
(log: log plot)

% LDLIII vs RLP-C: Patients
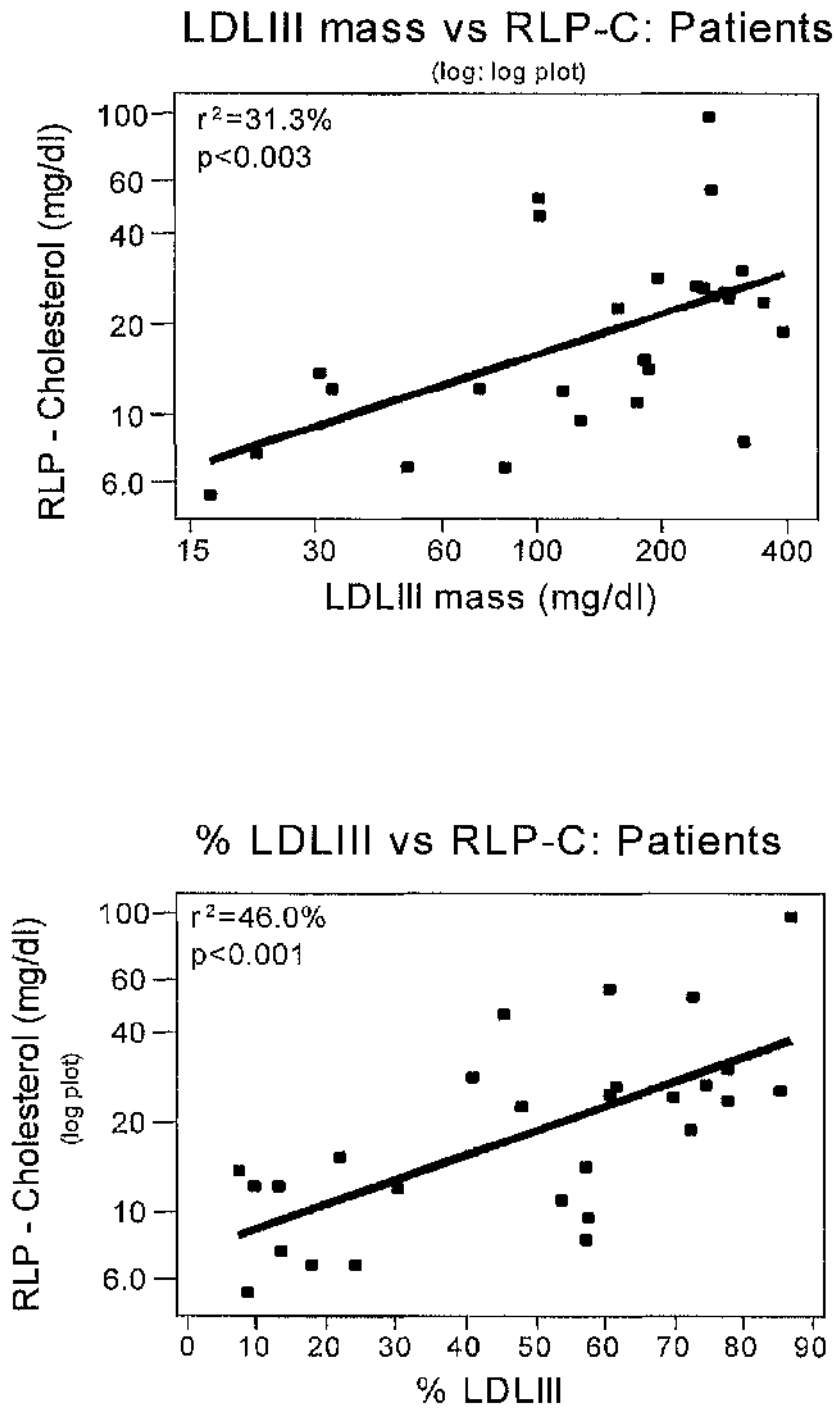
3.4. DISCUSSION

This study demonstrates that remnant lipoproteins are present in excess in patients with nephrotic range proteinuria. The data also confirms the original findings of our pilot study suggesting that small dense LDL is present in quantities that are associated with significantly increased cardiovascular risk in normal populations. This analysis has been extended, demonstrating that the excess of both RLP and LDLIII is not related to mild renal impairment and that no direct relationship was discernible with either urinary or plasma albumin. The results suggest that plasma triglyceride is the most important factor determining LDL size phenotype (i.e. % LDLIII) and RLP formation, with plasma triglyceride and total LDL determinants of LDLIII mass.

Comparing the RLP results with those obtained from the Framingham Heart Study, reveals that the proteinuric patients have levels of RLP-C and RLP-TG that are 2-3 fold higher than the reference value for males [170]. The control RLP-C data corresponded closely to reference values however the RLP-TG control data was lower than comparable male data. Four of the 5 women studied were premenopausal and the mean age of the patients was lower than the Framingham Offspring Study, given that younger patients and pre-menopausal women have lower RLP levels, this may account for the difference from reference males [170]. In keeping with other dyslipidaemic populations, plasma triglyceride was the most important determinant of RLP-C and RLP-TG mass present [80]. This excess of plasma triglyceride results from increased VLDL$_1$ and VLDL$_2$ concentration, which is primarily related to impaired clearance of these triglyceride-rich lipoproteins [108,171]. It is therefore likely, that the impaired clearance of VLDL$_1$ and VLDL$_2$ is related to the excess of RLP observed. As in vitro lipase activity was not reduced, it is unsurprising that lipase activity was not

associated with RLP concentration. It has been suggested that the defective clearance of VLDL may result from deficiencies in plasma or VLDL apolipoproteins [105].

The current theory describing the formation of atherogenic levels of LDLIII require the presence of excess triglycerides and adequate levels of hepatic lipase (found in males and post-menopausal females). Triglyceride is passed via CETP from $VLDL_1$ to LDL in return for cholesteryl ester. A triglyceride enriched LDL particle is formed which is hydrolysed by hepatic lipase, shrinking the particle and forming small dense LDL [56]. The results obtained in this study suggest that LDLIII formation in proteinuric patients probably follows similar lines. Increased triglyceride is related to LDLIII mass and %LDLIII. Plasma CETP activity is increased and related to the mass and percentage of LDLIII. It is likely that hepatic lipase was not rate limiting due to the extent of the hypertriglyceridaema. Finally $VLDL_1$ concentration is increased and is cholesteryl ester enriched. Moreover, the LDL present is triglyceride rich, a characteristic finding for small dense LDL [172]. Thus, in proteinuric patients it appears that the LDL phenotype is determined by the plasma triglyceride, with both plasma triglyceride and the total LDL mass determining the LDLIII mass.

There is increasing evidence to link both lipoprotein remnants and small dense LDL with coronary artery disease. RLP-C levels are increased in men with coronary artery disease [83], high RLP-C is a predictor for coronary artery stenosis [84] and sudden cardiac death [85]. RLP induces platelet aggregation [173], is associated with coronary artery endothelial cell dysfunction [174] and can promote lipid accumulation by macrophages [168]. Prospective and retrospective studies have established that increased small dense LDL is associated with enhanced cardiovascular risk [142,143],

and patients with an LDLIII mass of >100mg/dl have a seven fold increase in the risk of myocardial infarction [141]. Using this criteria, 20 of the 27 patients studied have a high risk of developing a myocardial infarction irrespective of other cardiovascular risk factors. A number of theories have been proposed regarding the cause of the increased atherogenicity of small dense LDL, these include: (i) decreased affinity for the LDL receptor with increased uptake by the scavenger receptor, [175] (ii) increased susceptibility of small dense LDL to oxidation, partly as a result of longer residence time in plasma due to decreased LDL receptor uptake [176,177], (iii) increased filtration by the endothelium due the smaller size of the LDL particles [56] and (iv) greater affinity for binding to arterial wall proteoglycans [178]. Each of the above factors will increase the likelihood of LDL being located in the subendothelial space and initiate the formation of the atherosclerotic plaque.

Patients with end-stage renal failure have increased small dense LDL [160] and RLP [82]. The data in this study does not suggest that mild renal dysfunction has contributed to the increase in small dense LDL or RLP observed in the proteinuric patients. The lack of correlation between either LDLIII or RLP and plasma or urinary albumin in this population is also noteworthy. The atherogenic lipoprotein phenotype consists of raised triglycerides, low HDL-C and increased small dense LDL. Plasma triglycerides have the closest association with LDLIII and reported relationship between plasma triglycerides and proteinuria vary significantly [105]. An inverse relationship was observed between albuminuria and HDL-C (r^2=21.2% p<0.02) suggesting that a relationship may exist between urinary albumin and one aspect of the atherogenic lipoprotein phenotype.

The central role of triglycerides and triglyceride rich lipoproteins in determining the extent of RLP and LDLIII formation is clearly demonstrated in this study. To our knowledge small dense LDL and atherogenic remnants have not been analysed in the same study population. The close correlation between RLP and both plasma triglycerides and LDLIII, suggest that RLP are likely to contribute to the increased cardiovascular risk attributed to the atherogenic lipoprotein phenotype. We suggest that the definition of the atherogenic lipoprotein phenotype may require modification to include RLP along with small dense LDL, mild hypertriglyceridaemia and a low HDL. The finding of excess small dense LDL and lipoprotein remnants, highlights the need to clarify the nature of the defective clearance of triglyceride rich lipoproteins that has been demonstrated in this population [108,171]. Clearly excess of both RLP and LDLIII add to the spectrum of cardiovascular risk factors that are likely to contribute to the increased atherogenesis present in proteinuric patients.

CHAPTER 4 -

Raised Very-Low Density Lipoprotein (VLDL) In Nephrotic Range Proteinuria Is Associated With Relative Deficiency Of Apolipoprotein C And E On VLDL Particles

4.1 INTRODUCTION

Patients with nephrotic range proteinuria have an increase in plasma concentration of both $VLDL_1$ and $VLDL_2$ [104,109]. Metabolic studies suggest that the increase in $VLDL_1$ results from delayed clearance of the lipoprotein whilst the rise in $VLDL_2$ results both from delayed clearance and enhanced hepatic production [108,171]. Excess plasma triglyceride in the form of $VLDL_1$ appears essential for the formation of small dense LDL [56,144], a form of LDL that is atherogenic [55] and associated with increased cardiovascular risk [139,140,142,143]. We have shown that patients with nephrotic range proteinuria possess excess quantities of both small dense LDL [109] and remnant lipoproteins, and postulate that the delayed $VLDL_1$ clearance and resultant hypertriglyceridaemia is a significant contributor to the atherogenic dyslipidaemia prevalent in patients with nephrotic range proteinuria.

The two methods of clearing $VLDL_1$ particles from the circulation are by hydrolysis using lipoprotein lipase to form $VLDL_2$ and then IDL, and by direct hepatic uptake of VLDL via the VLDL receptor which recognises apolipoprotein E [179]. Our laboratory has previously demonstrated impaired VLDL clearance but normal chylomicron clearance in the nephrotic syndrome [180], with normal in-vitro activity

of lipoprotein lipase [109,180]. Activation of lipoprotein lipase depends upon the cofactor apolipoprotein CII on the VLDL particle, whilst apolipoprotein CIII inhibits this process [181]. The aim of this study therefore, was to measure the apolipoproteins CII, CIII and E, in the plasma and $VLDL_1$ compartments, along with VLDL lipid compositions to clarify the potential cause of the impaired clearance leading to the excess $VLDL_1$ in the plasma of patients with nephrotic range proteinuria.

4.2 SUBJECTS AND METHODS

Subjects

Fasting plasma and the $VLDL_1$ fraction of plasma from the 27 patients and controls that were studied in Chapter 3, was stored at a temperature of -50^0C. After recruitment of all the patients and controls, apolipoproteins B, CII, CIII and E were analysed from the EDTA plasma and the $VLDL_1$ fraction.

Statistics

Statistical analyses were performed using MINITAB 10X for Windows (Minitab Inc.). Any factors that were not normally distributed were subjected to log transformation. These included the $VLDL_1$ concentration and the majority of apolipoprotein results. Results are shown as either median and interquartile range (IQR) or mean and standard deviation (SD). Samples were compared using the two-sample t-test. Simple regression analysis was performed to identify significant correlations. After fitting regression lines, the slopes for patients and controls were compared firstly to assess if they were parallel and then to see if their intercepts differed. This was done using a general linear model (GLM). Multivariate analysis of linear variables was performed

using stepwise regression. A GLM was used to perform multivariate analysis if a categorical variable was involved.

4.3 RESULTS

The data on renal function, urinary albumin, plasma albumin, lipids and lipoproteins is shown in chapter 3. There was a 4 fold increase in $VLDL_1$ concentration (patients 156mg/dl (57-212) vs controls 43mg/dl (24-74) $p<0.0001$, median + IQR) which persisted even if patients with hypoalbuminaemia or a creatinine clearance of <70ml/min were excluded.

Table 4.1: Plasma Apolipoproteins: Patients vs Controls (All Median + IQR)

	PATIENTS (n=27)	CONTROLS (n=27)	P
Apo B (mg/dl)	146 (112-179)	90 (68-98)	<0.0001
Apo CII (mg/dl)	6.2 (4.4-7.9)	3.1 (2.5-4.4)	<0.0001
Apo CIII (mg/dl)	30.0 (27.1-37.2)	17.7 (15.9-21.7)	<0.0001
Apo E (mg/dl)	4.3 (3.3-6.8)	3.5 (2.9-4.1)	<0.002
ApoCII / B (mg/mg $x10^{-2}$)	4.2 (3.3-5.6)	3.9 (2.8-4.4)	nsd
ApoCIII / B (mg/mg $x10^{-2}$)	21.1 (19.1-26.0)	22.2 (17.7-26.5)	nsd
ApoE / B (mg/mg $x10^{-2}$)	3.1 (2.8-3.8)	3.9 (3.5-4.8)	<0.03

Plasma Apolipoproteins.

Table 4.1 shows the results for the plasma apolipoproteins. In keeping with the increased in total lipoproteins present, the total mass of apolipoprotein B (apoB), apolipoprotein CII (apoCII), apolipoprotein CIII (apoCIII) and apolipoprotein E

(apoE) were all increased in the patients with proteinuria. Each VLDL, IDL and LDL particle carries one moiety of apoB, thus using this as a reference, a crude estimate of the amount of apoCII, CIII and E moieties per lipoprotein particle in the plasma was obtained, ignoring the apoC present on HDL. The results suggest that taking into account the excess of particles present, the amount of CII and CIII per lipoprotein particle did not differ between the patients and controls (table 4.1). However, the mass of apoE per particle was reduced in the patients compared with controls (p<0.03).

Table 4.2: Plasma Concentration of VLDL$_1$ Apolipoproteins: Patients vs Controls (All Median + IQR)

	PATIENTS (n=21)	CONTROLS (n=19)	P
Apo B (mg/dl)	13.0 (6.1-26.2)	3.7 (0.9-6.3)	<0.003
Apo CII (mg/dl)	1.20 (0.49-1.87)	0.51 (0.33-0.77)	<0.002
Apo CIII (mg/dl)	4.14 (2.43-5.42)	1.57 (0.94-2.83)	<0.001
ApoE (mg/dl)	0.14 (0.09-0.19)	0.13 (0.10-0.16)	nsd

VLDL$_1$ Apolipoproteins & Compositions: Patients vs Controls

The concentration of apolipoproteins present in the plasma VLDL$_1$ fraction are seen in table 4.2. The analysis of the VLDL$_1$ apolipoproteins was performed on 21 patients and 19 controls, due to the concentration of VLDL$_1$ being too low in some patients and controls to allow accurate measurement of either apoCII, apoCIII or apoE. If one of the VLDL$_1$ apolipoproteins was unrecordable due to low VLDL$_1$ concentration then all the apolipoprotein and composition results for that patient were ignored to avoid bias in any results. In keeping with the increase in total VLDL$_1$ concentration, apoB

(p<0.003), apoCII (p<0.002) and apoCIII concentration (p<0.001) were all increased in the patients compared with controls. However $VLDL_1$ apoE was not increased. To assess the quantity of apolipoprotein CII, CIII and E on each $VLDL_1$ particle, the molar ratio of each apolipoprotein to apolipoprotein B was calculated (table 4.3). This revealed each $VLDL_1$ particle in the patients with proteinuria to be deficient in apoCII, CIII and E. In the case of apoE, this deficiency was present to such an extent that it could be calculated that between only one in three and one in five $VLDL_1$ particles possessed an apoE moiety. The $VLDL_1$ particles in the proteinuric group also had a significantly lower triglyceride content (p<0.05) with a trend towards lower surface phospholipid (p<0.07). The total and free cholesterol content of the particles did not differ between the 2 groups.

The relative proportions of apolipoproteins to phospholipid on the surface of the $VLDL_1$ particles further demonstrated the relative deficiency of $VLDL_1$ apolipoproteins. The ratio of apoCII to phospholipid (3.7 ± 1.7 vs 5.8 ± 2.1 p<0.002), apoCIII to phospholipid (12.1 ± 4.9 vs 16.7 ± 6.6 p<0.03) and apoE to phospholipid (0.16 ± 0.13 vs 0.35 ± 0.22 p<0.02, all $\times10^{-3}$) were all significantly reduced compared with controls. In contrast the ratio of free cholesterol to phospholipid (FC:PL) was increased in the proteinuric patients (0.55 ± 0.17 vs 0.40 ± 0.18 p<0.002, all mean$\pm$SD).

Table 4.3: Apolipoprotein and Lipid content per VLDL$_1$ particle : Patients vs Controls (all moles per particle – Median + IQR)

	PATIENTS (n=21)	CONTROLS (n=19)	P
ApoCII	4.2 (3.1-8.2)	9.9 (7.4-23.2)	<0.0004
ApoCIII	16.6 (9.1-27.2)	29.3 (18.5-69.4)	<0.02
ApoE	0.17 (0.08-0.44)	0.48 (0.31-1.31)	<0.006
Triglyceride	4896 (3025-7912)	6979 (4590-15687)	<0.05
Total Cholesterol	1758 (1393-2808)	1667 (1312-2885)	nsd
Free Cholesterol	814 (515-1028)	759 (562-1474)	nsd
Phospholipid	1209 (977-2186)	1783 (1277-4091)	nsd

Relationship between VLDL$_1$ compositions and VLDL$_1$ concentration

Strong negative correlations were observed between the plasma VLDL$_1$ concentration and the number of moles of apoCII and apoCIII per VLDL$_1$ particle (fig 4.1). Thus for all the patients studied, as VLDL$_1$ mass increased from 25mg/dl to 1000mg/dl, the number of moles of apoCII per VLDL$_1$ particle decreased from 53 to 1 and apoCIII per VLDL$_1$ particle decreased from 150 to 3. A similar negative relationship was also observed between the moles per VLDL$_1$ of apoE and VLDL$_1$ concentration, with apoE per particle decreasing from 5 to 0.02 (i.e. one particle in fifty) as VLDL$_1$ mass increased (fig 4.1). A strong negative relationship was observed between the moles of triglyceride per VLDL$_1$ particle (an index of particle size) and the VLDL$_1$ mass (all subjects r^2=52.2% p<0.001, patients r^2=41.5% p<0.003, controls r^2=71.1% p<0.001), indicating that as VLDL$_1$ mass increased, the size of the VLDL$_1$ particles decreased. Weaker relationships were observed between VLDL$_1$ concentration and the FC:PL

ratio (all subjects $r^2=30.2\%$ p<0.001, patients $r^2=11.1\%$ p=nsd, controls $r^2=25.7\%$ p<0.03).

Table 4.4: Univariate (U) and Multivariate (M) Analysis for Regulation of ApoCII, apoCIII and ApoE per VLDL$_1$ particle - All Patients

	ApoCII		ApoCIII		ApoE	
	U $r^2\%$	M $r^2\%$	U $r^2\%$	M $r^2\%$	U $r^2\%$	M $r^2\%$
TG per particle	61.5 [a]	24.9 [a]	75.8 [a]	36.7 [a]	58.2 [a]	43.3 [a]
FC:PL	41.6 [a]	5.2 [b]	38.8 [a]	2.8 [c]	11.7 [c]	nsd
Subject Group	-	5.3 [b]	-	2.5 [c]	-	4.2 [d]

[a] p<0.001, [b] p<0.01, [c] p<0.05, [d] p=0.05 r^2=coefficient of determination

Regulation of VLDL$_1$ apolipoprotein CII and CIII composition

To assess determinants of apoCII and apoCIII content per VLDL$_1$ particle, the relationship between apoCII and apoCIII per VLDL$_1$ particle and lipoprotein composition was analysed. As triglyceride per particle increased from $2x10^3$ to $32x10^3$ moles so did apoCII and apoCIII per particle (fig 4.2, table 4.4). Conversely an inverse relationship was seen between FC:PL (increasing from 0.17 to 0.98) and both apoCII and apoCIII per particle (fig 4.3, table 4.4). An interaction was also observed between triglyceride per particle and FC:PL (all subjects $r^2=22.7$ p<0.003). We proceeded to multivariate analysis to assess factors determining apoC per particle. We analysed linear variables by stepwise regression and noted that for all subjects, triglyceride (TG) per particle and FC:PL were independent predictors of apoCII per particle (total $r^2=71.1\%$) and apoCIII per particle (total $r^2=80.5\%$). Multiple regression analysis

using a general linear model to include the categorical variable 'subject group', revealed that particle size, FC:PL and 'subject group' were all independent predictors of both apoCII and apoCIII per particle (table 4.4). From the equation obtained from multiple regression analysis, it could be estimated that the 'subject group' accounted for a mean difference of 1.5moles of apoCII per $VLDL_1$ and 1.3moles of apoCIII per $VLDL_1$.

Regulation of $VLDL_1$ apolipoprotein E composition

The relationship between apoE per particle and triglyceride per particle was similar to that found for apoC, indicating that as particle size increased, so did the apoE content (table 4.4, fig 4.2). However although analysing all subjects revealed a weak relationship between apoE per $VLDL_1$ and FC:PL ($r^2=11.7$ p<0.05), no relationship was seen if the analysis was performed for either patients or controls (fig 4.3). On multivariate analysis, triglyceride per particle and subject group but not FC:PL ratio were independent predictors of apoE per $VLDL_1$ particle. Despite the low coefficient of variation for subject group, from the equation obtained from multiple regression analysis it could be estimated that the 'subject group' had a profound effect on apoE per particle accounting for a mean difference of 1.7moles of apoE per $VLDL_1$ (range of apoE per $VLDL_1$: patients 0.04 -2.0, controls 0.02 -5.0moles/particle).

Effect of Renal Function and Hypoalbuminaemia

In the patients with proteinuria, the relationship between the $VLDL_1$ compositions and apolipoproteins did not seem to be affected by renal function (as measured by creatinine or creatinine clearance -- CrCl), or plasma albumin. Weak relationships were seen between urinary albumin and plasma apoB ($r^2=23.5\%$ p=0.01) and apoE

(r^2=23.5% p=0.03), whilst plasma albumin exhibited an inverse relationship with plasma apoB (r^2=25.3% p<0.009). However, no other relationships were observed and there was no correlation between renal function and either plasma or $VLDL_1$ apolipoprotein content. 15 patients had a CrCl greater than 70, with 12 less than 70, whilst amongst those patients whose $VLDL_1$ apolipoproteins could be measured, CrCl was greater than 70 in 12, and less than 70 in 9. Comparing the 2 subgroups revealed no differences in their plasma or $VLDL_1$ apolipoprotein content. Moreover comparing the subgroup with CrCl greater than 70 with the control group, revealed similar results to those obtained using all the proteinuric patients. Plasma albumin was >32g/dl in 21 patients and <32 in only 6, (>32 in 16 and <32 in only 5 in those patients whose $VLDL_1$ apolipoproteins could be accurately measured). Excluding the hypoalbuminaemic patients did not significantly change the comparison of patients and controls.

Fig 4.1: VLDL₁ mass (mg/dl) vs Apolipoprotein CII , CIII and E per VLDL₁ particle (all moles/particle)

VLDL1 mass vs ApoCII per VLDL1 particle

VLDL1 mass vs ApoCIII per VLDL1 particle

VLDL1 mass vs ApoE per VLDL particle
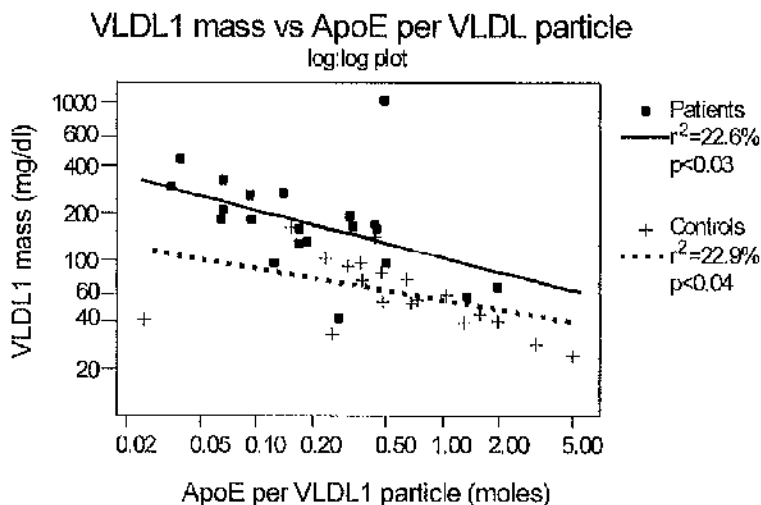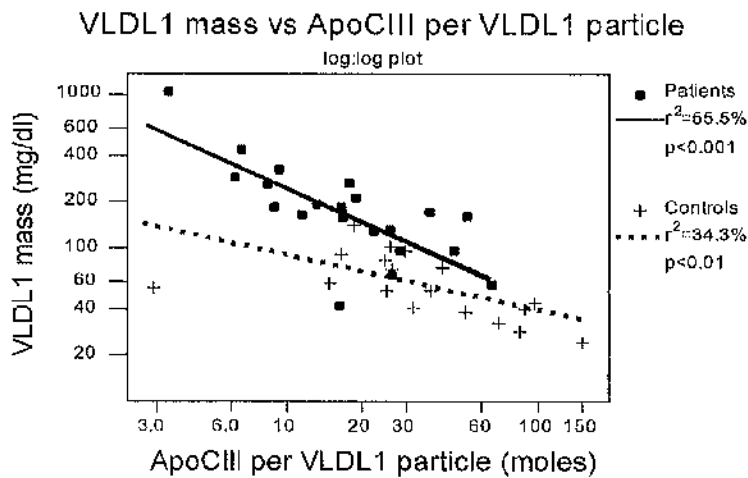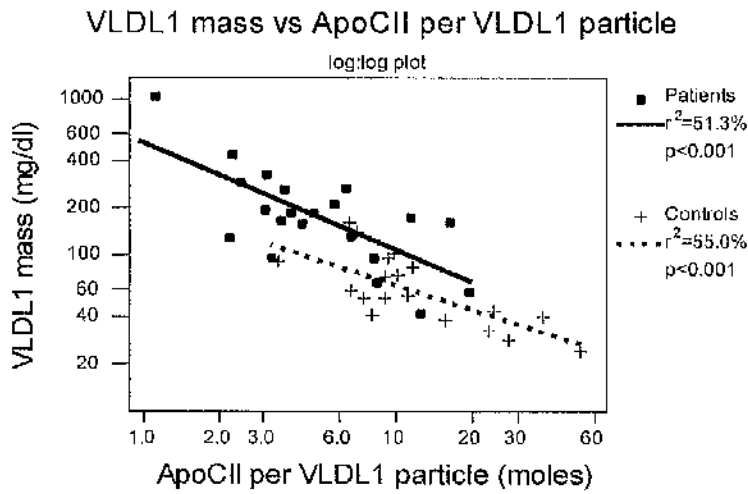
Fig 4.2:Triglyceride per VLDL$_1$ particle vs Apolipoprotein CII, CIII and E per VLDL$_1$ particle (all moles/particle)

ApoCII per VLDL1 vs TG per VLDL1

ApoCIII per VLDL1 vs TG per VLDL1

ApoE per VLDL1 vs TG per VLDL1
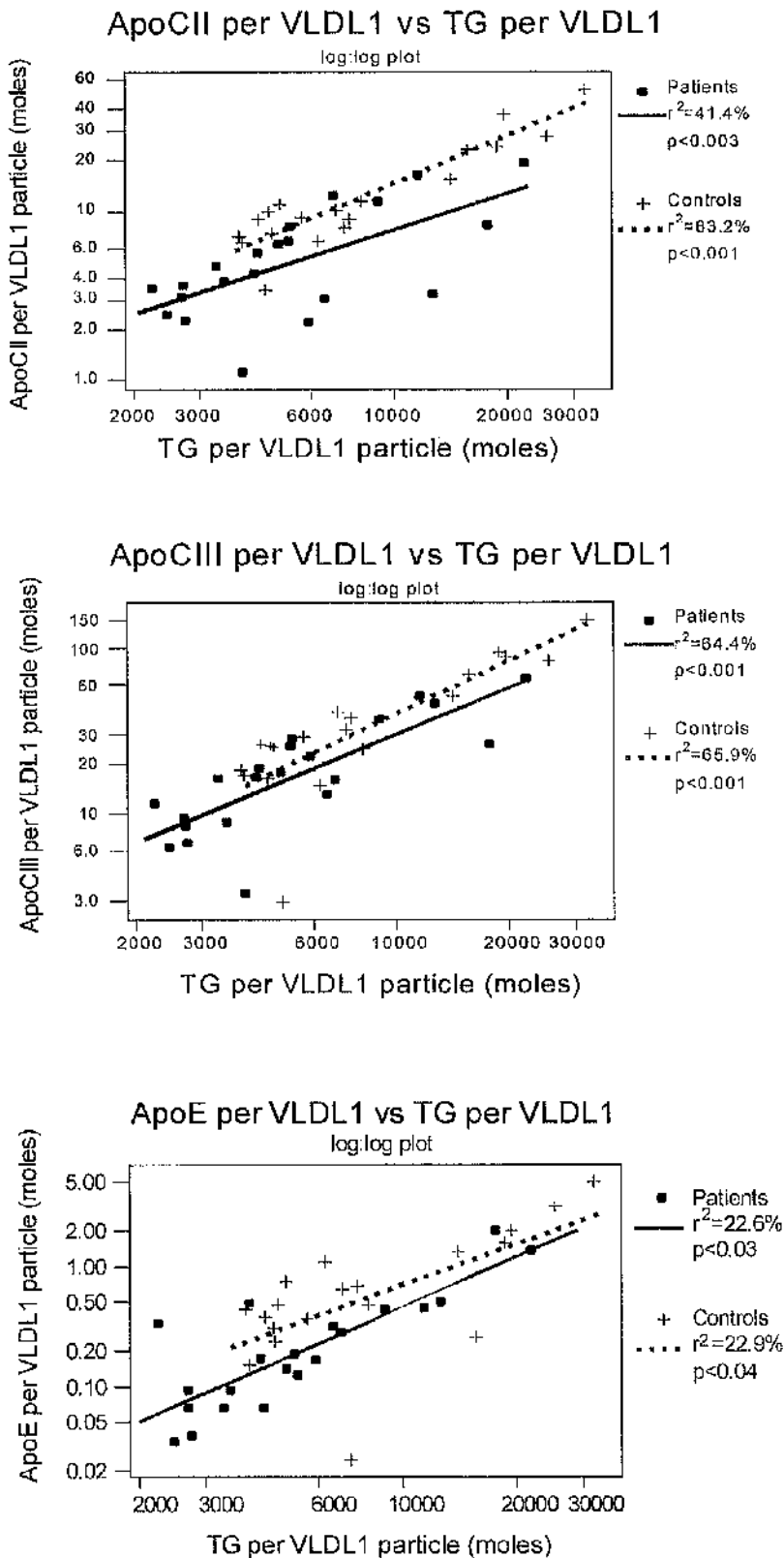
Fig 4.3: FC:PL ratio vs Apolipoprotein CII, CIII and E per VLDL₁ particle (all moles/particle)

ApoCII per VLDL1 vs Free Chol: Phospholipid

ApoCIII per VLDL1 vs Free Chol: Phospholipid

ApoE per VLDL1 vs Free Chol: Phospholipid
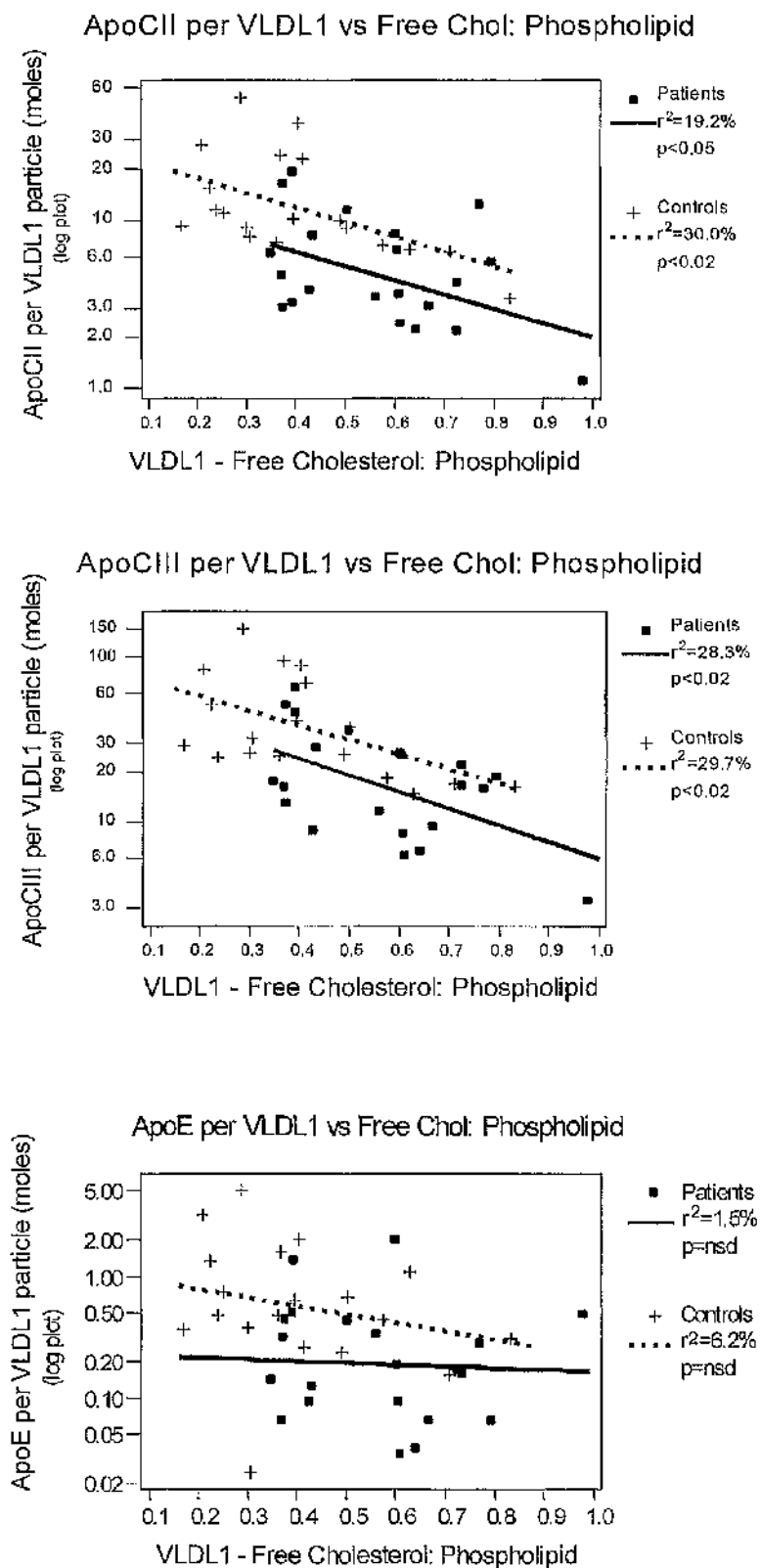
4.4 DISCUSSION

The data presented here suggest that patients with proteinuria have plasma apoCII and CIII levels that are increased but apparently proportionate to the excess of lipoprotein particles. However the data also suggest that there is a relative deficiency in circulating apoE. We planned to explore the causes of the VLDL difference. Given that we have demonstrated that heparin releasable lipoprotein lipase activity was not reduced in proteinuria [109], we hypothesised that the impaired clearance of $VLDL_1$ that has been noted previously [108,171] may result from a relative lack of apolipoproteins on the $VLDL_1$ particle, particularly apoCII the lipoprotein lipase cofactor. As a result, we have shown that the $VLDL_1$ particles in this population are deficient in apoCII, apoCIII and apoE. The study has also suggested that the deficiency in apolipoprotein C is related to a combination of the presence of smaller $VLDL_1$ particles, and $VLDL_1$ particles which are relatively enriched in free cholesterol, with an increased ratio of free cholesterol to phospholipid (FC:PL) on their surface. The VLDL apoE deficiency was related to the presence of smaller $VLDL_1$ particles but was not explained by altered lipid composition.

Patients with nephrotic range proteinuria are markedly heterogeneous in terms of their clinical diagnosis, renal function and plasma albumin. Indeed some studies looking at dyslipidaemia in the nephrotic syndrome require the patients only to have nephrotic range proteinuria, without necessarily being hypoalbuminaemic [156,182]. Metabolic studies have shown that $VLDL_1$ clearance is reduced and $VLDL_2$ production increased in a group of patients with nephrotic range proteinuria who were predominantly normoalbuminaemic [171]. More recently $VLDL_1$ concentration has been shown to be increased two-fold, with a four-fold increase in $VLDL_2$ and reduced $VLDL_1$, $VLDL_2$

and IDL clearance in a population with nephrotic syndrome and normal renal function [108]. In our study, neither the heterogeneity in renal function or plasma albumin, seem to have had a major influence on the lipoprotein apolipoprotein compositions. As a result the proteinuric patients were treated as a whole group.

A clear relationship is seen between the number of moles of apoCII, apoCIII and apoE per $VLDL_1$ particle and the $VLDL_1$ mass. Thus in both patients and controls, as the $VLDL_1$ mass increases, the apolipoprotein content of VLDL particles decreases. However, there is also a clear tendency for the patients with proteinuria to have smaller $VLDL_1$ particles compared with controls. Given the relationship between the apolipoprotein content of each particle and the particle size (i.e. triglyceride per particle), it is important to decide if particle size and the presence of proteinuria were independent factors. From our data we concluded that for apoCII, apoCIII, and apoE, particle size and lipid composition (i.e. FC:PL ratio) did not appear to explain all of the differences in apolipoprotein content between the patients and controls, and that there appeared that to be an additional effect of being in the proteinuric group. We proceeded to explore the relationships between lipoprotein size, lipid and apolipoprotein content not only for what they told us about hyperlipidaemia in nephrotic range proteinuria, but also to identify the factors that might regulate the apolipoprotein content of large triglyceride rich $VLDL_1$.

Despite apparently adequate plasma levels of apoC, both apoCII and apoCIII per $VLDL_1$ particle were reduced in the proteinuric patients. Moreover, as VLDL particle size decreased or FC:PL increased, the apoCII:CIII ratio remained constant. This is in contrast to the reported situation in other hypertriglyceridaemic conditions, where

apoCIII increases and apoCII decreases with a decrease in the apoCII:CIII ratio as plasma triglyceride and $VLDL_1$ mass increase [183,184]. It is also noteworthy that the compositional data obtained for the controls corresponded closely to those obtained by other authors [185]. The compositional abnormalities seen in the patients with nephrotic range proteinuria, may cast light on the nature of the hypertriglyceridaemia observed in this population. ApoCII is a cofactor which activates lipoprotein lipase (LpL). ApoCIII is thought to inhibit the lipolysis of triglyceride rich lipoproteins and may, if present in excess, cause hypertriglyceridaemia [181]. We have demonstrated that in nephrotic range proteinuria, inefficient lipolysis cannot be related to this as apoCIII is not increased. Therefore it is likely that the lack of apoCII causes the hypertriglyceridaemia associated with impaired catabolism of $VLDL_1$ to $VLDL_2$ [171].

These data also indicate that within the discrete density class of $VLDL_1$, across a wide range of $VLDL_1$ mass, the increase in $VLDL_1$ size (increased triglyceride per particle) leads to an increase in the surface area of the lipoproteins and a greater ability to accumulate apolipoprotein CII, CIII and E. Apart from apolipoproteins, the main surface constituents of lipoproteins are free cholesterol and phospholipid. The apoCII and apoCIII content per particle was independently associated with the surface FC:PL ratio. We hypothesise that the reduction in apoC observed, as FC:PL increases is due to the changing nature of the phospholipid layer as it becomes enriched in free cholesterol [186]. Free cholesterol will form a hydrogen bond with phospholipid and a phospholipid layer is saturated when FC:PL approaches 1.0 [187]. It is therefore interesting to observe that in fig 4.3 that as this ratio approaches unity, the number of apoCs per $VLDL_1$ reduces dramatically. Thus it may be that the lack of low molecular weight apolipoproteins in the patients $VLDL_1$ is also due to the saturation of the

phospholipid layer with free cholesterol. The mechanism underlying this association may be as follows. Small apolipoproteins such as apoC are known to bind to VLDL by penetrating the surface phospholipid matrix of lipoproteins and embedding their amphipathic helical structures in the phospholipid layer [181]. If the phospholipid layer is not fluid enough, due to the excess FC, then it is likely that apoCs will not be able to bind. We postulate therefore that even in the VLDL$_1$ range, the free cholesterol enrichment of the surface leads to an inability to incorporate apoC by exchange from HDL, and this, as a result, has consequences for efficient processing of VLDL$_1$ particles. There is evidence to suggest that free cholesterol is overproduced in the nephrotic syndrome [188], however indirect assessment of cholesterol synthesis does not support this evidence [189]. We hypothesise that patients with nephrotic range proteinuria may be secreting VLDL which given the smaller particle size, has a relative excess of surface free cholesterol and a high FC:PL. However further studies are required to assess whether this is related to excess free cholesterol production. It is interesting to note that despite the differences in particle size and FC:PL, having proteinuria remained an independent predictor of apoC per particle. Whether this is related to a relative deficiency of apoC, not identified by assessing the plasma apoC/B ratio, to the spread of the FC:PL results obtained in both patients and controls or to another as yet unidentified mechanism, remains to be determined.

Apolipoprotein E per VLDL$_1$ particle was determined by particle size and subject group. In the case of apoE, we suggest that the group effect is likely to reflect the relative plasma deficiency of apoE that was present in patients with proteinuria. The reduction in apoE per particle seen in the patients is likely to have major consequences for clearance of VLDL by the VLDL receptor, LDL-receptor related protein, or LDL

receptor, all of which are thought to use apoE as their ligand [179]. Indeed it can be calculated that between 67-80% of the $VLDL_1$ particles in the patients are devoid of apoE. It is unclear why a specific plasma deficiency of apoE was found in patients with nephrotic range proteinuria. All three apolipoproteins: apoE (34000 daltons), apoCII and apoCIII (8800 daltons), are smaller than albumin (65000 daltons) and thus could easily be lost in the urine. It may be that the plasma is deficient in all three apolipoproteins or that a specific deficit in plasma apoE is present due to varying affinity of the apolipoproteins to the lipoproteins, failure of the liver to produce apoE, or accelerated apoE catabolism. Elucidation of the precise mechanism will require further study. The observation that there was no independent association between apoE per particle and FC:PL suggests that inter-lipoprotein transfer in plasma is less important for apoE. It is interesting to note that recent evidence suggests that apoE has a role in VLDL production and apoE deficient hepatocytes produce smaller VLDL than controls [190]. It may be that the relative deficiency of apoE found in the proteinuric patients plays a role in the appearance of smaller $VLDL_1$ in this study.

In summary we have shown that patients with nephrotic range proteinuria possess $VLDL_1$ particles that are deficient in apolipoprotein CII, CIII and E. We have suggested two mechanisms that would potentially explain the hypertriglyceridaemia in patients with nephrotic-range proteinuria and explain previous observations that there is a primary clearance failure in this population [108,171]. The lack of apoCII on the VLDL particles will, it is predicted, result in inefficient lipolysis, and may effect not only $VLDL_1$ but also $VLDL_2$. The decreased plasma and VLDL apoE is likely to result in reduced receptor mediated catabolism of VLDL particles. The compositional analysis indicates that the abnormal generation in the liver of a smaller $VLDL_1$ particle

which is relatively enriched in free cholesterol and deficient in apoE might be sufficient explanation for the abnormalities observed in the VLDL$_1$ density range. The resulting hypertriglyceridaemia is of clinical importance as it is a major determinant of other aspects of lipoprotein abnormalities particularly the presence of both small dense LDL and remnant lipoproteins with their associated increased cardiovascular risk.

CHAPTER 5 -

Comparative Effects of Cerivastatin and Fenofibrate on the Atherogenic Lipoprotein Phenotype in Proteinuric Renal Disease

INTRODUCTION

It is now established that pharmacological lipid lowering with either HMG CoA reductase inhibitors (statins) or fibrates reduces cardiovascular morbidity and mortality [64-68, 70, 191]. However the majority of patients with coronary artery disease do not have hypercholesterolaemia, with a large proportion having a pattern of mild hypertriglyceridaemia, low HDL and excess small dense LDL [97] - the atherogenic lipoprotein phenotype [140]. In normal populations, statins will reduce the plasma concentration of atherogenic small dense LDL particles by lowering all 3 LDL subfractions in concert [153]. Fibrates promote a shift in LDL size towards larger lighter particles, with or without a change in total LDL [154,155], with recent data suggesting that fibrates reduce coronary events in patients with coronary heart disease, low HDL-C and raised triglycerides, without altering LDL-C concentration [191].

We have demonstrated that not only do patients with nephrotic range proteinuria possess atherogenic quantities of LDLIII [109], they also have excess quantities of atherogenic lipoprotein remnants. In patients with nephrotic range proteinuria, statins have been shown to reduce cholesterol and LDL-C [104]. This cholesterol lowering effect is accompanied by plasma triglyceride reduction and increased receptor mediated LDL clearance [156]. Given the link between increases in plasma

triglyceride and both small dense LDL formation [56] and concentrations of remnant lipoproteins [80], the triglyceride lowering effect of statins may have a beneficial effect not only on LDLIII concentration but also on the relative proportions of each LDL subfraction, and on remnant lipoproteins. The aim of this study therefore, was to compare the ability of a statin (cerivastatin) and a fibrate (fenofibrate) to lower levels of these atherogenic lipoproteins in patients with nephrotic range proteinuria. A secondary aim was to assess changes in VLDL subfractions and plasma apolipoproteins, to help identify mechanisms underlying any changes in the atherogenic lipoproteins.

SUBJECTS & METHODS

Subjects

12 patients (10 males, 2 females) were recruited from the out-patient clinic of the Glasgow Royal Infirmary Renal Unit according to the following inclusion criteria:

(a) primary glomerular disease

(b) urinary albumin excretion >1.5g/24hrs (equivalent to 3g proteinuria per 24hrs)

(c) serum creatinine <250umol/l.

(d) plasma cholesterol >6.5mmol/l

(e) plasma triglyceride >1.5mmol/l.

Any patient with a significant deterioration in their renal function during the 6 months prior to the study was excluded. Patients suffering from other diseases or on treatment that might influence their lipid profile were excluded, specifically patients with diabetes mellitus or amyloid, or taking thiazide diuretics, fat soluble beta blockers,

corticosteroids or other immunosuppressive agents. Treatment with other antihypertensives or diuretics was permitted. Any patient receiving lipid lowering therapy had their lipid lowering medication stopped for a period of 4 weeks prior to inclusion in the study. All patients had a biopsy proven diagnosis.

The study had a randomised, crossover design and lasted 5 months. 6 patients were randomised (using random numbers) to receive cerivastatin, 100micrograms (ug) for 1 month increasing to 200ug for the next month, or fenofibrate 200mg for 2 months. At the end of 2 months, the drug was stopped, patients were given a month off lipid lowering treatment as a wash-out period and then given the alternative treatment for 2 months. Patients were seen on a monthly basis, attending after an overnight fast. At each visit blood was sampled for creatinine, albumin, liver function tests (LFTs), creatine kinase (CK), lipids and lipoproteins, VLDL and LDL subfractions. A 24hr urine collection was taken at each visit for creatinine clearance and urinary albumin. The study was approved by the Ethics Committee of the Glasgow Royal Infirmary. All patients gave written consent before participating.

Statistics

Statistical analysis were performed by using MINITAB 10X for Windows (Minitab Inc.) and SPSS statistics package. Results are shown as means and standard deviations. Baseline and endpoint data was compared using ANOVA repeated measures of analysis. If this showed a significant difference then the 2 treatment modalities were further compared using paired *t*-test. Data before and after treatment was compared using paired *t*-test. Simple regression analysis was performed to identify significant correlations.

RESULTS

Baseline data

Ten males and 2 females were recruited. The diagnoses were IgA nephropathy (n=4), membranous nephropathy (n=3), minimal change nephropathy (n=2), mesangiocapillary glomerulonephritis (n=2) and focal and segmental glomerulosclerosis (n=1). The baseline data obtained before treatment with either cerivastatin or fenofibrate is shown in tables 5.1-5.4. Values at the beginning of each treatment phase (baseline and washout) did not differ for any parameter studied. The patients had a body mass index of 27.7 ± 3.6. Serum albumin was 35.8 ± 5.5g/l, 3 patients had a serum albumin <32g/l, however only one patient had significant oedema. Average blood pressure was 143/87mmHg, with 9 patients receiving antihypertensives. Only 3 of the 12 patients before cerivastatin and 2/12 before fenofibrate treatment had a serum creatinine >150umol/l. Compared to the normal population, the mean cholesterol, triglyceride, VLDL-C, and LDL-C were all raised, whilst HDL-C was reduced (Table 5.1). Total VLDL concentration and both VLDL subfractions were increased 4-5 times normal [144]. IDL concentration and total LDL concentration were increased 2 fold [144] (Table 5.2). LDLIII was the most prevalent LDL particle (Table 5.3) with a marked shift in subfraction distribution towards small dense LDL. Ten of the 12 patients in each group had an LDLIII concentration >100mg/dl. Compared with normal reference values, remnant lipoprotein cholesterol and triglyceride were both raised at baseline [170] (Table 5.4).

Biochemistry, Renal function, Blood pressure, Lipids & Lipoproteins

Changes in lipids and lipoproteins following treatment with cerivastatin and

fenofibrate are seen in Table 5.1. Cerivastatin produced a significant fall in cholesterol (21%), triglyceride (14%) and LDL-C (23%). The reduction in VLDL-C just failed to reach significance (16% reduction, 95% CI -36:4), and no change in HDL-C was observed. One patient developed myalgia on 200ug of cerivastatin which resolved on decreasing to a 100ug dose. No increment in CK was found. No change in serum creatinine, creatinine clearance, LFTs or CK was observed on cerivastatin treatment. Fenofibrate reduced plasma triglyceride (41%) and VLDL-C (52%). A significant reduction in plasma cholesterol (19%) was also seen, with a 19% increase in HDL-C. There was no consistent change in LDL-C. Fenofibrate treatment was associated with a 14% increase in serum creatinine (p<0.01) but no change in creatinine clearance was observed. There was also no significant change in CK following fenofibrate treatment (mean increase 78u/l, 95%CI: −105:261). One patient developed abnormal LFTs and a rise in CK after 2 months on fenofibrate. These values returned to normal after stopping the drug as planned.

Despite the greater reduction of LDL-C by cerivastatin, there was no significant difference in cholesterol or LDL-C at the end of each treatment period (Table 5.1). Plasma triglyceride (p<0.01) and VLDL-C (p<0.05), were lower after treatment with fenofibrate compared with cerivastatin. The absolute decrease in triglycerides (TG) and VLDL-C was also greater following fenofibrate compared with cerivastatin (mean difference TG 0.8mmol/l, 95% CI 0.4:1.3, VLDL-C 0.5mmol/l, 95% CI 0.1:0.9). HDL-C was higher after fenofibrate (p<0.05) with the response to fenofibrate being 0.15mmol/l greater than cerivastatin (95% CI 0.06:0.25). The on treatment serum creatinine was higher following fenofibrate, however there was no difference in creatinine clearance (Table 5.1).

VLDL, VLDL subfractions, IDL and LDL concentration

Cerivastatin produced a significant reduction in total VLDL concentration (26%) and total LDL concentration (18%). Changes in VLDL subfractions and IDL were less clear-cut with an 18% reduction in $VLDL_1$ (mean decrease 80mg/dl 95% CI 19:141), a 14% reduction $VLDL_2$ (mean decrease 29mg/dl 95% CI -4:61), and a non-significant reduction in IDL concentration (20%, table 5.2). Fenofibrate produced a much greater reduction in the triglyceride rich lipoproteins with large decreases in total VLDL (55%), $VLDL_1$ (57%), and $VLDL_2$ (47%). There was no significant reduction in IDL or LDL concentration.

Comparing the final results achieved (table 5.2) reveals that concentrations of total VLDL ($p<0.01$), $VLDL_1$ ($p<0.05$) and $VLDL_2$ ($p<0.05$) were all lower following treatment with fenofibrate compared with cerivastatin. The absolute reduction in total VLDL, $VLDL_1$ and $VLDL_2$ was also greater on fenofibrate, with a difference of 118mg/dl for total VLDL (95% CI 29: 207), and 39mg/dl for $VLDL_2$ (95% CI 12: 66). The difference in the absolute reduction of $VLDL_1$ concentration did not quite reach significance (mean difference 79mg/dl, 95% CI −13: 170). LDL concentration was lower following cerivastatin however the difference was not significant. The on treatment IDL results were similar for both treatments.

LDL Subfractions

Cerivastatin reduced LDLIII concentration by 27% (table 5.3). The quantity of LDLI present was very low and there was a negligible change in LDLII concentration. The relative percentage of each LDL subfraction did not change. Following fenofibrate

treatment LDLIII concentration decreased by 49% with a corresponding 138% increase in the concentration of LDLI. The relative proportions of each LDL subfraction therefore changed on this drug with a shift in particle size towards larger lighter particles. %LDLIII decreased from 60 to 33% (95% CI 12:41, p<0.01), whilst the %LDLI and %LDLII both increased (%LDLI: 8% to 19%, 95% CI 3:19, p<0.05; %LDLII 32% to 48%, 95% CI 1:30, p<0.05).

On treatment LDLIII concentration was lower (p<0.05) and LDLI concentration higher (p<0.01) on fenofibrate compared to cerivastatin. The absolute response to treatment also differed for LDLI and LDLIII. Fenofibrate increased whilst cerivastatin decreased LDLI concentration (mean difference 33mg/dl, 95% CI 4:62). The absolute decrease in LDLIII concentration following fenofibrate was greater than that achieved with cerivastatin (although not quite reaching statistical significance - mean difference 63mg/dl, 95% CI -134:7, p<0.07). A similar finding was seen with the relative proportions of each LDL subfraction. At the end of each treatment, the %LDLI was higher (p<0.03) and the %LDLIII lower (p<0.03) following fenofibrate compared with cerivastatin (fig 5.1).

A close correlation was observed between the plasma triglyceride reduction and both the decrease in $VLDL_1$ ($r^2=70.5\%$ p<0.001) and LDLIII concentration ($r^2=67.5\%$ p<0.001, fig 5.2) following fenofibrate. The correlation between $VLDL_1$ reduction and the reduction in LDLIII concentration just failed to be statistically significant ($r^2=30\%$ p<0.07). No correlation was noted between reduction in LDLIII and either LDL-C (fig 5.2) or LDL concentration. Following cerivastatin the triglyceride reduction was not associated with the decrease in either $VLDL_1$ or LDLIII concentration (fig 5.2),

neither was $VLDL_1$ reduction associated with the change in LDLIII concentration. However, there was an association between the reduction in LDL-C and LDLIII concentration (r^2=33.7% p<0.05, fig 5.2), with weaker associations between $VLDL_2$ reduction and LDLIII (r^2=23% p=0.1) and between $VLDL_2$ reduction and LDL-C (r^2=31% p=0.06).

Remnant Lipoproteins

Cerivastatin treatment did not result in a significant reduction in levels of remnant lipoprotein cholesterol (RLP-C mean decrease 9mg/dl, 95%CI -8:26) or triglyceride (RLP-TG, mean decrease 20mg/dl, 95%CI -32:73, Table 5.4). The 65% increase in RLP-TG noted in table 5.4 was heavily skewed by one outlier. However if this outlier was removed, no difference in the results was obtained (RLP-C 13% decrease 95%CI –21:47, RLP-TG 1% decrease 95%CI -54:56). The change of both RLP-C and RLP-TG on cerivastatin treatment was associated with the change in $VLDL_1$ concentration (RLP-C r^2=39.5% p<0.03 (fig 5.3), RLP-TG r^2=40.0% p<0.04), but not triglyceride reduction (RLP-C r^2=15.0% p=nsd (fig 5.3), RLP-TG r^2=12.1% p=nsd). Fenofibrate reduced RLP-C by 35% (mean decrease 16mg/dl, 95%CI 8:25) and RLP-TG by 44% (43mg/dl 95%CI 9:77). Fenofibrate induced RLP reduction was associated with $VLDL_1$ reduction (RLP-C r^2=60.7% p<0.004 (fig 5.3), RLP-TG r^2=68.3% p<0.002), and plasma triglyceride reduction (RLP-C r^2=58.2% p<0.005 (fig 5.3), RLP-TG r^2=55.5% p<0.005). A relationship was also observed between the reduction in LDLIII concentration and both RLP-C (r^2=44.6% p<0.02, fig 5.4) and RLP-TG (r^2=35.0% p=0.05) following fenofibrate. Despite fenofibrate appearing to produce a greater reduction in RLP-C and RLP-TG, the on treatment RLP-C and RLP-TG concentrations did not differ between the two drugs.

Apolipoproteins

Cerivastatin treatment reduced plasma apoB (21%) and apoE (20%). Smaller reductions were seen in plasma apoCII (10%) and apoCIII (7%). Fenofibrate resulted in a reduction in plasma apoB (27%), apoE (32%), apoCII (29%) and apoCIII (26%). The on treatment plasma apoB and apoE concentrations did not differ between the two drugs, however plasma apoCII ($p<0.01$) and apoCIII ($p<0.01$) were lower following fenofibrate compared with cerivastatin. No significant correlations were observed between plasma apolipoprotein reduction and reduction in either LDLIII or remnant lipoproteins, however weak associations were noted between RLP-C reduction on fenofibrate and the decrease in plasma apoCII (r^2=31.5% $p<0.06$) and plasma apoCIII (r^2=26.8% $p<0.09$).

Table 5.1: Basic Lipid & Lipoproteins: Cerivastatin vs Fenofibrate (Mean±SD)

	Baseline	Cerivastatin Final Result (% decrease)	Fenofibrate Final Result (% decrease)
Creatinine (umol/l)	128 ± 38	127 ± 38 (2%)	145 ± 49[b] (-14%[d])
Creatinine Clearance (ml/min)	71 ± 27	74 ± 31 (-4%)	70 ± 30 (0.6%)
UAE (g/24hrs)	2.7 ± 1.4	2.8 ± 1.3 (-4%)	2.3 ± 1.1 (2%)
Plasma Cholesterol (mmol/l)	7.7 ± 1.0	6.0 ± 0.9 (21%[d])	6.3 ± 1.0 (19%[d])
Plasma Triglyceride (mmol/l)	3.6 ± 2.6	2.8 ± 2.0 (14%[c])	2.2 ± 1.7[b] (41%[d])
VLDL-C (mmol/l)	1.6 ± 1.1	1.1 ± 0.8 (16%)	0.7 ± 0.6[a] (52%[d])
LDL-C (mmol/l)	5.2 ± 1.2	3.9 ± 0.8 (23%[d])	4.4 ± 0.8 (8%)
HDL-C (mmol/l)	0.96 ± 0.21	0.99 ± 0.23 (-4%)	1.12 ± 0.26[a] (-19%[d])

[a] $p<0.05$, [b] $p<0.01$ for final result cerivastatin vs fenofibrate

[c] $p<0.05$, [d] $p<0.01$ for response to treatment cerivastatin and fenofibrate

Table 5.2: Lipoprotein Concentration: Cerivastatin vs Fenofibrate

	Baseline	Cerivastatin	Fenofibrate
		Final Result (% decrease)	Final Result (% decrease)
Total VLDL (mg/dl)	433 ± 291	311 ± 232 (26%[d])	220 ± 214 [b] (55 %[d])
VLDL$_1$ (mg/dl)	287 ± 281	200 ± 217 (18%)	135 ± 184 [a] (57%[d])
VLDL$_2$ (mg/dl)	147 ± 61	112 ± 62 (14%)	85 ± 53 [a] (47%[d])
IDL (mg/dl)	97 ± 29	77 ± 38 (20%)	84 ± 23 (8%)
Total LDL (mg/dl)	447 ± 90	349 ± 48 (18%[d])	382 ± 80 (14%)

[a] $p<0.05$, [b] $p<0.01$ for final result cerivastatin vs fenofibrate

[c] $p<0.05$, [d] $p<0.01$ for response to treatment cerivastatin and fenofibrate

Table 5.3: LDL Subfractions: Cerivastatin vs Fenofibrate

	Baseline	Cerivastatin	Fenofibrate
		Final Result (% decrease)	Final Result (% decrease)
LDLI (mg/dl)	42 ± 42	36 ± 35 (24%)	67 ± 45 [b] (-138% [c])
LDLII (mg/dl)	145 ± 93	125 ± 62 (-2%)	183 ± 68 (-87%)
LDLIII (mg/dl)	259 ± 105	187 ± 85 (27% [d])	133 ± 95 [a] (49% [d])
%LDLI	9 ± 7	10 ± 9 (9%)	19 ± 17 [b] (-171% [d])
%LDLII	31 ± 16	36 ± 16 (-23%)	48 ± 15 (-95% [c])
%LDLIII	60 ± 23	54 ± 24 (11%)	33 ± 23 [b] (39% [d])

[a] $p<0.05$, [b] $p<0.01$ for final result cerivastatin vs fenofibrate

[c] $p<0.05$, [d] $p<0.01$ for response to treatment cerivastatin and fenofibrate

Table 5.4: Plasma Lipoprotein Remnants and Apolipoproteins: Cerivastatin vs Fenofibrate

	Baseline	Cerivastatin		Fenofibrate	
		Final Result	(% decrease)	Final Result	(% decrease)
RLP- C (mg/dl)	49 ± 24	39 ± 22	(-2%)	33 ± 21	(35%[d])
RLP-TG (mg/dl)	107 ± 93	80 ± 71	(-65%)	70 ± 101	(44%[d])
ApoB (mg/dl)	178 ± 34	134 ± 33	(21%[d])	135 ± 38	(27%[d])
ApoCII (mg/dl)	8.6 ± 3.8	7.3 ± 3.4	(10%[c])	6.4 ± 3.4 [b]	(29%[d])
ApoCIII (mg/dl)	35.1 ± 8.8	31.7 ± 7.8	(7%[c])	26.7 ± 7.5 [b]	(26%[d])
ApoE (mg/dl)	6.7 ± 3.8	5.0 ± 3.0	(20%[c])	4.5 ± 2.6	(32%[d])

[a] $p<0.05$, [b] $p<0.01$ for final result cerivastatin vs fenofibrate

[c] $p<0.05$, [d] $p<0.01$ for response to treatment cerivastatin and fenofibrate

Fig: 5.1

Absolute Change in %LDL Subfraction:
Cerivastatin vs Fenofibrate (Mean+SE)
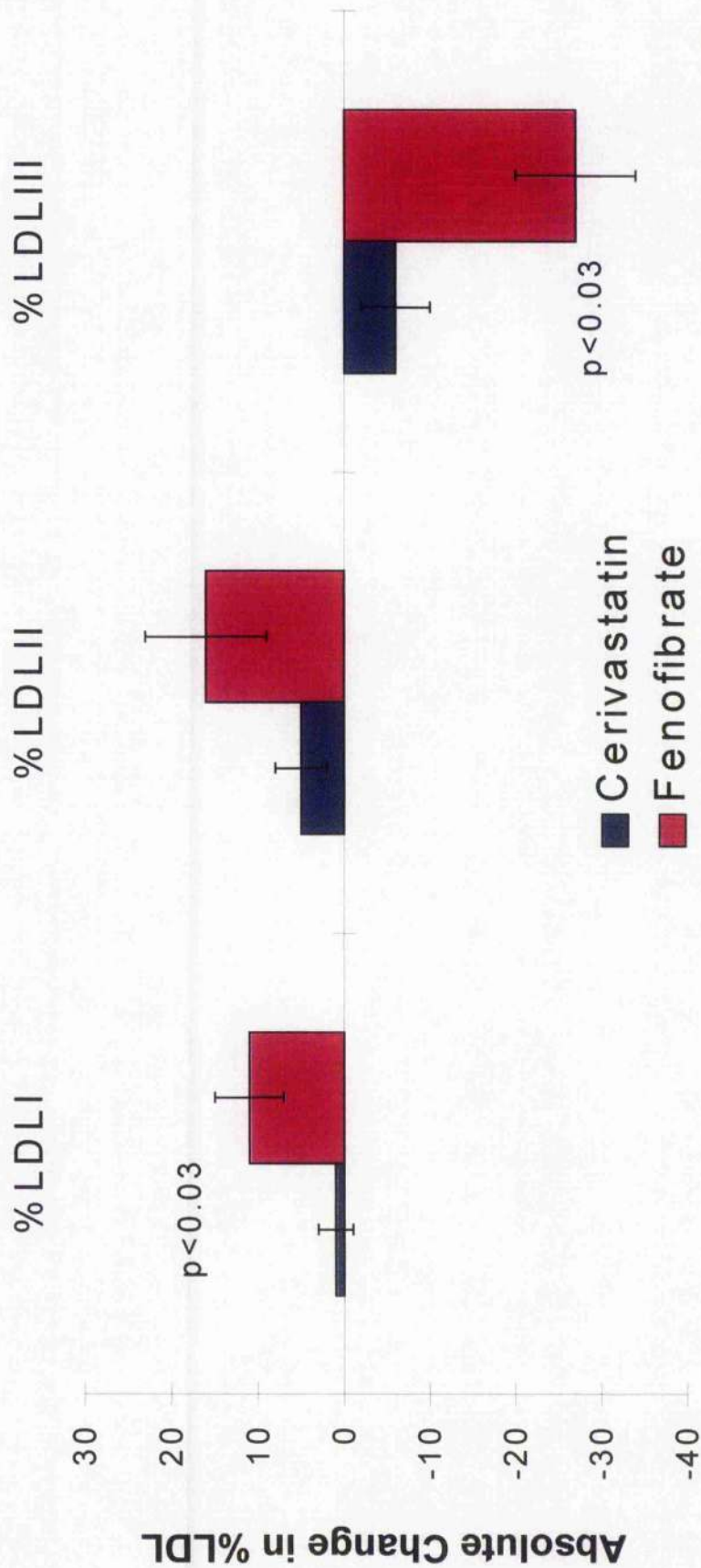
Fig: 5.2. Percentage Change in Plasma Triglyceride (TG) and LDL-Cholesterol (LDL-C) vs LDLIII mass for Fenofibrate and Cerivastatin.

% Change: TG vs LDLIII mass – Fenofibrate

$r^2 = 67.5\%$
$p < 0.001$

% Change Plasma Triglyceride

% Change LDLIII mass

% Change: TG vs LDLIII mass – Cerivastatin

$r^2 = 0.5\%$
$p = nsd$

% Change Plasma Triglyceride

% Change LDLIII mass

% Change: LDL-C vs LDLIII mass – Fenofibrate

$r^2 = 1.1\%$
$p = nsd$

% Change LDL- Cholesterol

% Change LDLIII mass

% Change - LDL-C vs LDLIII mass : Cerivastatin

$r^2 = 33.7\%$
$p < 0.05$

% Change LDL- Cholesterol

% Change LDLIII mass

Fig 5.3: Percentage Change in Plasma Triglyceride (TG) and VLDL₁ mass vs Remnant Lipoprotein Cholesterol (RLP-C) for Fenofibrate and Cerivastatin.

Fig 5.4:

% Change: LDLIII vs RLP - C
- Fenofibrate

r^2=44.6%
p<0.02

% Change RLP - C

% Change LDLIII mass
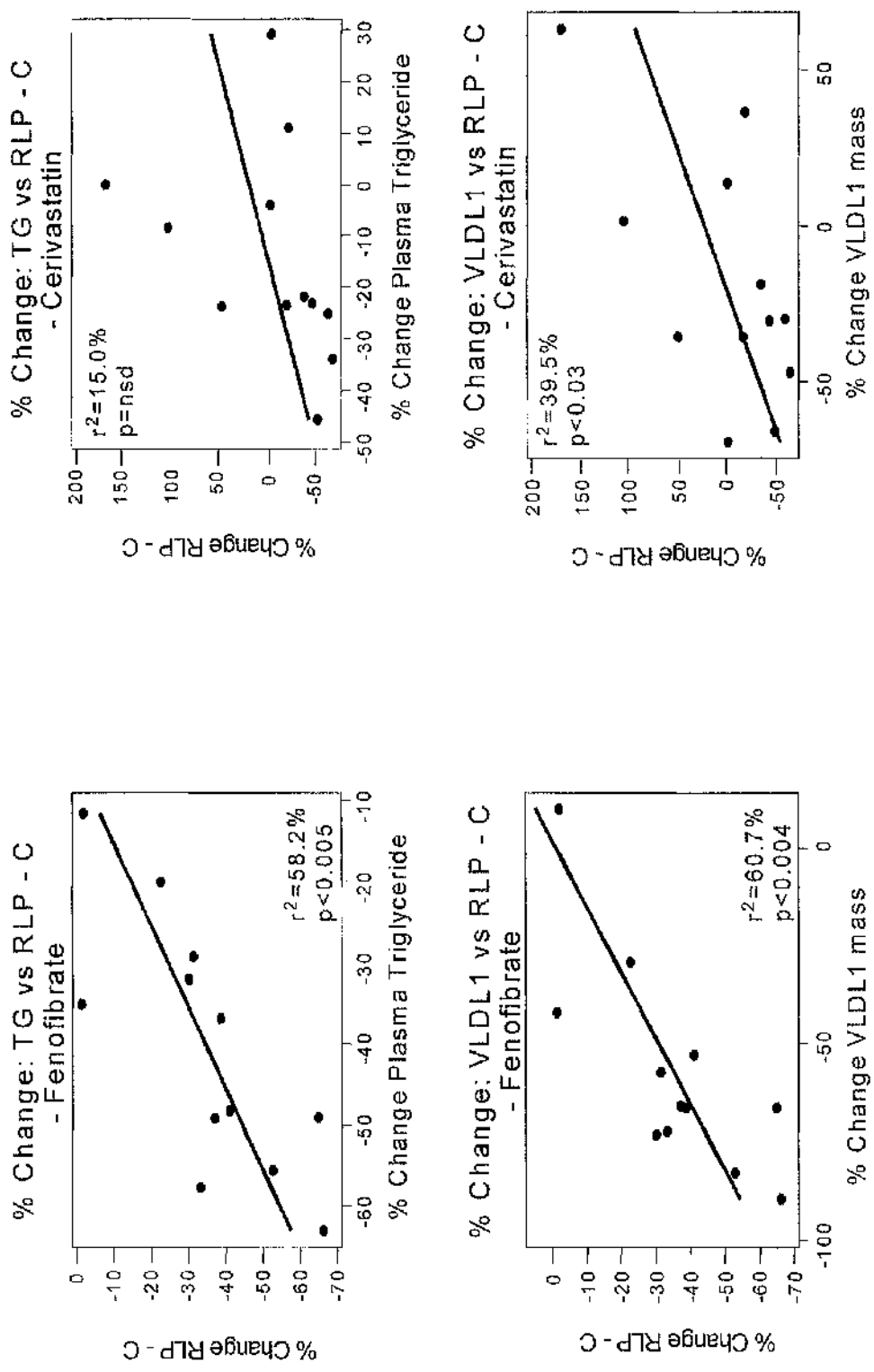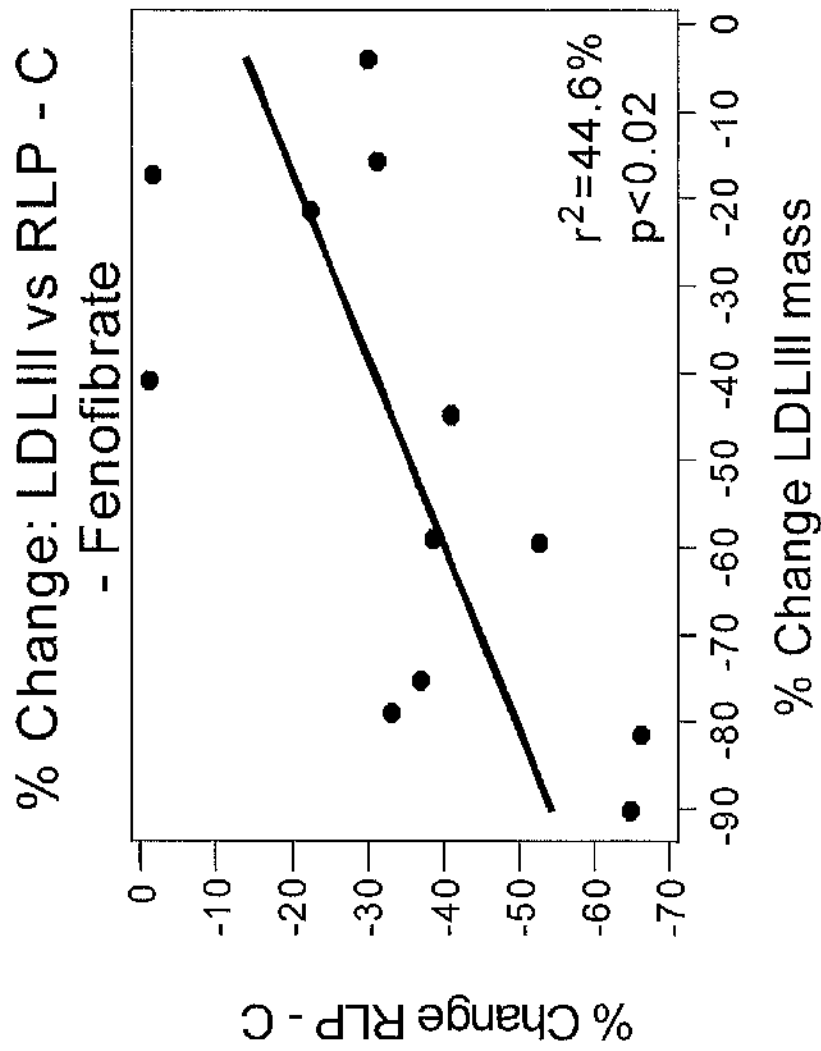
DISCUSSION

In this study we have demonstrated that in patients with nephrotic range proteinuria, both fenofibrate and cerivastatin significantly reduce plasma concentrations of small dense LDL. However the two treatments seem to differ in the means by which they alter LDL subfractions. Cerivastatin reduced LDLIII concentration by reducing circulating LDL concentration, without any effect on the LDL pattern. Fenofibrate however, had no overall effect on LDL concentration but produced marked LDLIII reduction by shifting LDL size towards less atherogenic larger lighter particles. Fenofibrate also resulted in a lower final concentration of LDLIII and proved more effective in lowering potentially atherogenic remnants of triglyceride rich lipoproteins. Despite successful lipid reduction, with 27% and 49% reductions in LDLIII, atherogenic levels of LDLIII remain on both drug regimens.

The main effect of fenofibrate was on plasma triglyceride, VLDL-C and HDL-C levels, with a significant reduction in plasma cholesterol, but not LDL-C. This response is similar to that found in non-proteinuric populations where it is known that the ability of fibrates to lower LDL-C is related to baseline triglyceride concentrations, with the greatest decreases in LDL seen in patients with initially normal plasma triglyceride levels [154,192]. In mixed hyperlipidaemia, as here, fibrates often fail to reduce LDL-C [192,193]. This response has been reported with other fibrates in nephrotic dyslipidaemia [194]. Cerivastatin reduced cholesterol and LDL-C, with a lesser, but still significant effect on triglycerides. This lipid lowering effect follows a similar pattern to that found with the drug in non-proteinuric populations, and with other statins in the large scale intervention studies [65,66].

In keeping with the marked reductions in triglycerides and VLDL-C, fenofibrate decreased both VLDL subfractions by 50%. It is thought that the main effect of fibrates is to suppress apolipoprotein CIII (apoCIII) synthesis and promote lipoprotein lipase activity [152]. ApoCIII inhibits lipolysis of VLDL by lipoprotein lipase and interferes with hepatic uptake of VLDL via apolipoprotein E. Thus fibrate therapy is likely to improve lipolysis and increase hepatic uptake of VLDL. In this context it is interesting to note that fenofibrate reduced potentially atherogenic lipoprotein remnants by 35-45%. This supports the theory that fibrates improve lipolysis and clearance of triglyceride rich lipoproteins. Metabolic studies suggest that the effect of statins on VLDL subfractions is partly dependent upon the dyslipidaemia present. In hypercholesterolaemic populations (with raised $VLDL_2$ but not $VLDL_1$), statins generally increase the fractional catabolic rate of $VLDL_2$ and therefore lower $VLDL_2$ concentration. $VLDL_1$ metabolism is generally unchanged [153]. However in mixed hyperlipidaemia, of which proteinuria is an example, statins appear to increase catabolism of all VLDL subfractions [195, Packard CJ - unpublished data]. Our study is in keeping with this hypothesis with cerivastatin treatment resulting in a reduction in triglyceride rich $VLDL_1$ with a modest (but not quite significant) reduction in cholesteryl ester enriched $VLDL_2$. That the statin failed to effect significantly the remnant lipoproteins is surprising since these drugs are effective in type III hyperlipidaemia, where remnants accumulate in the circulation [196]. Changes in remnant lipoproteins following statin treatment were associated with the changes in $VLDL_1$ concentration. Given the observed association between the reduction in $VLDL_1$ and RLP on both drug regimens, it is likely that the lack of significant remnant reduction observed following statin treatment results from the heterogeneity of the change in $VLDL_1$ (ranging from a 70% decrease to a 63% increase following

cerivastatin treatment).

Fenofibrate, while not consistently reducing total LDL concentration, did provide a dramatic shift in the quality of the LDL in the circulation. The predominant LDL subfraction at the beginning of therapy was LDLIII, however following treatment, the proportion that was LDLIII was reduced so that larger lighter LDLII became the major species present. Cerivastatin in contrast despite producing a decrease in both triglycerides and $VLDL_1$, did not shift the size distribution. All three subfractions decreased in concert. This is possibly because the $VLDL_1$ concentration, which according to published models [56], is critical in determining LDLIII, was less affected than on fenofibrate. Thus, comparing treatments, fenofibrate had a greater effect on LDLIII with a larger reduction and lower final LDLIII concentration compared with the statin.

Potential mechanisms underlying the change in LDLIII following treatment with fenofibrate and cerivastatin are further elucidated by examining the relationship between the changes in plasma triglycerides, LDL-C, VLDL subfractions and LDLIII concentration. Following fenofibrate therapy there is a strong association between triglyceride reduction and the decrease in both $VLDL_1$ and LDLIII concentration, a weak relationship was also seen between $VLDL_1$ reduction and the decrease in LDLIII concentration. Excess triglycerides (in the form of $VLDL_1$) promote formation of small dense LDL, by allowing the triglyceride enrichment of LDL (via CETP mediated exchange), thus making LDL a better substrate for lipolysis by hepatic lipase [56]. The association between triglyceride reduction and LDLIII reduction, supports the role of hypertriglyceridaemia in LDLIII formation. Following cerivastatin

treatment, the reduction in LDL-C is associated with the decrease in LDLIII. This suggests that the drug is reducing LDLIII by reducing the amount of LDL available for conversion to LDLIII and therefore lowering small dense LDL by a different mechanism to fenofibrate.

The changes in remnant lipoproteins (RLP) following treatment parallel those changes seen with the LDL phenotype. It is recognised that plasma triglycerides are the best marker for high levels of RLP [80]. Fenofibrate, by improving lipolysis, reduces remnants by 35-45%, with a close association between RLP reduction and triglyceride reduction. This hypothesis is further supported by noting the association between the reduction in RLP on fenofibrate, and the fenofibrate induced reduction in plasma VLDL$_1$, apolipoprotein CII and apolipoprotein CIII. A clear link is also established between RLP reduction and a decrease in LDLIII concentration. This is unsurprising given the common link of hypertriglyceridaemia but does suggest that some of the cardiovascular risk attributed to the atherogenic lipoprotein phenotype, may result from excess lipoprotein remnants.

Although fenofibrate treatment resulted in an increase in serum creatinine, no change in creatinine clearance was observed. Serum creatinine has been shown previously to increase in patients with impaired renal function treated with fenofibrate, however this increment is not associated with changes in either creatinine clearance or GFR and therefore does not seem to represent any loss of renal function [197]. Given that urinary creatinine also increases [197], it is likely that this increment represents an increase in the production of creatinine from muscle. Fenofibrate did not result in any myalgia and no significant increase in creatine kinase was observed. It remains to be

seen if this change in serum creatinine is of clinical importance. It is noteworthy that previous dose response studies have noted that increases in serum creatinine are transient despite continued therapy [198].

It is now accepted that heavy proteinuria increases the risk of vascular disease. It is evident that simply looking at the plasma cholesterol and LDL-C does not allow full assessment of the cardiovascular risk posed by dyslipidaemia in patients with nephrotic range proteinuria. In addition to hypercholesterolaemia, raised plasma triglyceride is well recognised in nephrotic range proteinuria [199], with reports of 67% [104] and 71% [106] of patients having a plasma triglyceride greater than 2mmol/l and 2.3mmol/l respectively. Thus the atherogenic lipoprotein phenotype is a common finding that is not necessarily expressed as an increase in LDL, but is nevertheless highly atherogenic. We have demonstrated that both statins and fibrates successfully reduce LDLIII concentration in a selected population with nephrotic range proteinuria and dyslipidaemia and further provided evidence that increased remnant lipoproteins are a component of the atherogenic lipoprotein phenotype that responds to fibrate but not to statin therapy. Despite significant lowering of lipids, lipoproteins and reductions in the concentration of LDLIII of 27% and 49% on the statin and fibrate respectively, atherogenic levels of small dense LDL remain. Ten patients had an LDLIII concentration >100mg/dl at baseline. Of these ten, only 3 patients following fenofibrate treatment, and no patients following cerivastatin had their LDLIII concentration reduced to less than 100mg/dl. The differing relationships between LDLIII reduction and (i) triglyceride reduction following fenofibrate and (ii) LDL-C reduction following cerivastatin in addition to the differing effects on LDL phenotype suggest a potential role for combined therapy to achieve greater reduction

in LDLIII concentration. We suggest that cardiovascular protection should aim not only to reduce total cholesterol but also reduce concentrations of small dense LDL and remnant lipoproteins.

CHAPTER 6 -

The Association of Lipoproteins with Progression of Renal Failure in Glomerular Disease

INTRODUCTION

It is now clearly established that dyslipidaemia contributes to increased atherosclerosis. There are many similarities between atherosclerosis and glomerulosclerosis [147] and it has been hypothesised that 'any condition resulting in albuminuria and hyperlipidaemia could establish self perpetuating renal damage' [148]. Experimental data has demonstrated that mesangial cells are susceptible to lipoprotein induced damage [148,200] and a number of animal models have demonstrated an association between dyslipidaemia and glomerular injury [114]. Studies looking at the relationship between dyslipidaemia and progression of renal disease in humans are less numerous. In diabetic nephropathy, increases in both cholesterol and triglyceride are independently associated with increased risk of renal failure [149,150,201]. Data in non-diabetic disease is less extensive, however recently, baseline levels of cholesterol, triglyceride, LDL-C, apoB and apoE have all been associated with progression of renal insufficiency in patients with glomerular disease [151]. These observations have been extended, with evidence to suggest that triglyceride rich apoB containing lipoproteins promote progression of human chronic renal failure [202].

Given the close association between excess triglyceride rich lipoproteins and progression, the importance of hypertriglyceridaemia in the production of excess

quantities of small dense LDL, we aimed to assess if dyslipidaemia and particularly small dense LDL and the subfractions of VLDL were associated with progression of renal failure in patients with proteinuric glomerular disease.

SUBJECTS AND METHODS

Subjects

30 patients with chronic glomerulonephritis were recruited from the out-patient clinics of the Royal Infirmary and Western Infirmary Renal Units in Glasgow. To be included, patients had to have, at the time of study:

(a) urinary albumin excretion >1.0g/24hrs,

(b) a creatinine clearance between 10ml/min and 80ml/min,

(c) a reduction in creatinine clearance over the previous year of follow-up.

Our aim was to investigate patients with a broad range of initial creatinine clearance, all of whom had deteriorating renal function as a result of glomerular disease. Patients whose glomerulonephritis was being actively treated with corticosteroids or immunosuppressive agents were excluded. Patients suffering other diseases or on treatment that might influence their lipid profile were excluded, specifically patients with underlying liver disease, diabetes mellitus, amyloid, systemic lupus erythematosis or any neoplastic disorder. Patients receiving lipid lowering therapy, thiazide diuretics or fat soluble beta blockers were included if their therapy was unchanged (i.e. either the drug or dose) over period of retrospective and prospective analysis (¬examination period). All patients gave written consent before participating. The study was approved by the Ethics Committee of Glasgow Royal Infirmary. On the

study date (month = 0, fig 6.1), patients provided a fasting blood sample for lipid and lipoprotein analysis, measurement of LDL and VLDL subfractions, serum creatinine and albumin, and a 24 hour urine for urinary albumin excretion. (The date of lipoprotein analysis is referred to as the study date, month = 0, the entire period of study, retrospective and prospective is referred to as the examination period.)

Rate of Progression

Rate of progression of renal failure was calculated using linear regression from the slope of the plot of creatinine clearance vs time and is expressed in ml/min/month. A minimum of 5 serial measurements of creatinine clearance and a minimum examination period of 1 year was used to calculate a slope. Creatinine clearance was calculated from the serum creatinine using the Cockcroft and Gault formula [169]. Systolic blood pressure, diastolic blood pressure and 24hr urinary albumin data was also collected over the same period. The examination period was terminated 9 months after recruitment of the last patient, with prospective data restricted to a maximum of 18 months after the study date for each individual patient. If any patient was commenced on, or had a change in the dose of lipid lowering therapy, was prescribed nephrotoxic medication or if they developed an intercurrent illness which resulted in an additional deterioration in their renal function, the examination period was terminated early. To minimise the effect of changes in renal function or proteinuria on plasma lipids and lipoproteins over the examination period, the time over which data was extracted was restricted to a maximum of 2.5yrs. To minimise the effect of malnourishment from advanced uraemia, patients with a creatinine clearance <10ml/min at the time of study were excluded.

Statistics

Statistical analyses were performed by using MINITAB 10X for Windows (Minitab Inc.) and Microsoft Excel for Windows 95 (Microsoft Corp.). Results are shown as means and standard deviations or as median and interquartile range (IQR). Samples were compared using the two-sample t-test. Variables that were not normally distributed were compared by Kruskall-Wallis test. Changes in albuminuria or blood pressure over follow-up were compared using analysis of variance and paired t-test for paired samples. Linear regression analysis was performed to identify significant correlations. P values less than 0.05 were considered significant. Stepwise regression and a general linear model was used for multivariate analysis. For regression analysis, any factors that were not normally distributed were subjected to log transformation.

RESULTS

Clinical data, renal function, blood pressure and albuminuria

30 patients with progressive chronic renal failure and albuminuria >1g/24hrs (equivalent to 1.5-2g of total urinary protein/day) were recruited. Clinical data for the patients is shown in table 6.1. Diagnoses were: IgA nephropathy (n=11), membranous nephropathy (n=6), chronic glomerulonephritis (n=5), focal and segmental glomerulosclerosis (n=4), mesangiocapillary glomerulonephritis (n=4). There were 27 males and 3 females. Fig 6.1 shows the length of prospective and retrospective periods of study for the 30 patients. The average total length over which creatinine clearance was examined was 1.9yrs (range 1.2-2.5yrs), with a examination period retrospectively of 1.1yrs, and prospectively of 0.8yrs. Four patients were commenced on lipid lowering therapy after measurement of their lipid profiles, and as a result the

rate of progression was analysed using retrospective data only. The other 26 patients had almost identical lengths of prospective and retrospective periods (11.5months (8-13) vs 11.0months (11-13) respectively - median and IQR). Creatinine clearance (CrCl) at the study date was 40 ± 21ml/min. The mean earliest recorded CrCl during the retrospective period was 47 ± 22ml/min, with the final CrCl at the end of the prospective period 33 ± 19ml/min (all mean+SD). The percentage reduction in creatinine clearance (CrCl) over the examination period was 32% (range 10-60%). Rates of progression ranged from -0.14 to -1.7ml/min/month, with a skewed distribution (mean -0.59ml/min/mth, median -0.52ml/min/mth). The median correlation coefficient for the regression line used to calculate rate of progression was 0.82 (interquartile range 0.67-0.93), and 93% of the regression lines had a correlation coefficient >0.5.

Fig 6.2 shows the data for albuminuria and blood pressure across the entire period of examination. Urinary albumin did not significantly differ across the period of study, with no difference between the first and last urinary albumin measurements available for individual patients as analysed by paired t-test (mean change: -26mg/24hrs 95% CI -602: 549). Urinary albumin averaged over the entire period of examination correlated with the rate of progression ($r^2=22.0$ $p<0.01$, fig 6.3a, table 6.1) and was found to be more accurate in predicting the rate of progression than the earliest available urinary albumin or the urinary albumin measured at the study date ($r^2=11.2$ $p<0.07$). Dividing patients into slow and fast progressors based on the median rate of progression of 0.5ml/min/mth, revealed similar results. The patients with the faster rate of progression had a higher urinary albumin over the period of follow-up ($p=0.005$) and a higher urinary albumin at the study date ($p<0.03$).

From earliest retrospective data, 24 of the 30 patients were hypertensive or receiving antihypertensive drugs; the mean number of antihypertensive drugs taken at this point was 1.1, compared to 1.6 at the end the examination period. Eleven patients at the beginning of the examination period and 18 at the end were receiving angiotensin converting enzyme inhibitors. Systolic blood pressure varied little over the examination period (fig 6.2) and the average of the first 2 readings did not differ from the last 2 readings (by paired t-test). Overall mean DBP did not differ across the entire examination period, there was however a significant reduction in the diastolic BP of individual patients (by paired t-test - mean reduction 3.7mmHg (95% CI 0.6-6.8mmHg). This is consistent with the increased use of antihypertensives. No correlation was noted between the rate of progression of renal failure and the mean systolic or diastolic blood pressure (table 6.1), and no there was no difference in blood pressure between slow and fast progressors.

Lipids, Lipoproteins and VLDL subfractions.

The results of the lipid and lipoprotein profiles are shown in table 6.2. The lipid profiles were typical for patients with proteinuria, normal serum albumin levels and mild to moderate chronic renal failure. Plasma cholesterol was marginally elevated, plasma triglyceride was increased and accompanied by a low HDL-C. LDL-C was normal. As a result, the ratio of cholesterol to HDL-C was markedly increased. Total VLDL and both VLDL subfractions showed a 2 fold increase compared to normal male values [144]. Similarly plasma concentration of IDL was increased 2 fold, whereas LDL concentration was normal.

Plasma cholesterol correlated with the rate of progression of renal failure, with a stronger relationship observed with the non HDL-cholesterol fraction and progression (r^2=18.4% p<0.02, fig 6.3b). The lipoprotein fraction that appeared to account for the relationship between plasma lipids and rate of progression was $VLDL_2$ (fig 6.4a), with no significant correlation noted between the other lipoproteins and the rate of progression. Dividing the patients into slow and fast progressors resulted in no significant differences in the lipids or lipoproteins between the 2 groups (table 6.2). There was no observed trend in non HDL-C or $VLDL_2$ across the range of creatinine clearance studied. No correlation was identified between either non HDL-C or $VLDL_2$ and urinary albumin or creatinine clearance at the study date.

Plasma concentration of LDL and LDL Subfractions

Table 6.3 shows the plasma concentration of LDL and LDL subfractions in the patients studied. The plasma concentration of LDLIII was increased 2 fold compared with normal male values [144] and the relative distribution of LDL was shifted towards the atherogenic smaller denser particles. Forty-six percent of the LDL present was in the form of LDLIII and 17 of the 30 patients studied (57%) were found to have an LDLIII concentration greater than 100mg/dl, a level associated with a marked increase in coronary heart disease risk. There was no correlation between LDLIII concentration and either CrCl or urinary albumin. Further, no correlation was found between either the concentration or percentage of LDLIII and the rate of progression of renal failure (fig 6.4b, table 6.3). There was also no association between the larger lighter LDL subfractions and the rate of progression. Dividing the patients into slow and fast progressors resulted in no significant differences between the two groups (table 6.3).

Parameters Determining Rate of Progression

Multivariate analysis using stepwise regression identified mean urinary albumin over the examination period and non-HDL cholesterol at the study date to be independent predictors of the rate of progression of renal failure (total r^2=33.9%, albuminuria 22.0%, non HDL-C 11.9%). Both these parameters were significant using a general linear model (albuminuria $p<0.02$, non HDL-C $p<0.04$). The association between the rate of progression of renal failure and both albuminuria and non HDL-C was independent of renal function (either measured at the study date or on the earliest visit during the examination period). The correlation between $VLDL_2$ and rate of progression was not independent of albuminuria ($p=0.085$).

Table 6.1: Clinical and Biochemical Data over Examination Period - Mean ± SD (* Median + Interquartile Range)

Parameter	All Patients (n=30)	Univariate Correlation with Rate of Progression (r^2%)	p for regression coefficient	Slow Progressors (< -0.5ml/min/mth) (n=15)	Fast Progressors (> -0.5ml/min/mth) (n=15)
Rate of Progression (ml/min/mth)*	-0.52 (-0.27 to -0.81)	-	-	-0.27 (-0.22 to -0.43)	-0.79 (-0.65 to -1.01)
Systolic BP (mmHg)	141 ± 15	0.5	<0.8	140 ± 16	141 ± 14
Diastolic BP (mmHg)	85 ± 9	5.2	<0.3	84 ± 9	86 ± 9
Body Mass Index	26.6 ± 5.0	3.1	<0.4	25.2 ± 2.9	28.3 ± 6.4
Urinary Albumin over Examination Period (g/24hrs)*	2.6 (1.7- 4.0)	22.0	<0.01	2.0 (1.4-2.6)	3.1 (2.7-4.5) [a]
Urinary Albumin at Study Date (g/24hrs)*	2.7 (1.9- 3.9)	11.6	<0.07	2.1 (1.8-3.5)	3.8 (2.2-6.2) [b]
Creatinine Clearance (ml/min)	40 ± 21	0.5	<0.8	36 ± 23	43 ± 18
Albumin (g/l)	37 ± 5	9.4	<0.2	38.8 ± 4.0	36.0 ± 5.7

[a] p=0.005 [b] p<0.03   for fast vs slow progressors (Kruskall Wallis)

Table 6.2: Plasma Concentrations of Lipids, Lipoproteins and VLDL subfractions at Study Date - Median + Interquartile Range

Parameter	All Patients (n=30)	Correlation with Rate of Progression (r^2%)	p for regression coefficient	Slow Progressors (<-0.5ml/min/mth) (n=15)	Fast Progressors (>-0.5ml/min/mth) (n=15)
Plasma Cholesterol (mmol/l)	6.1 (5.2-6.9)	14.7	<0.04	6.1 (5.1-6.8)	6.0 (5.2-7.5)
Plasma Triglyceride (mmol/l)	1.9 (1.4-3.2)	10.5	<0.09	1.8 (1.4-2.3)	2.2 (1.7-4.9)
VLDL-C (mmol/l)	0.9 (0.6-1.6)	4.0	<0.3	0.7 (0.4-1.1)	0.9 (0.6-2.5)
LDL-C (mmol/l)	3.9 (3.1-4.7)	5.8	<0.3	3.9 (3.2-4.8)	3.9 (3.0-4.7)
HDL-C (mmol/l)	1.0 (0.8-1.1)	7.3	<0.2	1.0 (0.9-1.2)	0.8 (0.7-1.1)
Non HDL-C (mmol/l)	5.0 (4.4-5.9)	18.4	<0.02	4.9 (4.1-5.3)	5.1 (4.4-6.6)
Total VLDL (mg/dl)	210 (134-332)	7.5	<0.2	188 (134-301)	250 (159-634)
$VLDL_1$ (mg/dl)	119 (59-180)	2.2	<0.5	119 (60-162)	145 (57-423)
$VLDL_2$ (mg/dl)	96 (75-140)	13.2	<0.05	90 (57-120)	100 (81-159)
IDL (mg/dl)	72 (54-89)	8.8	<0.2	75 (49-90)	72 (64-82)
LDL (mg/dl)	280 (222-367)	1.5	<0.6	279 (249-397)	281 (205-351)

Table 6.3: Plasma concentration of LDL Subfractions at Study Date - Median + IQR

LDL Subfraction	All Patients (n=30)	Correlation with Rate of Progression (r²%)	p for regression coefficient	Fast Progressors (> -0.5ml/min/mth) (n=15)	Slow Progressors (< -0.5ml/min/mth) (n=15)
LDLI concentration (mg/dl)	28 (11-52)	3.2	<0.4	20 (7-43)	33 (16-71)
LDLII concentration (mg/dl)	129 (59-203)	2.4	<0.5	105 (28-213)	134 (62-200)
LDLIII concentration (mg/dl)	108 (55-186)	2.4	<0.5	102 (73-204)	122 (49-184)
%LDLI	11 (5-16)	5.5	<0.3	8 (3-17)	11 (8-16)
%LDLII	38 (22-62)	2.2	<0.5	35 (19-64)	46 (24-60)
%LDLIII	48 (18-73)	4.1	<0.3	57 (22-78)	37 (17-71)

Fig 6.1: Distribution of Examination Period for Patients Studied:
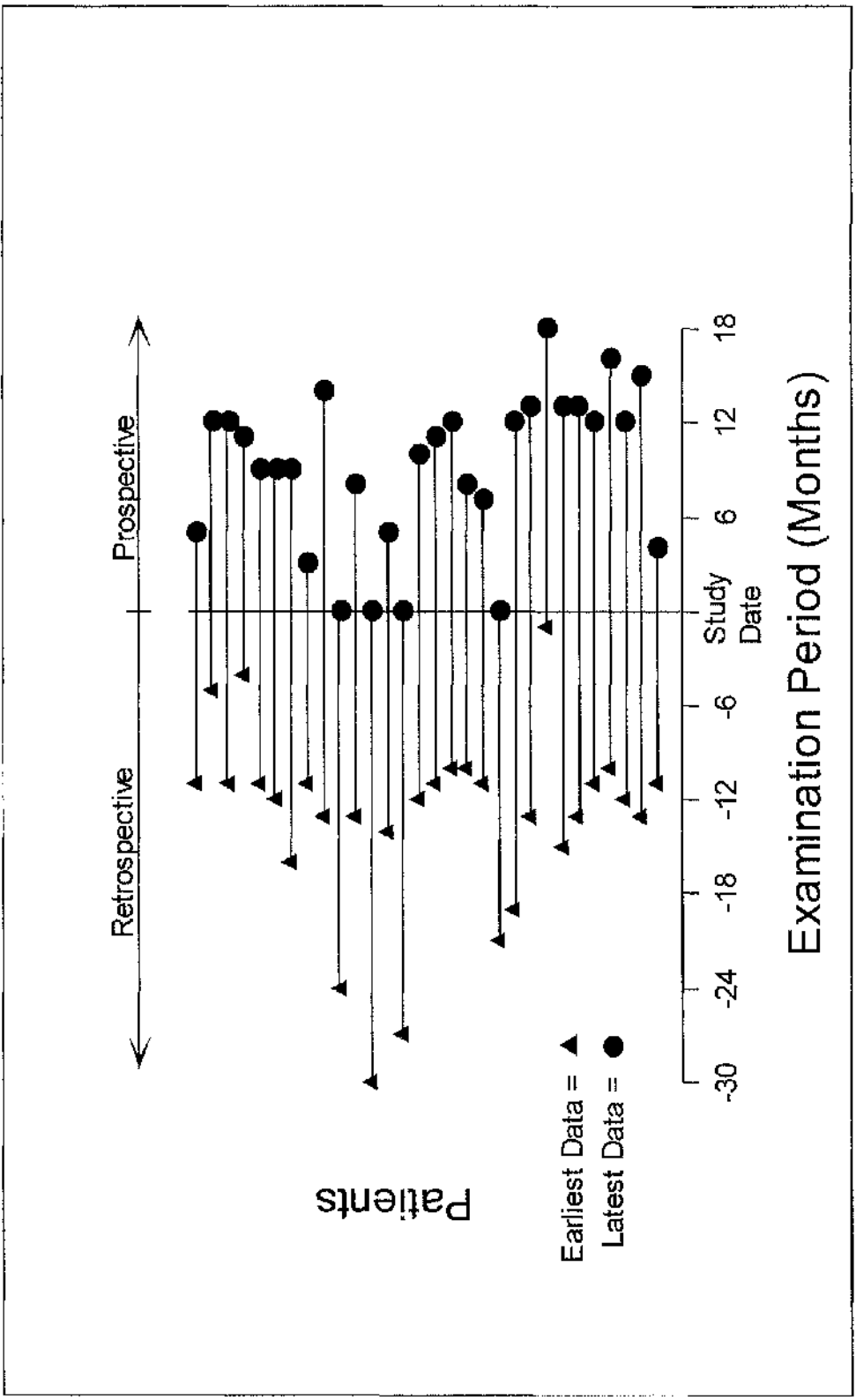
Fig 6.2: Urinary Albumin (Median+IQR) and Blood Pressure (Mean+SD) over Examination Period

Blood Pressure (mmHg)

Albuminuria (g/24hrs)

UAE
SBP
DBP

n=   27   30   30   22

< -6mths   -6: 0mths   0-6mths   >6mths

6 month periods
(Retrospective (-) and Prospective)
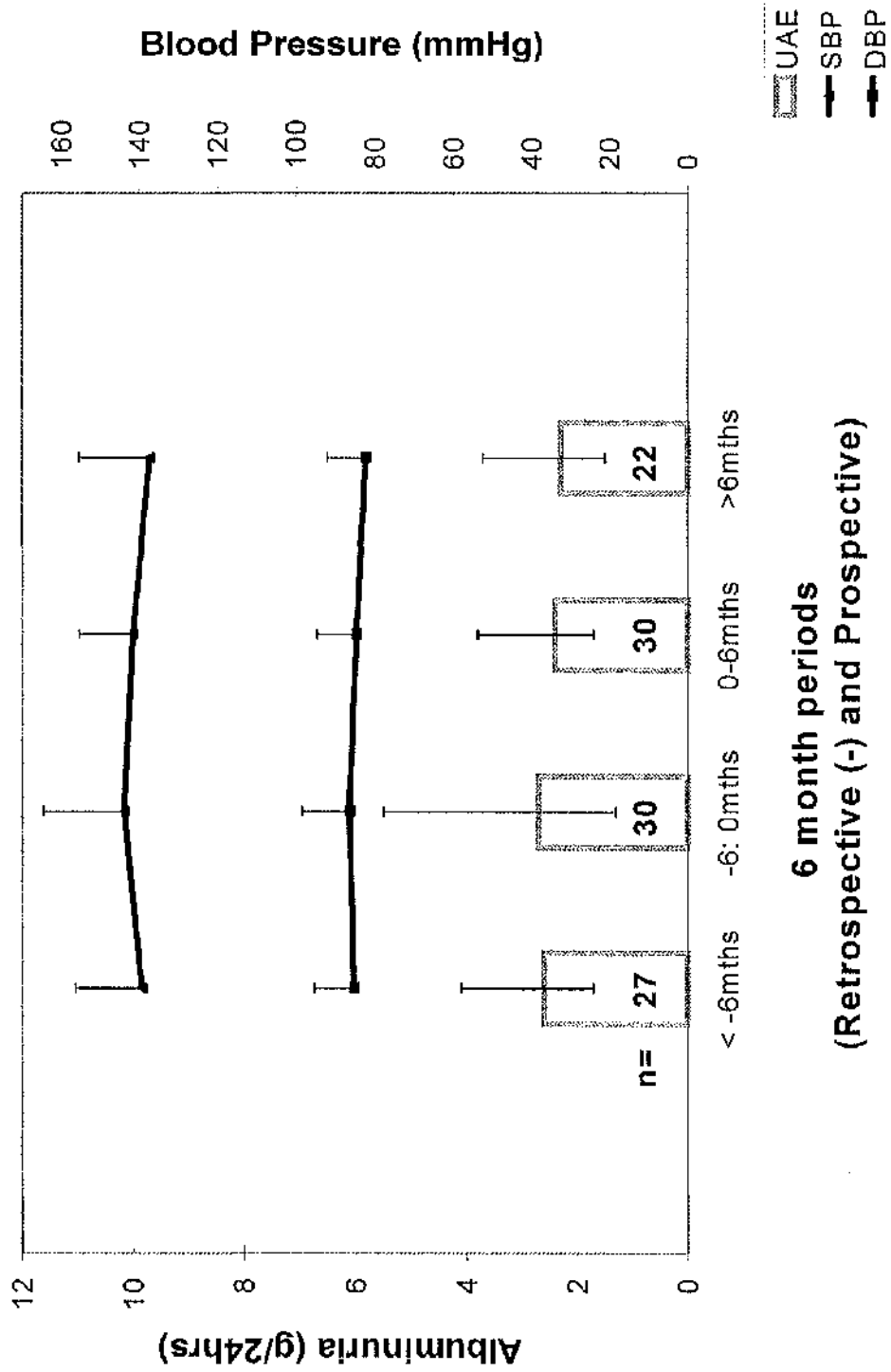
Rate of Progression vs Mean Urinary Albumin over Examination Period and Non HDL-C

Mean Urinary Albumin vs Rate of Progression
(log:log plot)
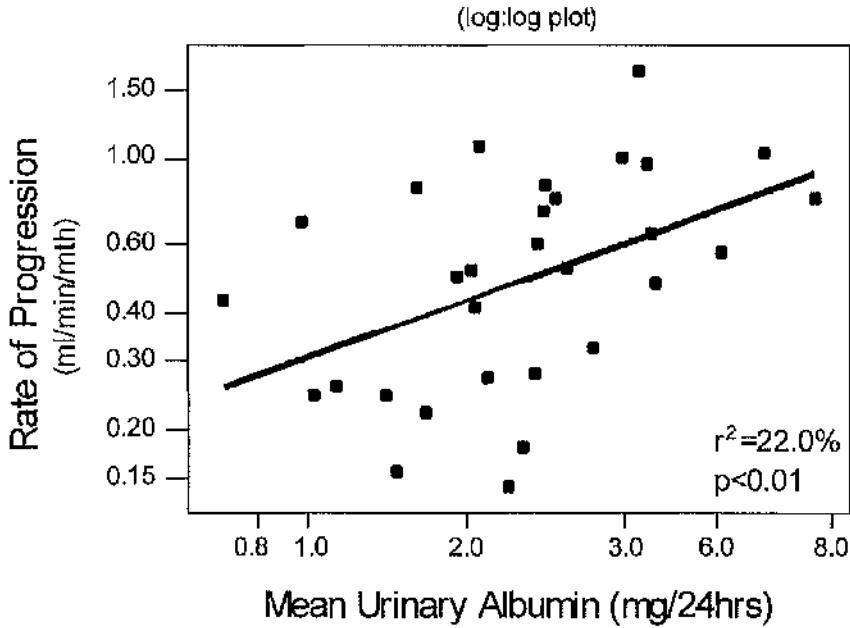
Non HDL-C vs Rate of Progression
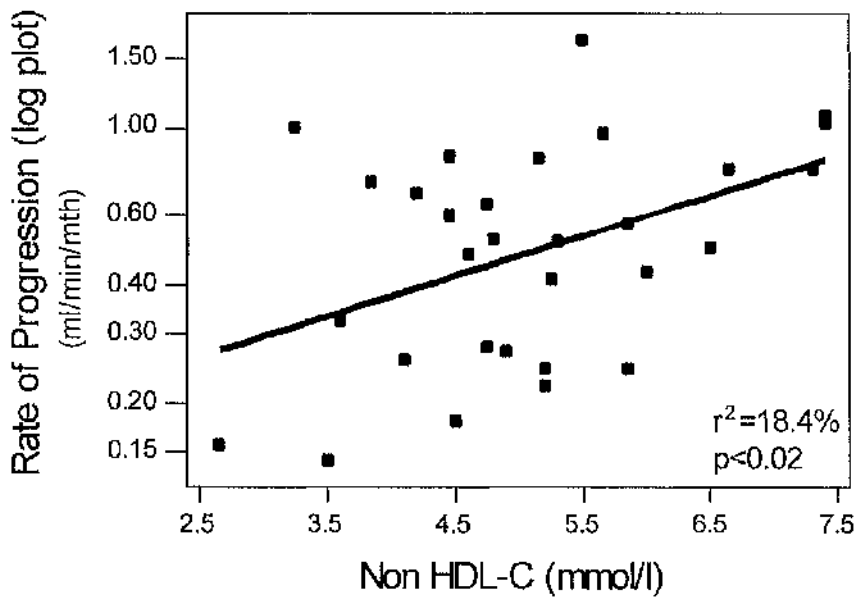
Fig 6.4: Rate of Progression vs VLDL₂ and LDLIII

VLDL2 vs Rate of Progression
(log:log plot)

VLDL2 (mg/dl)

LDLIII mass vs Rate of Progression
(log:log plot)

LDLIII mass (mg/dl)
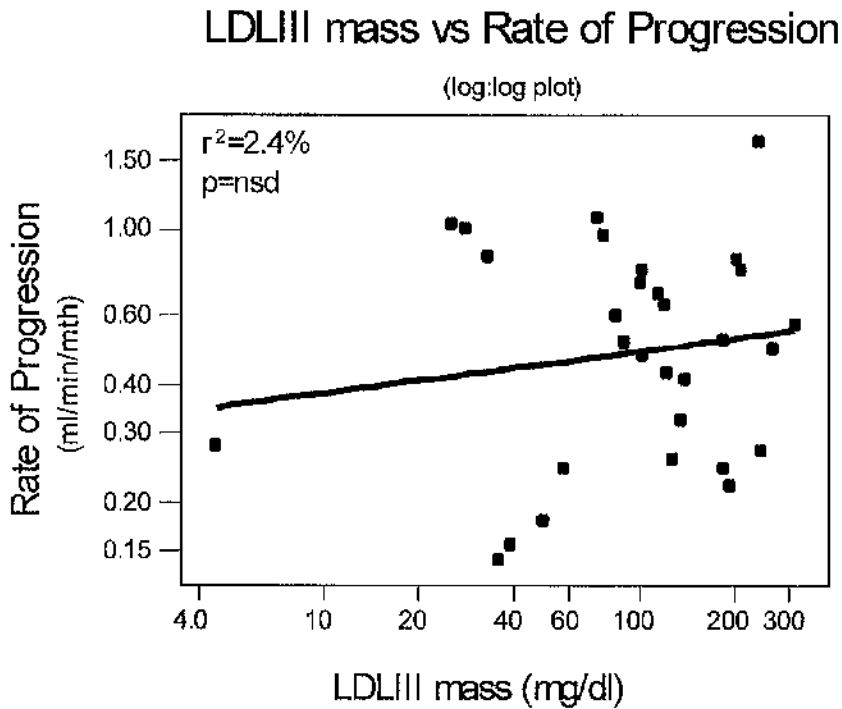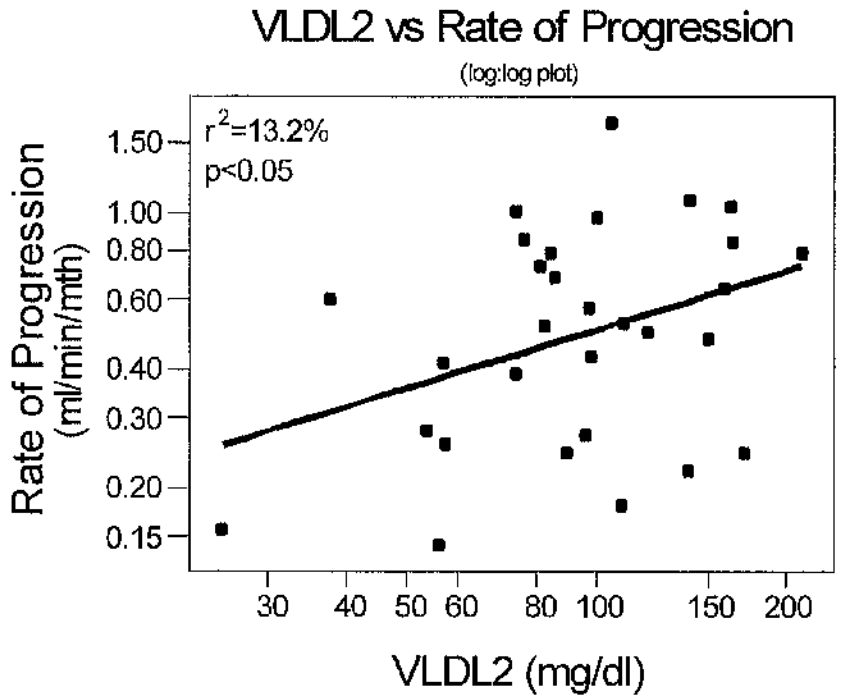
DISCUSSION

In this study, involving patients with glomerular disease, proteinuria and a broad spectrum of renal function, we have demonstrated a relationship between dyslipidaemia and the rate of progression of renal failure. Non HDL-C correlated with the rate of progression, a relationship that was independent of both the degree of proteinuria and renal function as measured at the study date. Non HDL-C includes all apoB containing lipoproteins (i.e. VLDL, IDL and LDL). Within this spectrum the lipoprotein fraction associated most clearly with progression was $VLDL_2$. Contrary to our initial hypothesis, there was no observable relationship between small dense LDL and progression of renal failure.

One of the difficulties in establishing an association between dyslipidaemia and progression of renal failure is that the quantity and quality of lipoproteins will change as the glomerular filtration rate (GFR) falls. In glomerular disease, as GFR falls, less albumin is filtered (and therefore albuminuria may fall), plasma triglyceride and triglyceride rich lipoproteins increase and lipase activity is reduced [203]. As a result initial lipoprotein profiles may bear little resemblance to the lipoprotein profiles during long term follow-up, and the contribution of lipoproteins to renal failure progression may differ as GFR falls. To limit the effect of profound changes in lipids over follow-up, we limited the maximal period of examination to 2.5 years and collected data on renal function both retrospectively before and prospectively after the study date at which lipoproteins were evaluated. Thus the lipid and lipoprotein profiles represent approximate mid-point values during the period of renal function decline. This hopefully allowed the lipoprotein analysis to be representative of the

lipoprotein profile over the whole period of examination and also allowed a long enough period to calculate the rate of progression. In addition, to limit the effect of severe uraemia, no patients with a creatinine clearance of less than 10ml/min were studied and at the end of the examination period only one patient had a creatinine clearance which had fallen to <10ml/min. Over the examination period, we found no significant difference in urinary albumin or systolic blood pressure, and although diastolic blood pressure fell over the examination period, the rate of progression of renal failure remained linear, with good correlation coefficients obtained for the slopes of creatinine clearance vs time.

Proteinuria is the most consistent risk factor for progression of renal failure in glomerular disease. We have previously demonstrated that patients with nephrotic range proteinuria have atherogenic levels of LDLIII and therefore we hypothesised that in the context of pre-existing glomerular damage, small dense LDL (LDLIII) may contribute to renal injury. For these reasons we chose to study small dense LDL in a population who had evidence of progression of renal failure resulting from glomerular disease associated with significant albuminuria i.e. a fairly representative population with progressive glomerular injury. However, a relationship between LDLIII and progression was not identified. There are theoretical reasons and experimental evidence to implicate LDLIII in causing glomerular damage. Due to its smaller size, it is more easily filtered by damaged endothelium and can therefore access the subendothelial space [204]. It is readily oxidised [177] and not only has oxidised LDL been implicated in the pathogenesis of glomerulosclerosis [200] but it has been identified in human renal biopsies, with increased oxidised LDL accumulation associated with more advanced renal disease [205]. Possible explanations for the lack

of association between LDLIII and progression in this study are (1) the small number of patients studied and (2) the study population investigated.

The number of patients studied was indeed small. Clearly this can result in false negative results. It is notable in this context that diastolic blood pressure which is well recognised to contribute to progression of renal failure [206], was not correlated with progression in this study. This may partly be explained by the extensive use of ACE inhibitors, however it does suggest that the small numbers involved may have hidden weaker but important associations. The study population was chosen within a specific range of proteinuria, and it is recognised that if the range of data in a study population is restricted, apparent associations and correlations may be missed. There was however, a wide variation in both rates of progression (-0.14 to -1.7ml/min/mth) and LDLIII concentration (5 to 310mg/dl), and therefore it is unlikely that the selection criteria applied to the study population resulted in a significant association between LDLIII concentration and rate of progression being overlooked.

Although no association was observed with LDLIII, there was an independent relationship between non HDL-C and the rate of progression. Non HDL-C consists of the entire spectrum of apolipoprotein B containing lipoproteins and apolipoprotein E rich remnant lipoproteins. Within this spectrum, increased $VLDL_2$ was found to be associated with faster progression. Plasma triglyceride has been shown to be independently associated with progression in nephropathy secondary to non-insulin dependent diabetes mellitus [150] and recently published data has suggested that 'complex' triglyceride rich apoB containing lipoproteins are associated with progression of renal failure in non diabetic renal disease [202]. Complex triglyceride

rich apoB containing lipoproteins are generally found in the density range of VLDL (d <1.006g/ml), with cholesterol rich apoB containing lipoproteins generally in the density range of IDL and LDL. This recent study is therefore in agreement with the present data.

In glomerular disease, endothelial damage and areas of basement membrane denudation allow filtration of macromolecules including lipoproteins and result in interaction between lipoproteins and both mesangial and visceral epithelial cells [207]. Most experimental studies have concentrated on the effect of LDL or oxidised LDL on glomerular mesangial or epithelial cells. However glomerular apoE deposition has been identified in human renal biopsies [208], and given that LDL does not possess apoE, the apolipopoprotein must originate from larger lighter lipoproteins. A number of studies have reported on the interaction between both VLDL and triglyceride rich lipoprotein remnants on glomerular mesangial and epithelial cells. Human mesangial [209] and visceral epithelial cells [210] possess receptors for apoE containing lipoproteins. ApoE rich lipoproteins have a greater affinity for glomerular cells and inhibit uptake of apoB containing lipoproteins [209]. Cholesteryl ester rich VLDL stimulates proliferation and expansion of mesangial cells [211] and accumulation of apoE rich lipoproteins by glomerular cells exceeds their ability to degrade the lipoproteins [212]. Thus mechanisms exist where glomerular mesangial and epithelial cells can accumulate triglyceride rich apoB and apoE containing lipoproteins (such as $VLDL_2$), resulting in glomerular lipid deposition, macrophage uptake and ultimately glomerulosclerosis. The association between triglyceride rich lipoproteins and renal injury has also been noted in animal models. In the obese Zucker rat increased VLDL is associated with glomerulosclerosis [213] and increased

lipid content in VLDL and IDL is associated with the development of proteinuria in the female analbuminaemic rat [214].

In conclusion, this study has demonstrated that there is an independent association between non HDL-C and progression of renal failure. The data suggests that triglyceride rich lipoproteins may be particularly implicated, with a correlation between $VLDL_2$ and progression. No association was observed between excess small dense LDL and progression of renal failure. Further study is required to assess the role that VLDL subfractions and partially metabolised atherogenic apoE rich VLDL remnant particles may have in contributing to progressive renal injury.

CHAPTER 7 -

The Effect of Proteinuria on Small Dense LDL Concentration in Type 2 Diabetes Mellitus

INTRODUCTION

The increased risk of coronary heart disease in patients with type 2 diabetes mellitus [24] is attributed to an increase in the prevalence of established risk factors including hypertension, dyslipidaemia, obesity and insulin resistance [25]. A strong relationship exists between vascular disease and both plasma triglyceride and LDL-cholesterol in this population [123,124,157], and subgroup analysis of large scale intervention studies indicates that lipid lowering with HMGCoA reductase inhibitors reduces the rate of death from coronary heart disease and non-fatal MI in patients with type 2 diabetes [28]. The development of diabetic nephropathy, with macroalbuminuria, results in a further increase in cardiovascular risk compared to diabetic patients who exhibit microalbuminuria or normal urinary albumin levels [6].

The characteristic dyslipidaemia of type 2 diabetes consists of raised plasma triglyceride and low HDL-cholesterol, with plasma cholesterol and LDL-cholesterol generally within the normal range [157,215]. This dyslipidaemia results from increased production and impaired clearance of triglyceride rich lipoproteins [157], and is closely related to both insulin resistance and glycaemic control [157]. Patients with type 2 diabetes typically have smaller LDL and excess quantities of LDLIII [157,158], and therefore possess the

atherogenic lipoprotein phenotype. It has recently been suggested that patients with diabetic nephropathy and macroalbuminuria possess LDL that is smaller than normal diabetic controls [159]. The aim of this study therefore, was to quantify the extent of small dense LDL (LDLIII) formation in patients with diabetic nephropathy and macroalbuminuria, comparing these patients with both microalbuminuric and normal type 2 diabetic patients with similar glycaemic control, and also with non-diabetic controls. A secondary aim was to identify factors determining the extent of LDLIII formation in type 2 diabetes with and without nephropathy, whilst assessing if these factors accounted for differences in small dense LDL between diabetic and non-diabetic populations.

METHODS

Subjects

Four groups of patients were studied. 12 patients with type 2 diabetes, diabetic nephropathy and macroalbuminuria (diabetic nephropathy group), 12 with type 2 diabetes and microalbuminuria, 12 normal type 2 diabetic controls and 24 non-diabetic controls. The diabetic patients were recruited consecutively from the joint diabetic-renal clinic and diabetic clinic of Glasgow Royal Infirmary. The normal controls were either relatives of patients attending the renal unit or members of the laboratory staff. All four groups were matched for age and sex, with the diabetic groups also matched for HbA1c. Type 2 diabetes was defined as having diabetes diagnosed above the age of 40 or having a minimum of 6 months between the diagnosis of diabetes and commencement of insulin. Diabetic nephropathy was diagnosed as either: (1) the presence of macroproteinuria with

retinopathy, normal size kidneys on ultrasound and the absence of significant haematuria or other multisystem disease; or (2) macroproteinuria and biopsy confirmation of diabetic nephropathy. Microalbuminuria was defined as a urinary albumin:creatinine ratio of 3-30mg/mmol [216] (approximately equivalent to a urinary albumin level of 20-200mg/l).

Patients were studied before commencement of lipid lowering therapy. Patients suffering from other diseases or on other treatment that might influence their lipid profile were excluded, specifically patients with underlying liver disease, amyloid, any neoplastic disorder, systemic lupus erythematosis or taking thiazide diuretics, fat soluble beta blockers, corticosteroids or other immunosuppressive agents. Patients were excluded if the serum creatinine was >180umol/l (2mg/dl). All patients were free of acute illness at the time of study. No patient had suffered a vascular event in the previous 4 months. The study was approved by the Ethics Committee of Glasgow Royal Infirmary. All patients and controls gave written consent before participating. Patients and controls attended after an overnight fast and samples were taken for the estimation of lipids and lipoproteins, LDL and VLDL subfractions, serum creatinine and albumin. All patients with diabetic nephropathy collected a 24 hour urine for analysis of urinary albumin excretion. Urinary albumin in microalbuminuric or control diabetic patients was measured on aliquots of early morning urine. In the patients with normal urinary albumin and microalbuminuria, all recent urinalysis at routine follow-up had been consistently within the normal or microalbuminuric range respectively.

Statistics

Statistical analysis were performed by using MINITAB 10X for Windows (Minitab Inc.). Any factors that were not normally distributed were subjected to log transformation. These included: BMI, plasma TG, VLDL-C, HDL-C, total VLDL and VLDL subfractions, total LDL and LDL subfractions. Results are shown as means and standard deviations or as median and inter-quartile range. Samples were compared using a one-way analysis of variance (ANOVA) with Fisher's comparison employed to identify differences among the groups if the ANOVA was significant. Simple regression analysis was performed to identify significant correlations. Stepwise regression was used for multivariate analysis involving linear variables. If a categorical variable was involved then multivariate analysis was performed using a general linear model.

RESULTS

Patient Characteristics

Age, body mass index (BMI) and renal function are given in Table 7.1. The ages of all 4 groups were closely matched, with the ratio of males to females in each group being 1:1. The body mass index (BMI) of all 3 diabetic groups was greater than the normal controls, however the increase in the microalbuminuric patients did not reach significance. Renal function in the patients with nephropathy was well preserved, with a mean creatinine clearance (CrCl - calculated from the Cockcroft and Gault formula [169]) of 60ml/min. Only 3 patients had a CrCl <50 (range 30-94). The serum creatinine however, was increased in the patients with nephropathy compared with those with microalbuminuria

(p<0.005), diabetic controls and normal controls (both p<0.001). Renal function in the microalbuminuric patients, diabetic controls and normal controls did not differ.

Four of the 12 diabetic nephropathy patients, 4 of the 12 microalbuminuric patients and one of the diabetic controls were receiving insulin. As measured by HbA1c, overall glycaemic control was good and did not differ between the three diabetic groups (table 7.1). Urinary albumin/creatinine ratio in the patients with nephropathy ranged from 52-755mg/mmol (urinary albumin concentration range 500-5800mg/l), in the microalbuminuric group from 3.1-26.1mg/mmol (32-200mg/l) and in the diabetic controls from 0.5-2.7mg/mmol (6-14mg/l). Despite being well within the normal range, mean serum albumin was reduced in the patients with nephropathy (40±3.5g/l) compared with both microalbuminuric (44±3g/l p<0.005) and diabetic control patients (44±2g/l p<0.001), however no patient had a serum albumin <35g/l.

Lipids and Lipoproteins

Plasma cholesterol did not differ across the four groups (table 7.1). Plasma triglyceride (TG) and VLDL-C were substantially increased in the patients with nephropathy compared with all three other groups. Although plasma triglyceride was higher in the microalbuminuric and diabetic control patients compared with the normal controls, the difference was not significant (both p=0.11). LDL-C was similar across the four groups. HDL-C was reduced in the nephropathy patients compared with all three other groups. In addition, both the microalbuminuric patients and diabetic controls exhibited a significantly lower HDL-C compared with the normal controls. As a result of these

differences, the patients with diabetic nephropathy had a substantial increase in cholesterol:HDL ratio compared with the other study populations.

Total VLDL mass was increased in the patients with diabetic nephropathy compared with microalbuminuric patients, diabetic controls and normal controls (table 7.2). This resulted primarily from a 2 fold increase in $VLDL_2$, which was present in greater quantities in the patients with nephropathy compared with the three other groups (fig 7.1). In contrast although the median $VLDL_1$ was also increased 2 fold in the nephropathy patients, the results were very heterogeneous and as a result, this increase was only significant when compared with the non-diabetic controls. The masses of IDL and LDL were similar across all four study groups.

LDL Subfractions

The mass of LDLIII in the patients with diabetic nephropathy was increased 2-3 fold compared with the microalbuminuric and diabetic control patients (both $p<0.05$), with a 5 fold increase compared with the normal controls ($p<0.001$, table 7.2, fig 7.2). There was a concomitant reduction in the concentration of large light LDLI ($p<0.001$ vs normal controls, $p<0.05$ vs both microalbuminuric and diabetic control patients, fig 7.2) and LDLII ($p<0.001$ vs normal controls, $p<0.05$ vs diabetic control patients, fig 7.2). 75% of patients with nephropathy had an LDLIII mass greater than 100mg/dl (a level associated with greatly increased risk of coronary heart disease [141]) compared with 33% of microalbuminuric patients, 25% of diabetic controls and 4% of normal controls. Compared to the normal controls, both microalbuminuric ($p<0.05$) and diabetic control

patients (p<0.05) had a 2 fold increase in LDLIII concentration, with a reduction in larger lighter subfractions.

The relative proportions of the LDL subfractions followed along similar lines. The patients with nephropathy had an increase in the percentage of LDLIII compared with all three other groups. Similarly, %LDLI was reduced in the diabetic nephropathy patients compared with all three other groups, whilst the %LDLII was reduced compared with the diabetic controls and normal controls. Both the microalbuminuric (p<0.01) and the diabetic control patients (p<0.05) had an increase in %LDLIII compared with the normal controls (table 7.2, fig 7.2).

Lipoprotein composition

Within the density range of triglyceride rich $VLDL_1$, the patients with nephropathy possessed $VLDL_1$ particles that were cholesterol enriched and triglyceride deplete compared with both microalbuminuric patients and normal controls (table 7.3). In contrast, the LDL particles in the patients with nephropathy were triglyceride enriched, and all three diabetic groups possessed LDL that was relatively cholesterol deplete compared with the non-diabetic controls. There was no observed difference in the cholesterol or triglyceride content of $VLDL_2$ or IDL.

Urinary Albumin and Dyslipidaemia

Amongst all 36 diabetic patients, urinary albumin/creatinine ratio correlated with chol/hdl-c ratio (r^2=31.5% p<0.001), total cholesterol (r^2=16.1% p=0.02), VLDL-C

($r^2=17.5\%$ p<0.02), and inversely with HDL-C ($r^2=16.8\%$ p<0.02). Urinary albumin/creatinine ratio was also correlated with serum creatinine ($r^2=21.0\%$ p=0.005), and inversely related to serum albumin ($r^2=43.6\%$ p<0.001). Assessing the relationships between albuminuria and the lipoprotein subfractions revealed that urinary albumin/creatinine ratio was associated with the concentration of the $VLDL_2$ subfraction ($r^2=26.9\%$ p=0.001, fig 7.3), but not that of $VLDL_1$ ($r^2=9.3\%$ p=nsd). An increasing urinary albumin/creatinine ratio correlated with increasing LDLIII mass ($r^2=14.6\%$ p<0.03, fig 7.3) and % LDLIII ($r^2=18.0\%$ p=0.01).

Determinants of Small Dense LDL in NIDDM and Diabetic Nephropathy

Factors regulating the concentration of small dense LDL in the diabetic patients were assessed by performing regression analysis on the entire diabetic study population. This revealed a strong correlation between LDLIII and plasma triglyceride ($r^2=46.1\%$ p<0.001,fig 7.4), $VLDL_1$ ($r^2=49.2\%$ p<0.001, fig 7.4) and $VLDL_2$ ($r^2=40.3.\%$ p<0.001, fig 7.4), with an inverse correlation to HDL-C ($r^2=28.9\%$ p<0.002). The %LDLIII was similarly correlated to plasma triglyceride ($r^2=65.9\%$), $VLDL_1$ ($r^2=63.2\%$), and $VLDL_2$ ($r^2=49.2\%$, all p<0.001), with an inverse relationship seen with HDL-C ($r^2=21.2\%$ p=0.005). The relationship between small dense LDL and urinary albumin/creatinine ratio has already been noted. No correlation was observed between either LDLIII concentration or %LDLIII and creatinine clearance, BMI, albumin or HbA1c.

Stepwise regression revealed that in the 36 diabetic patients, plasma triglyceride, HDL-C and LDL mass accounted for 65.7% of the variance of LDLIII concentration (TG 46.1%,

HDL-C 10.6%, LDL mass 10.0%), with all three parameters significant on multivariate analysis using a general linear model (TG $p<0.001$, HDL-C $p=0.003$, LDL mass $p=0.007$). Plasma triglyceride ($p<0.001$) and HDL-C ($p<0.05$) were independent determinants of %LDLIII in the diabetic patients, accounting for 69.7% of the variance on stepwise regression. After accounting for the differences in plasma triglyceride and HDL-C, the development of nephropathy did not independently effect either LDLIII concentration or %LDLIII in NIDDM. A plasma triglyceride of 1.8mmol/l had a sensitivity of 87% and a specificity of 80% for atherogenic concentrations of small dense LDL (>100mg/dl), reducing the triglyceride to 1.5mmol, increased the sensitivity to 94% but decreased the specificity to 70%.

Small Dense LDL: Type 2 Diabetes vs Non-Diabetic Controls

To assess the effect of having a diagnosis of type 2 diabetes on LDLIII concentration, we repeated the regression analysis using all 60 subjects studied. The univariate analysis was similar to that found in the diabetic population, with a strong correlation between LDLIII concentration and plasma triglyceride ($r^2=50.4\%$), VLDL$_1$ ($r^2=47.3\%$) and VLDL$_2$ ($r^2=44.2\%$, all $p<0.001$), and an inverse correlation to HDL-C ($r^2=36.0\%$). A relationship was also observed between LDLIII mass and BMI ($r^2=18.6\%$ $p<0.002$). On multivariate analysis, plasma triglyceride ($p<0.001$), LDL mass ($p=0.003$), HDL-C ($p<0.05$) and BMI ($p<0.05$) were independent determinants of LDLIII concentration, accounting for 66.2% of the variance (TG 51.1%, HDL-C 6.8%, LDL mass 4.6%, BMI 3.7%). Without the inclusion of body mass index into the regression equation, there was an independent effect of having a diagnosis of type 2 diabetes on the concentration of LDLIII ($p<0.04$),

with the relationship between LDLIII and both plasma TG (p<0.02, fig 5) and HDL-C (p<0.04, fig 5) differing between the diabetic and non-diabetic patients. After adding BMI into the multiple regression equation, the independent effect of having type 2 diabetes no longer achieved statistical significance (p=0.06).

Table 7.1: Clinical Characteristics and Lipid Profiles of Each Group: Mean±SD (*Median + IQR)

	Type 2 Diabetes & Macroalbuminuria (n=12)	Type 2 Diabetes & Microalbuminuria (n=12)	Type 2 Diabetic Controls (n=12)	Normal Controls (n=24)	ANOVA
Male : Female	6:6	6:6	6:6	12:12	-
Age (years)	62.7±7.0	62.6±7.8	63.1±7.8	59.6±6.7	nsd
BMI	31.7±4.5[a]	30.3±4.3	31.5±7.1[a]	27.0±3.7	<0.02
Creatinine (umol/l)*	125 (110-157)[b,d,e]	105 (90-117)	97 (91-110)	95 (85-120)	=0.001
HbA1c (%)	7.0±1.8	7.7±1.3	7.1±1.2	-	nsd
Cholesterol (mmol/l)	6.1±1.4	5.2±1.4	5.2±0.8	5.6±0.8	nsd
Triglyceride (mmol/l)*	2.6 (1.4-4.1)[b,c,e]	1.6 (1.1-2.0)	1.5 (1.2-2.3)	1.3 (0.9-1.6)	=0.001
VLDL-C (mmol/l)*	1.0 (0.6-2.1)[a,d,e]	0.4 (0.2-0.7)	0.4 (0.3-0.8)	0.4 (0.2-0.6)	=0.007
LDL-C (mmol/l)	3.8±1.2	3.5±0.8	3.6±0.8	3.8±0.7	nsd
HDL-C (mmol/l)*	0.9 (0.8-1.0)[b,c,e]	1.1 (1.0-1.3)[a]	1.0 (1.0-1.2)[b]	1.3 (1.0-1.6)	<0.001
Cholesterol / HDL	7.0±1.6[b,d,f]	4.7±1.0	5.1±1.6	4.4±1.2	<0.001

[a] p<0.05 - <0.01, [b]p<0.005 - p<0.001   vs normal control patients,
[c] p<0.05 - <0.01, [d]p<0.005 - p<0.001   vs diabetic control patients,
[e] p<0.05 - <0.01, [f]p<0.005 - p<0.001   vs microalbuminuric patients.

Table 7.2: Plasma Concentrations of Lipoproteins and Lipoprotein Subfractions: Median and IQR (* mean ± SD)

	Type 2 Diabetes & Macroalbuminuria (n=12)	Type 2 Diabetes & Microalbuminuria (n=12)	Type 2 Diabetic Controls (n=12)	Normal Controls (n=24)	ANOVA
VLDL Total (mg/dl)	297 (151-497) [b,c,e]	126 (70-224)	127 (78-239)	112 (61-165)	=0.001
VLDL$_1$ (mg/dl)	145 (99-332) [b]	58 (39-160)	86 (43-159)	61 (30-92)	<0.03
VLDL$_2$ (mg/dl)	113 (71-157) [b,d,f]	56 (38-76)	54 (27-76)	43 (29-63)	<0.001
IDL (mg/dl)	72 (56-79)	59 (43-69)	51 (36-67)	50 (37-68)	nsd
LDL mass (mg/dl)	302 (219-354)	283 (256-342)	308 (258-351)	327 (263-374)	nsd
LDLI mass (mg/dl)	29 (11-48) [b,c,e]	42 (27-89)	39 (34-62) [a]	68 (47-103)	<0.001
LDLII mass (mg/dl)	123 (56-158) [b,c]	132 (89-181) [a]	155 (134-199)	197 (140-262)	=0.001
LDLIII mass (mg/dl)	150 (96-202) [b,c,e]	57 (36-178) [a]	61 (52-159) [a]	33 (27-64)	<0.001
%LDLI *	9.9 ± 5.3 [b,c,e]	19.8 ± 13.0	15.5 ± 6.1 [a]	22.9 ± 10.6	=0.003
%LDLII *	37.3 ± 19.1 [b,c]	46.9 ± 18.2 [a]	54.4 ± 13.2	61.6 ± 10.4	<0.001
% LDLIII *	52.8 ± 23.3 [b,c,e]	33.3 ± 26.3 [a]	30.1 ± 18.1 [a]	15 ± 11	<0.001

[a] p<0.05 - <0.01, [b] p<0.005 - p<0.001   vs normal control patients,
[c] p<0.05 - <0.01, [d] p<0.005 - p<0.001   vs diabetic control patients,
[e] p<0.05 - <0.01, [f] p<0.005 - p<0.001   vs microalbuminuric patients.

Table 7.3: Lipoprotein Compositions: Total Cholesterol and Triglyceride as Percentage of Lipoprotein Mass (Mean±SD)

	Type 2 Diabetes & Macroalbuminuria (n=12)	Type 2 Diabetes & Microalbuminuria (n=12)	Type 2 Diabetic Controls (n=12)	Normal Controls (n=24)	ANOVA
$VLDL_1$ %Total Cholesterol	9.9 ± 3.2 [c]	8.2 ± 1.4	8.5 ± 2.4	7.1 ± 2.1	<0.02
$VLDL_1$ %Triglyceride	63.3 ± 4.6 [b,f]	67.9 ± 3.5	66.3 ± 2.6	67.4 ± 4.7	<0.03
$VLDL_2$ %Total Cholesterol	21.8 ± 3.8	21.1 ± 5.6	20.2 ± 2.4	19.5 ± 3.0	nsd
$VLDL_2$ %Triglyceride	40.4 ± 5.2	40.2 ± 8.0	41.1 ± 2.8	43.2 ± 4.6	nsd
IDL %Total Cholesterol	33.8 ± 3.0	33.7 ± 5.1 [a]	33.5 ± 4.2 [a]	36.5 ± 3.3	nsd
IDL %Triglyceride	18.5 ± 4.2 [b]	18.2 ± 5.9	17.5 ± 5.9	15.0 ± 3.5	nsd
LDL %Total Cholesterol	36.6 ± 3.0 [d]	37.8 ± 1.8 [a]	37.2 ± 2.0 [c]	39.7 ± 1.5	<0.001
LDL %Triglyceride	9.1 ± 3.2 [d,e]	7.1 ± 1.6	7.8 ± 1.0	6.6 ± 1.3	<0.005

[a] p<0.05, [b] p<0.01, [c] p<0.005, [d] p<0.001,   vs normal control patients,

[e] p<0.05, [f] p<0.01   vs microalbuminuric patients.

Fig 7.1: Dot-Plot showing distribution of plasma triglyceride, $VLDL_1$ and $VLDL_2$ in diabetic nephropathy, microalbuminuria, diabetic controls and normal controls.

1 = Diabetic Nephropathy
2 = Microalbuminuria
3 = Normal Controls
4 = NIDDM Controls

▬ = Median
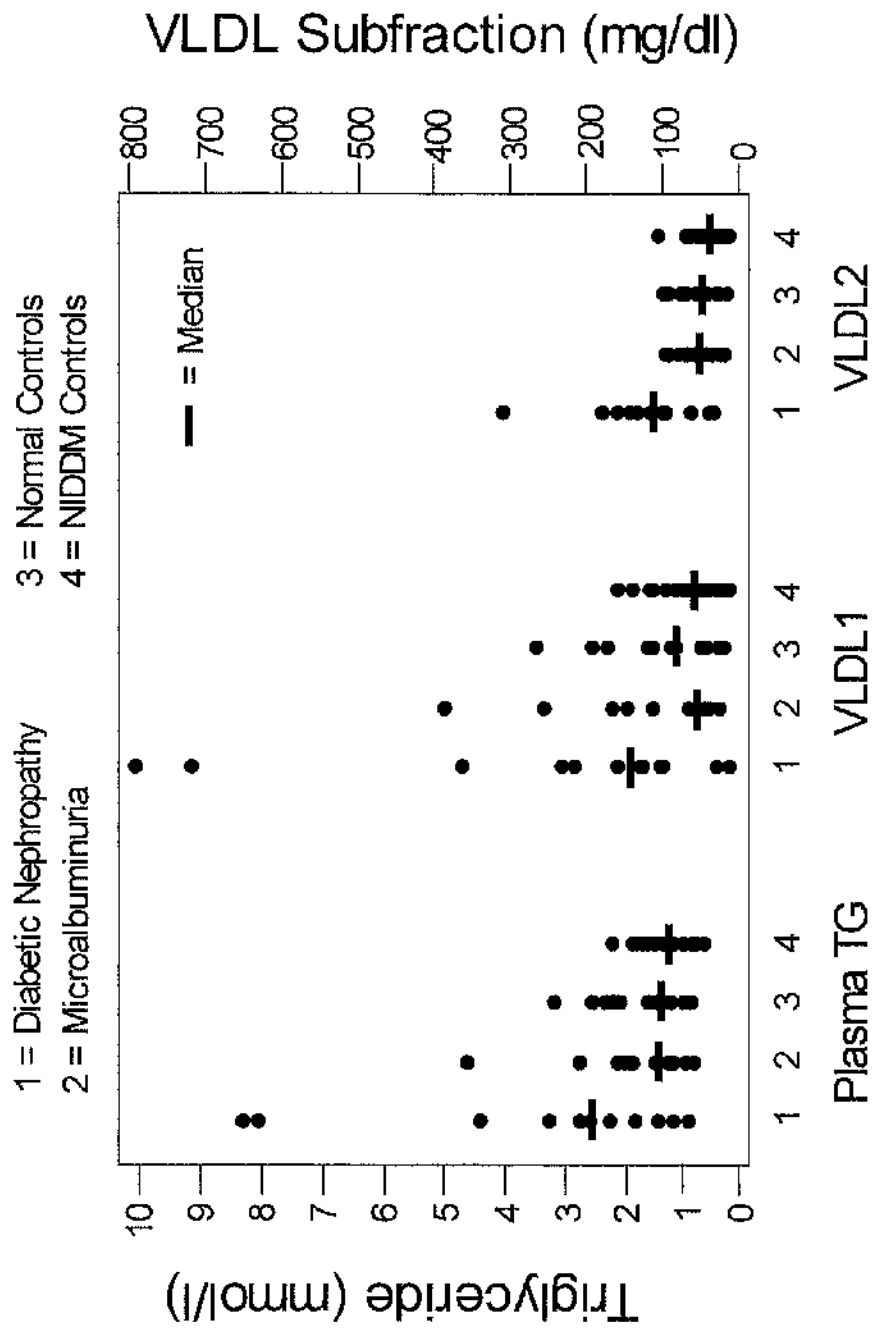
Fig 7.2: Boxplot of LDLI, II and III in diabetic nephropathy (macroalb), microalbuminuria (microalb), diabetic controls (normoalb) and normal controls

[a] p<0.001, [b] p<0.01, [c] p<0.05 vs normal controls
[d] p<0.05 vs diabetic controls
[e] p<0.05 vs microalbuminuric patients
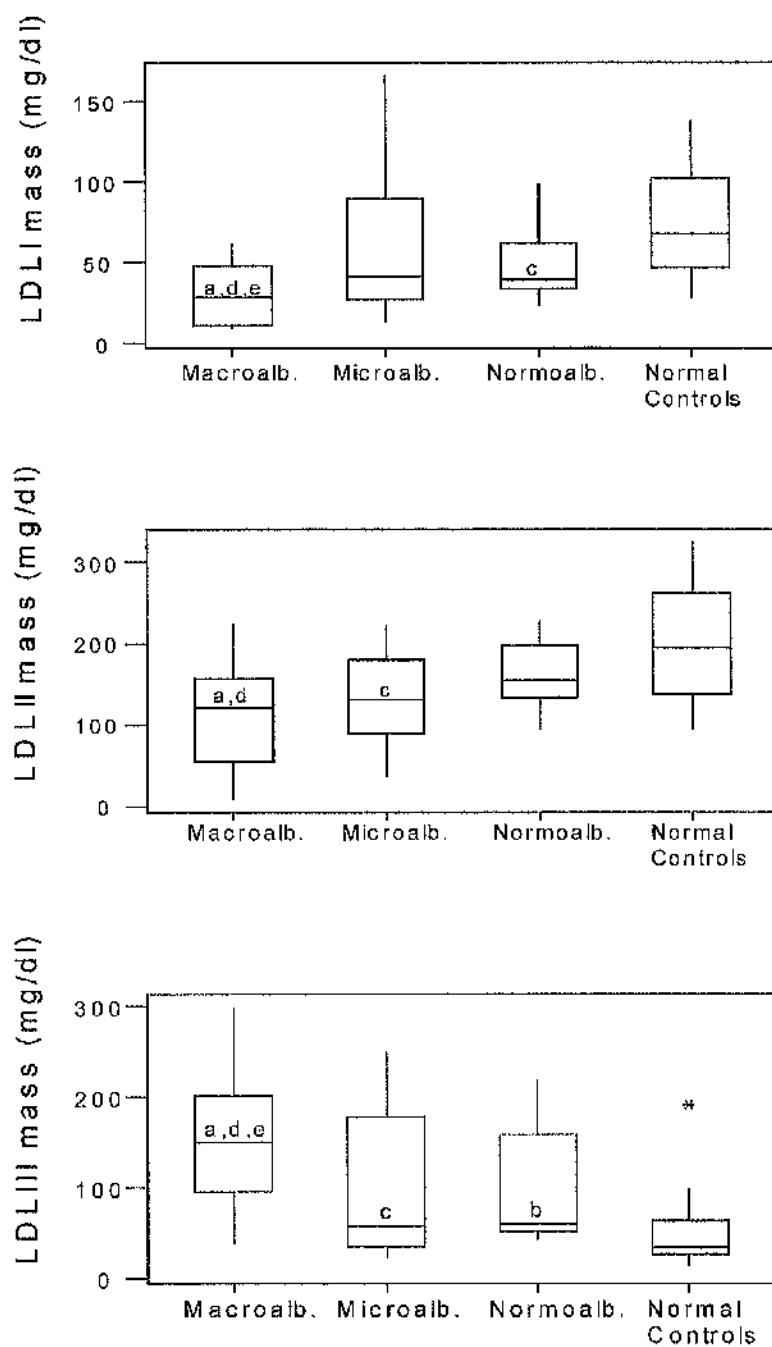
Fig 7.3: Plasma Concentration of VLDL$_2$ and LDLIII vs Urinary Albumin: Creatinine Ratio

LDLIII mass vs Urinary Albumin:Creatinine
- Diabetic Patients

(log : log plot)

r^2=14.6%
p<0.03

Albumin/Creatinine Ratio (mg/mmol)

VLDL2 vs Urinary Albumin:Creatinine
- Diabetic Patients

(log : log plot)

r^2=26.9%
p=0.001

Albumin/Creatinine Ratio (mg/mmol)

Fig 7.4: Plasma Triglyceride, VLDL₁ and VLDL₂ Concentration vs LDLIII Concentration

Fig 7.4: Plasma Triglyceride, $VLDL_1$ and $VLDL_2$ Concentration vs LDLIII Concentration

Triglyceride vs LDLIII mass: Diabetic Patients
(log : log plot)

$r^2 = 46.1\%$
$p < 0.001$

LDLIII mass (mg/dl)

Plasma Triglyceride (mmol/l)

VLDL1 vs LDLIII mass: Diabetic Patients
(log : log plot)

$r^2 = 49.2\%$
$p < 0.001$

LDLIII mass (mg/dl)

VLDL1 (mg/dl)

VLDL2 vs LDLIII mass: Diabetic Patients
(log : log plot)

$r^2 = 40.3\%$
$p < 0.001$

LDLIII mass (mg/dl)

VLDL2 (mg/dl)

Fig 7.5: Plasma Triglyceride and HDL-C vs LDLIII for Diabetic Patients vs Controls

TG vs LDLIII mass: Diabetic Patients vs Controls
(log : log plot)
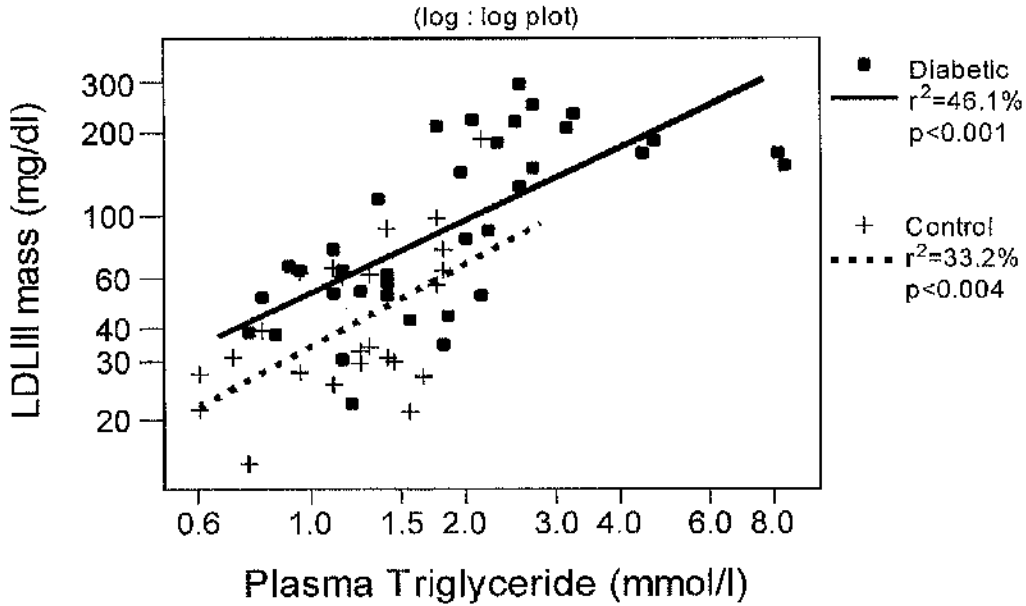
LDLIII mass (mg/dl)

Plasma Triglyceride (mmol/l)

Diabetic
$r^2 = 46.1\%$
$p < 0.001$

Control
$r^2 = 33.2\%$
$p < 0.004$

HDL-C vs LDLIII mass: Diabetic Patients vs Controls
(log : log plot)

LDLIII mass (mg/dl)

HDL-C (mmol/l)

Diabetic
$r^2 = 28.9\%$
$p = 0.001$

Controls
$r^2 = 13.6\%$
$p < 0.08$
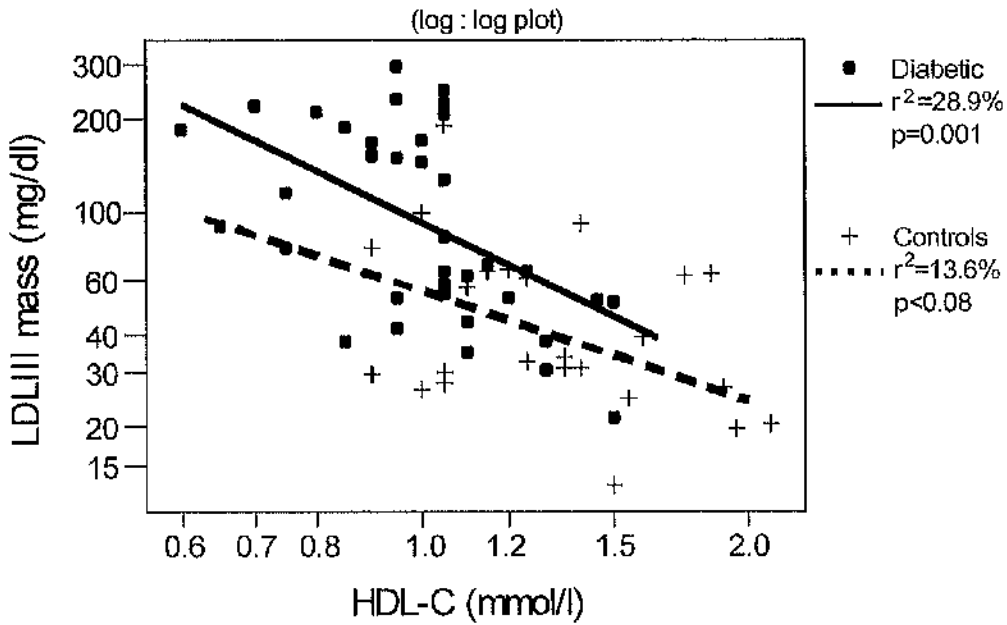
DISCUSSION

This study demonstrates that in type 2 diabetes, the presence of diabetic nephropathy and macroalbuminuria is associated with a 2-3 fold increase in LDLIII mass when compared with both microalbuminuric patients and diabetic controls matched for age, sex and HbAlc. Moreover, when compared with non-diabetic controls, patients with diabetic nephropathy have a 5 fold increase in LDLIII. This increase in small dense LDL occurs despite patients with diabetic nephropathy having levels of total cholesterol and LDL-cholesterol that are comparable to the other diabetic and control populations. As found previously in both normal and type 2 diabetic populations [139,142,144,158], the main determinant of LDLIII concentration was plasma triglyceride, with both HDL-C and total LDL concentration being additional factors. After accounting for differences in lipids, the degree of proteinuria was not independently related to LDLIII concentration. However, as the diabetic groups were matched for age, BMI and glycaemic control, it is likely that the variation in plasma lipids, especially triglyceride, resulted from the differing levels of proteinuria and so proteinuria was ultimately responsible for the high levels of small dense LDL. Determinants of LDLIII mass also differed between diabetic and non-diabetic patients suggesting that additional factors contribute to formation of small dense LDL in type 2 diabetes.

The current theories of lipoprotein metabolism suggest that both plasma triglyceride and hepatic lipase activity are rate limiting factors in the formation of LDLIII [56]. The data obtained from the patients with proteinuria secondary to glomerular disease (chapter 3) suggested that the processes involved in formation of LDLIII in proteinuria were similar to those seen in normal populations. This study provides

further support for these conclusions with plasma triglyceride being the most important determinant of LDLIII concentration in all subjects. Moreover VLDL₁ in the nephropathy patients was cholesterol rich and triglyceride deplete, whilst LDL was triglyceride rich and LDL deplete, consistent with extensive CETP mediated neutral lipid exchange. However we also found that the microalbuminuric and normal diabetic control patients had an increase in plasma LDLIII concentration despite having small, non-significant differences in VLDL₁ and plasma triglyceride. There also appeared to be an independent effect of having diabetes when all 60 subjects were included in the multivariate analysis assessing determinants of LDLIII concentration. Addition of body mass index to the statistical model accounted for some, if not all, of this effect. Body mass index and other markers of obesity (e.g. waist: hip ratio) are known to be associated with insulin resistance. They are also correlated with hepatic lipase activity [144,217]. It has previously been suggested that the delayed post-prandial clearance of triglyceride rich lipoproteins in insulin resistant subjects contributes to small dense LDL formation in NIDDM [158]. This may explain the findings in our diabetic patients. However, we favour the possibility that BMI is acting as a marker of increased hepatic lipase activity, which would promote LDLIII formation once the elevated plasma triglyceride has made the LDL particles triglyceride rich.

The concentration and percentage of LDLIII present in the diabetic patients was related to urinary albumin, with a marked increase in LDLIII in the patients with diabetic nephropathy and macroalbuminuria. There was also a strong correlation between VLDL₂ and both urinary albumin/creatinine ratio and LDLIII. It is notable from figure 1, that there is a marked increase in VLDL₂ in the patients with

macroalbuminuria, with the distribution of $VLDL_2$ in the other 3 groups being very similar. The differences in $VLDL_1$ distribution between the diabetic groups are less distinct. Admittedly, there is a trend for $VLDL_1$ to increase when non-diabetic and diabetic patients are compared, however no clear correlation was observed between $VLDL_1$ and urinary albumin, and any difference in $VLDL_1$ concentration in the patients with diabetic nephropathy compared with the other diabetic patients can be accounted for by the 2 outlying patients with an extremely high $VLDL_1$ concentration. We suggest that the rise in plasma triglyceride that results in the increase in LDLIII concentration present in the patients with diabetic nephropathy compared with the other diabetic patients, is due to the increase in VLDL2 concentration, and that this increase in $VLDL_2$ is a result of the development of macroalbuminuria. In normal populations $VLDL_2$ is rapidly metabolised to LDL, with increased $VLDL_2$ production the main cause of the excess LDL found in most types of hypercholesterolaemia [56]. In patients with glomerular disease and nephrotic range proteinuria, $VLDL_2$ has been shown to be increased due to a combination of increased production and impaired clearance [108,171]. In this situation, we speculate that excess $VLDL_2$ results in increased LDLIII either as a result of increased total LDL formation or from $VLDL_2$ providing an alternative source of triglyceride for neutral lipid exchange via CETP.

The plasma LDLIII concentrations in this study are similar to those obtained in the 27 non-diabetic patients with proteinuria secondary to glomerular disease. However in comparison to the patients with glomerular disease, the concentration of LDLIII may be underestimated as this current study had a higher percentage of females (and it is recognised that females have lower concentrations of LDLIII [144]) and urinary albumin was lower in the diabetic patients compared with the patients with glomerular

disease (chapter 3). The diabetics patients studied also had better than average glycaemic control. Given that glycaemic control is closely correlated with plasma triglyceride, this study may also underestimate the extent of LDLIII formation in the general population of type 2 diabetic patients with diabetic nephropathy.

Patients with type 2 diabetes have a marked increase in coronary artery disease compared with age and sex matched non-diabetic patients [24]. A raised plasma triglyceride is strongly associated with increased coronary risk in type 2 diabetes [124]. The strong association between plasma triglyceride and small dense LDL demonstrated, suggests that the excess small dense LDL contributes to the excess cardiovascular risk seen with increasing plasma triglyceride. The development of diabetic nephropathy and proteinuria leads to a further increase in cardiovascular mortality, independent of age, blood pressure and cholesterol [6]. Not only did the patients with diabetic nephropathy have a marked increase in LDLIII compared with non-diabetic controls but a further 2-3 fold increase in LDLIII was observed in comparison to both microalbuminuric patients and diabetic controls. This 'double jeopardy' was primarily related to the marked increase in triglyceride, and was present despite the microalbuminuric and diabetic control patients having comparable glycaemic control, body mass index and LDL mass.

We observed no difference between the microalbuminuric patients and normal diabetic controls with respect to any parameter studied. Although more patients in the microalbuminuric group were treated with insulin compared with the normal diabetic controls, the overall glycaemic control and BMI in the two groups was not different. These results are in keeping with the majority of studies, where lipid profiles do not

differ between microalbuminuric and diabetic patients with normal urinary albumin, if the populations are matched in terms of age, BMI and glycaemic control or insulin levels [218,219]. It has been suggested that microalbuminuric patients have smaller LDL than normal diabetic controls [159], however this finding is not universal [220]. Our data suggests that once glycaemic control and BMI are controlled for, the development of microalbuminuria is not associated with excess LDLIII. Although our numbers are small, the observation that in large scale studies of type 2 diabetes [218], plasma triglyceride does not differ between microalbuminuric patients and diabetic controls supports this conclusion.

In summary, in this study we have demonstrated that the development of diabetic nephropathy with its associated macroalbuminuria results in large increases in the plasma concentration of small dense LDL. The excess of this atherogenic lipoprotein is not expressed as an increase in LDL-C but is associated with increased plasma triglyceride and a low HDL-C (the atherogenic lipoprotein phenotype). The primary determinant of the LDLIII concentration is plasma triglyceride, however we suggest that the excess triglyceride and LDLIII in the patients with nephropathy results from an increase in the concentration of VLDL$_2$. There also seemed to be an independent effect of having type 2 diabetes. Previous data has suggested that patients with type 2 diabetes continue to have a preponderance of small dense LDL even after removal of patients with raised triglycerides (TG >2.25) [159]. The relationship between triglyceride and LDLIII in this study suggests that LDLIII becomes predominant at levels of plasma triglyceride greater than 1.5mmol/l, i.e. at levels not normally regarded as hypertriglyceridaemic. Clearly measurement of total cholesterol and LDL-C is insufficient in addressing the cardiovascular risk posed by the dyslipidaemia in

this population, and the excess LDLIII is likely to be a major contributor to the increase in cardiovascular morbidity and mortality present in patients with type 2 diabetes and nephropathy.

CHAPTER 8 -

The Atherogenic Lipoprotein Phenotype in End-Stage Renal Failure: Origin and Extent of Small Dense LDL Formation

INTRODUCTION

The characteristic dyslipidaemia of end-stage renal failure (ESRF) consists of raised plasma triglyceride and low HDL-C [114,115]. VLDL-C is increased however levels of total cholesterol and LDL-C are generally normal and may be low [114,115]. The primary metabolic defect is thought to be impaired catabolism of triglyceride rich lipoproteins (mostly VLDL) by the enzymes lipoprotein and hepatic lipase. However the precise mechanism underlying the impaired lipolytic activity remains to be established [114,116,117]. The lipoprotein abnormalities also differ according to modality of renal replacement therapy with patients on peritoneal dialysis tending to have higher total cholesterol and LDL-C [118].

Dyslipidaemia is an independent risk factor for atherosclerosis in ESRF [8,134,221], however with cholesterol and LDL-C levels being normal in ESRF, coupled with the lack of long term lipid lowering studies in this population, the debate continues as to the role of lipid lowering therapy in ESRF and particularly to whom it should be targeted. Two published studies have demonstrated that LDL size is smaller in uraemic patients compared with control populations [160,161]. To date however, the analysis of LDL phenotype in this population has been qualitative in nature and clearly in the context of normal or low levels of LDL, the quantity of small dense LDL

present requires to be estimated in order to fully evaluate the extent to which LDL phenotype may influence cardiovascular risk in end-stage renal failure.

The primary aim of our study was to identity the extent to which small dense LDL (LDLIII) is formed in patients with ESRF and by analysing VLDL subfractions and post-heparin lipase activity, to identify the factors contributing to LDLIII formation in this population. A secondary aim was to clarify possible origins of the differences in lipids and lipoproteins between patients treated with peritoneal and haemodialysis.

SUBJECTS AND METHODS

Subjects

75 patients (45 male, 30 female) and 40 controls (24 male, 16 female) were recruited for the study. 50 patients were receiving renal replacement therapy (25 treated by haemodialysis (HD) and 25 treated by peritoneal dialysis (PD)) another 25 patients were 'pre-dialysis' (PreD - defined as patients with chronic renal failure and a serum creatinine greater than 500umol/l, but not yet receiving renal replacement therapy). Patients on renal replacement therapy had been stable on their form of dialysis for more than 6 months. Patients suffering from other diseases or on treatment that might influence their lipid profile were excluded, specifically patients suffering from diabetes mellitus, amyloid, or any neoplastic disorder or patients taking thiazide diuretics, fat soluble B blockers, corticosteroids or immunosuppressants. Patients taking lipid lowering medication were also excluded. However, at the time of the study only 2 patients out of a dialysis population of about 130 were on lipid lowering medication, therefore it was not felt that this would bias any results. These patients

were compared to 40 normal controls who were either relatives of patients attending the renal unit or members of the laboratory staff. The mean ages and proportion of males: females of the two populations were identical. All patients were free of acute illness at the time of study and no patient had suffered an acute vascular event in the preceding 3 months. The haemodialysis patients were all receiving between 12 and 15 hours a week dialysis and were seen immediately before their regular dialysis session. CAPD patients were all receiving four exchanges of dextrose based dialysis solutions per day. All subjects were seen after an overnight fast. An intravenous cannula was inserted and samples taken for analysis of lipids, LDL and VLDL subfractions, serum creatinine and albumin. 70 units/kg of intravenous heparin was administered followed by sampling 10 minutes later for assessment of lipoprotein and hepatic lipase activity. The study was approved by the Ethics Committee of Glasgow Royal Infirmary.

Statistical analysis

Statistical analyses were performed using Minitab version 10X (Minitab Inc). Variables that were not normally distributed were subject to log transformation namely: BMI, plasma TG, VLDL-C, HDL-C, total VLDL concentration, $VLDL_1$, $VLDL_2$, IDL concentration, LDL concentration, LDLI, II and III concentration. Tabulated results are shown as median + interquartile range (IQR) or as mean $\pm$ standard deviations. Patients and controls were compared using two sample t-test. Patient subgroups and controls were compared using a one-way analysis of variance (ANOVA) with Fisher's comparison employed to identify differences among the groups if the ANOVA was significant. Regression analysis was performed to identify univariate correlations, multiple regression analysis was performed using a general linear model.

RESULTS

Anthropometric data, lipids, lipoproteins and lipases.

All Patients

The results for anthropometric data, lipids, lipoproteins and lipase activity are shown in table 8.1.

Table 8.1: Anthropometric data, Lipids, Lipoproteins and Lipase activity: Patients vs Controls (Median + IQR)

	Patients (n=75)	Controls (n=40)	p
Sex (M:F)	45 : 30	24 : 16	nsd
Age	51 (38-59)	53 (36-60)	nsd
Body Mass Index	24.4 (21.8-27.5)	25.0 (23.0-29.3)	nsd
Cholesterol (mmol/l)	5.1 (4.4-6.2)	5.1 (4.6-5.8)	nsd
Triglyceride (mmol/l)	1.7 (1.2-2.3)	1.0 (0.8-1.4)	< 0.0001
VLDL-C (mmol/l)	0.75 (0.5-1.1)	0.32 (0.2-0.4)	< 0.0001
LDL-C (mmol/l)	3.4 (2.5-3.9)	3.4 (2.8-4.0)	nsd
HDL-C (mmol/l)	0.9 (0.8-1.2)	1.3 (1.0-1.5)	< 0.0001
Chol / HDL	5.4 (4.1-6.9)	3.7 (3.1-4.7)	< 0.0001
Lipoprotein Lipase (umolFA/ml/hr)	3.5 (2.2-4.2)	3.9 (3.2-5.2)	< 0.002
Hepatic Lipase (umolFA/ml/hr)	9.8 (5.9-14.3)	11.4 (9.3-15.4)	< 0.02

The age and body mass index (BMI) of the patients was comparable to that of the control population. Patients had higher plasma triglyceride, VLDL-C and Chol/HDL

ratio (all p<0.0001). HDL-C (p<0.0001) and activity of both lipoprotein and hepatic lipase enzymes was lower in renal failure (LpL p<0.002, HL p<0.02). There was no difference in cholesterol or LDL-C between the two groups.

Patient Subgroups vs Controls

Table 8.2 shows the anthropometric data, lipids, lipoproteins and lipase activity divided according to patient subgroup. There were differences in the ratio of males to females in the treatment groups, with patients on PD having the highest ratio of females: males. Plasma triglycerides and VLDL-C were raised in all three patient groups compared with controls, similarly HDL-C was reduced in all three groups. Cholesterol and LDL-C were only raised in patients treated with PD with LDL-C actually being reduced in HD patients. The reduction in lipoprotein lipase activity was significant in the two groups on renal replacement therapy, whereas hepatic lipase was only significantly reduced in the pre-dialysis patients.

Effect of Mode of Dialysis

Comparing patients treated with PD and HD revealed that there were more females in the peritoneal dialysis group. The mean age, BMI, plasma triglyceride, VLDL-C, HDL-C, lipoprotein and hepatic lipase activities were all similar (table 8.2), however plasma total cholesterol and LDL-C were higher in patients on PD compared with haemodialysis patients (both p<0.001).

Table 8.2: Anthropometric data, Lipids, Lipoproteins and Lipases : Patient Subgroups vs Controls (Median + IQR)

	Peritoneal Dialysis (n=25)	Haemodialysis (n=25)	Pre- Dialysis (n=25)
Sex M/F[+]	11 / 14	18 / 7	16 / 9
Age	53 (43-58)	47 (30-58)	54 (39-61)
BMI	25.2 (21.8-29.5)	23.0 (20.6-26.2)*	24.6 (22.3-27.5)
Cholesterol (mmol/l)	5.9 (5.1-6.5) ***	4.6 (3.6-5.3)	5.1 (4.4-6.3)
Triglyceride (mmol/l)	2.0 (1.3-3.0) ****	1.7 (1.5-2.0) ****	1.3 (1.0-2.3) ***
VLDL-C (mmol/l)	0.8 (0.5-1.5) ****	0.7 (0.6-1.0) ****	0.6 (0.4-1.1) ***
LDL-C (mmol/l)	3.9 (3.5-4.3) *	2.8 (2.0-3.6) **	3.0 (2.4-4.2)
HDL-C (mmol/l)	0.8 (0.7-1.1) ****	1.0 (0.8-1.2) ****	1.0 (0.8-1.4) *
Chol / HDL	6.7 (5.4-8.3) ****	4.6 (3.7-5.6) *	5.1 (3.4-6.7) **
Lipoprotein Lipase (umolFA/ml/hr)	3.8 (2.0-4.4) *	3.0 (1.9-3.8) ****	3.6 (2.8-4.4)
Hepatic Lipase (umolFA/ml/hr)	9.3 (6.5-14.9)	10.4 (5.1-14.0)	9.7 (6.1-12.6) *

****p<0.001 ***p<0.005 **p<0.01 *p<0.05 comparing subgroups vs control

[+] X^2 test for comparison of sex

VLDL, VLDL Subfractions, IDL & LDL Subfractions

<u>All Patients</u>

There was a two fold increase in total VLDL, $VLDL_1$, $VLDL_2$ and IDL concentration in the patients compared with controls (table 8.3). The total concentration of LDL did

not differ between the patients and controls, however the concentration and percentage of the LDL subfractions differed markedly. The concentration of LDLIII was increased in the patients whereas the concentration of the larger lighter LDLI & LDLII subfractions was reduced (table 8.3). Similarly the percentage of LDL that was small & dense (LDLIII) increased in the patient group (30 ± 26 vs $14\pm10\%$ p<0.0001), with a corresponding decrease in the percentage of LDLII (47 ± 18 vs $62\pm11\%$ p<0.0001, fig 8.1).

Table 8.3: VLDL subfractions, IDL & LDL subfractions: Patients vs Controls: Median + IQR ([+]Mean + SD)

	PATIENTS	CONTROLS	p
VLDL Total (mg/dl)	178 (107-238)	80 (55-112)	< 0.0001
VLDL$_1$ (mg/dl)	80 (40-137)	47 (27-70)	< 0.0005
VLDL$_2$ (mg/dl)	84 (58-125)	34 (25-52)	< 0.0001
IDL (mg/dl)	71 (51-94)	44 (35-60)	< 0.0001
LDL Total (mg/dl)	279 (213-325)	286 (243-345)	nsd
LDLI (mg/dl)	55 (25-93)	59 (45-98)	< 0.04
LDLII (mg/dl)	134 (85-183)	173 (134-220)	< 0.0001
LDLIII (mg/dl)	50 (25-122)	31 (26-54)	< 0.03
%LDLI [+]	23 ± 15	24 ± 11	nsd
%LDLII [+]	47 ± 18	62 ± 11	<0.0001
%LDLIII [+]	30 ± 26	14 ± 10	<0.0001

Patient Subgroups vs Controls

The results comparing patient subgroups with controls are seen in table 8.4. The

concentration of total VLDL, $VLDL_2$ and IDL were increased in all three patient subgroups. $VLDL_1$ was increased in the PD and HD groups but not in the predialysis patients. LDL concentration was reduced in the patients treated with haemodialysis. The LDL subfraction distribution was similar for each patient group with an increase in the percentage of LDLIII and a decrease in the percentage LDLII. The concentration of each LDL subfraction followed a similar pattern, but was clearly also influenced by the total LDL concentration. Therefore the concentration of LDLIII was only significantly increased in the PD patients with a non-significant increase in the patients treated with haemodialysis due to the lower total LDL concentration in this group. There was however, marked heterogeneity in the plasma concentration of LDLIII in each group. As a result, 40% of patients treated by peritoneal dialysis, 28% treated by haemodialysis and 28% of predialysis patients possessed a plasma LDLIII concentration >100mg/dl compared with only 2.5% of the control population (p=0.002, X^2 test). The concentration of larger lighter LDLII was lower in the HD and pre-dialysis patients.

Effect of Mode of Dialysis

Comparing PD and HD patients revealed that the $VLDL_2$ subfraction was increased in PD patients (p<0.005), with levels of total VLDL, $VLDL_1$ and IDL being similar. Total LDL concentration was also increased in PD patients, however the LDL subfraction distribution did not differ between the treatment modalities.

Table 8.4: VLDL subfractions, IDL & LDL subfractions: Patients Subgroups vs Controls -Median + IQR ($^+$Mean±SD)

	Peritoneal Dialysis (n=25)	Haemodialysis (n=25)	Pre- Dialysis (n=25)
VLDL Total (mg/dl)	208 (109-333) ****	185 (120-210) ****	122 (79-224) ***
VLDL$_1$ (mg/dl)	88 (40-190) ****	102 (59-134) ***	51 (30-119)
VLDL$_2$ (mg/dl)	113 (68-164) ****	68 (57-102) ****	82 (48-99) ****
IDL (mg/dl)	73 (60-95) ****	65 (48-84) ***	73 (41-98) ***
LDL Total (mg/dl)	310 (268-336)	243 (172-300) ***	249 (186-355)
LDLI (mg/dl)	46 (22-97)	41 (24-78) *	72 (31-102)
LDLII (mg/dl)	162 (109-193)	115 (78-151) ****	126 (73-170) ***
LDLIII (mg/dl)	84 (29-160) *	64 (25-114)	30 (18-120)
% LDLI$^+$	19 ± 13	21 ± 15	29 ± 17
% LDLII$^+$	48 ± 21 ***	49 ± 15 ***	45 ± 17 ***
% LDLIII$^+$	33 ± 29 ***	30 ± 22 **	26 ± 27 **

****$p < 0.001$ ***$p < 0.005$ **$p < 0.01$ *$p < 0.05$ comparing subgroups vs control

Factors regulating LDLIII production

The results of univariate and multivariate analysis for factors regulating LDLIII concentration are seen in table 8.5. Figs 8.2-8.4, show the correlation between either plasma triglyceride or hepatic lipase and LDLIII concentration. For all patients with chronic renal failure, there is a close correlation with the plasma triglyceride (fig 8.2, p<0.001), with a weaker but significant correlation with hepatic lipase (fig 8.2,

p<0.03). On multivariate analysis plasma triglyceride (p<0.001) and hepatic lipase (p<0.04) were seen to independently correlate with LDLIII concentration. The correlation with plasma triglyceride was seen across each of the patient subgroups (fig 8.3), with plasma triglycerides being an independent predictor of LDLIII in PD and HD patients (p<0.001). The correlation between LDLIII concentration and hepatic lipase was only present in PD and PreD (fig 8.4), with hepatic lipase activity rather than plasma triglyceride being an independent predictor of LDLIII concentration in the predialysis patients.

Table 8.5 Univariate (U) and Multivariate (M) Analysis for Regulation of LDLIII Concentration (r^2%)

	All Patients		Peritoneal Dialysis		Haemodialysis		PreDialysis	
	U r^2%	M r^2%	U r^2%	M r^2%	U r^2%	M r^2%	U r^2%	M r^2%
Triglyceride	38.4*	35.5*	45.2*	32.8*	47.9*	47.3*	25.8[+]	11.1
Hepatic Lipase	6.7**	3.8[+]	19.7**	7.3	3.3	2.7	28.4[+]	13.8**

*p<0.001   [+]p<0.01   **p<0.03   [++]p<0.04

Lipoprotein Compositions

The lipoprotein compositions obtained are shown in table 8.6. The most consistent changes seen were in the cholesteryl ester and triglyceride compositions of the lipoproteins. Patients with renal failure had $VLDL_1$ and $VLDL_2$ that was cholesteryl ester rich and triglyceride deplete compared with controls. In contrast IDL and LDL were cholesterol deplete and triglyceride enriched in the patients compared with controls. There were no major differences in the percentage of protein or phospholipid

in each lipoprotein fraction. The lipoprotein compositions of each patient subgroup followed a similar pattern to the entire renal failure group. Lipoprotein compositions in patients treated with PD and HD were identical with the exception of VLDL$_2$ which was cholesteryl ester rich in PD patients (PD 23.7±3.6% vs HD 20.8±2.6% p<0.005) and IDL which was cholesteryl ester rich and triglyceride deplete in PD patients (Cholesteryl ester: PD 34.2±3.8% vs HD 32.0±4.2% p<0.05, Triglyceride: PD 18.6±4.0% vs HD 21.1±4.2% p<0.05) .

Table 8.6: Plasma Lipoprotein Compositions: Patients vs Controls (Mean±SD)

	PATIENTS	CONTROLS	p
VLDL$_1$% Total Cholesterol	9.8 ± 3.3	6.9 ± 2.1	< 0.0001
VLDL$_1$% Triglyceride	65.1 ± 4.0	67.4 ± 3.9	< 0.005
VLDL$_2$ % Total Cholesterol	22.7 ± 3.7	18.7 + 3.6	< 0.0001
VLDL$_2$ % Triglyceride	39.8 ± 5.1	42.5 ± 6.8	< 0.03
IDL % Total Cholesterol	34.0 ± 4.1	35.8 ± 3.3	< 0.001
IDL % Triglyceride	18.5 ± 4.6	15.6 ± 3.7	< 0.001
LDL % Total Cholesterol	36.2 ± 3.9	39.5 ± 1.5	< 0.0001
LDL % Triglyceride	10.4 ± 3.1	6.9 ± 1.4	< 0.0001

Fig 8.1: LDL Subfractions as Percentage of Total LDL: Patients vs Controls (mean + standard deviation)

Plasma Triglyceride vs LDLIII mass: All Patients
(Log:Log Plot)

$r^2 = 38.4\%$
$p < 0.001$

Hepatic Lipase vs LDLIII mass: All Patients
(Log:Log Plot)

$r^2 = 6.7\%$
$p < 0.03$
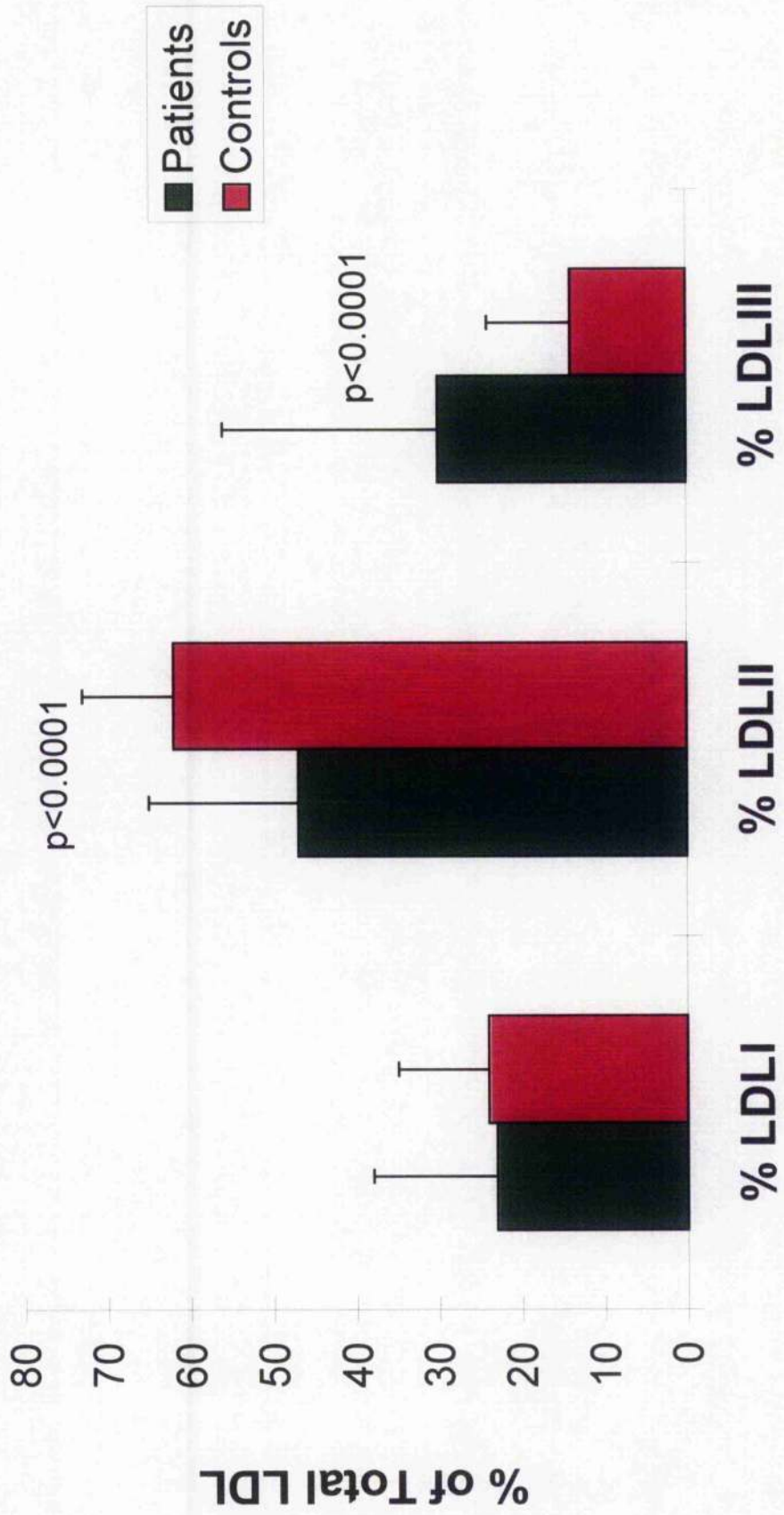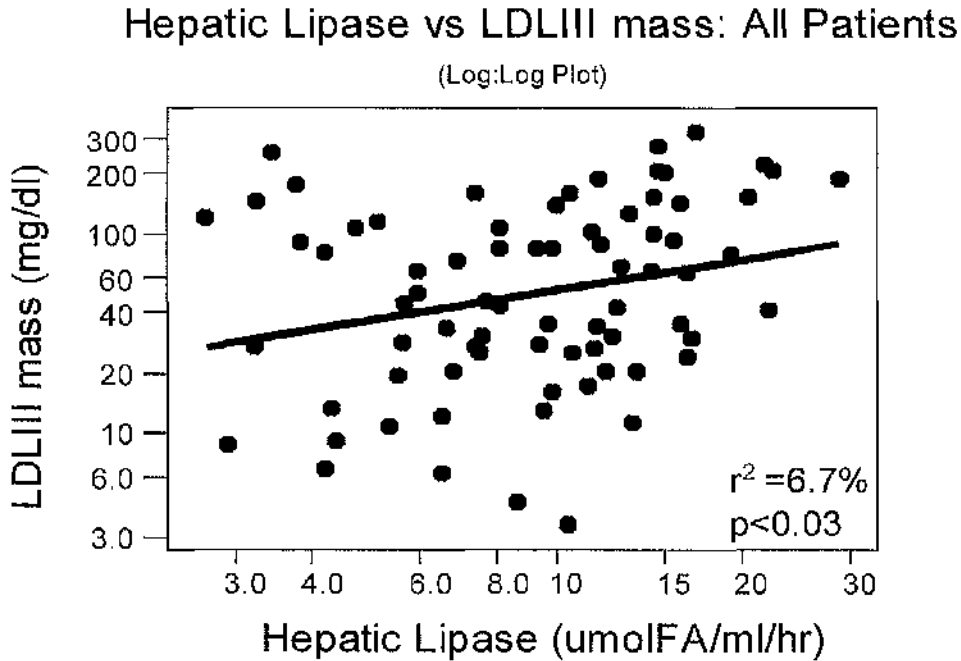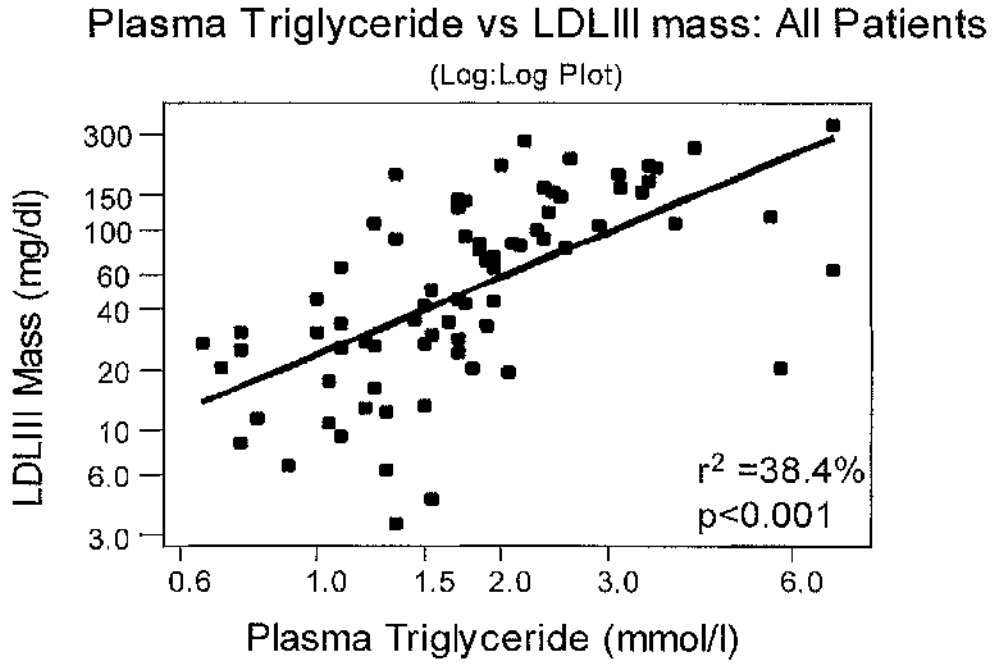
Fig 8.3: Plasma Triglyceride vs LDLIII concentration: Patients Subgroups

Plasma Triglyceride vs LDLIII mass: Haemodialysis
(Log:Log Plot)

$r^2 = 47.9\%$
$p < 0.001$

Plasma Triglyceride (mmol/l)

Plasma Triglyceride vs LDLIII mass: Peritoneal Dialysis
(Log:Log Plot)

$r^2 = 45.2\%$
$p < 0.001$

Plasma Triglyceride (mmol/l)

Plasma Triglyceride vs LDLIII mass: 'Pre-Dialysis'
(Log:Log Plot)

$r^2 = 25.8\%$
$p < 0.01$

Plasma Triglyceride (mmol/l)
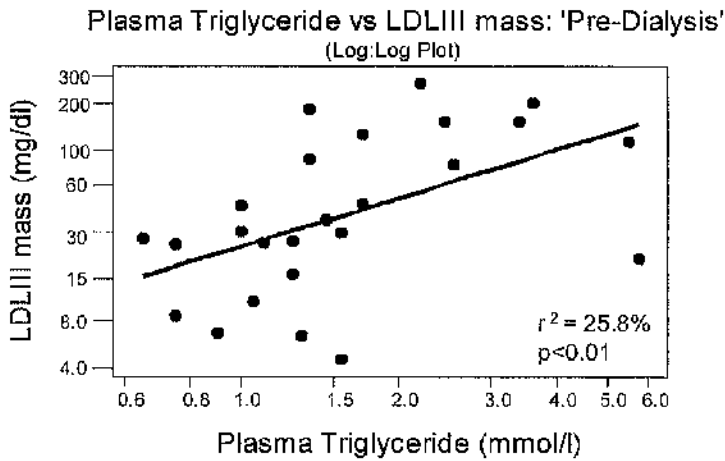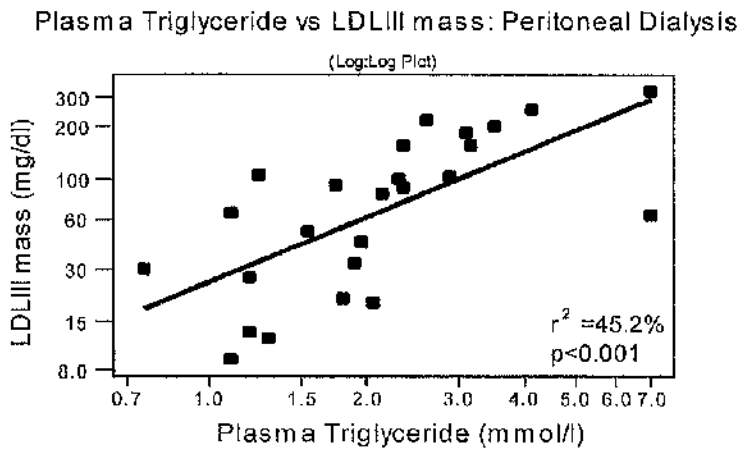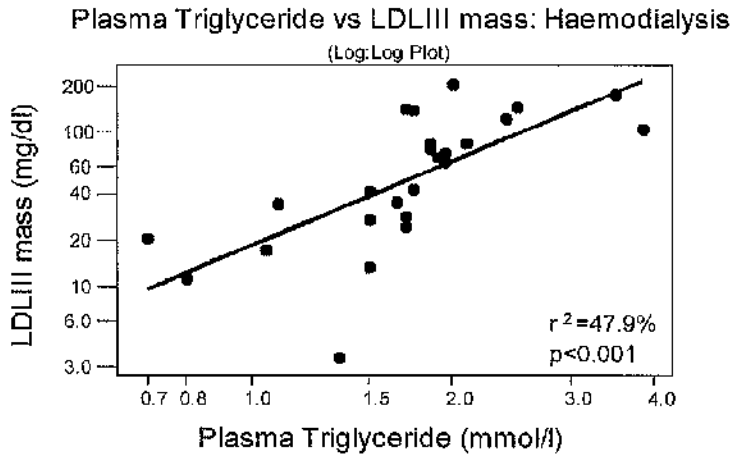
Fig 8.4: Hepatic Lipase vs LDLIII concentration: Patients Subgroups

Hepatic Lipase vs LDLIII mass: Haemodialysis
(Log:Log Plot)

$r^2 = 3.3\%$ (-)
p=nsd

LDLIII mass (mg/dl)

Hepatic Lipase (umolFA/ml/hr)

Hepatic Lipase vs LDLIII mass: Peritoneal Dialysis
(Log:Log Plot)

$r^2 = 19.7\%$
p<0.03

LDLIII mass (mg/dl)

Hepatic Lipase (umolFA/ml/hr)

Hepatic Lipase vs LDLIII mass: 'Pre-Dialysis'
(Log:Log Plot)

$r^2 = 28.4\%$
p<0.01

LDLIII mass (mg/dl)

Hepatic Lipase (umolFA/ml/hr)
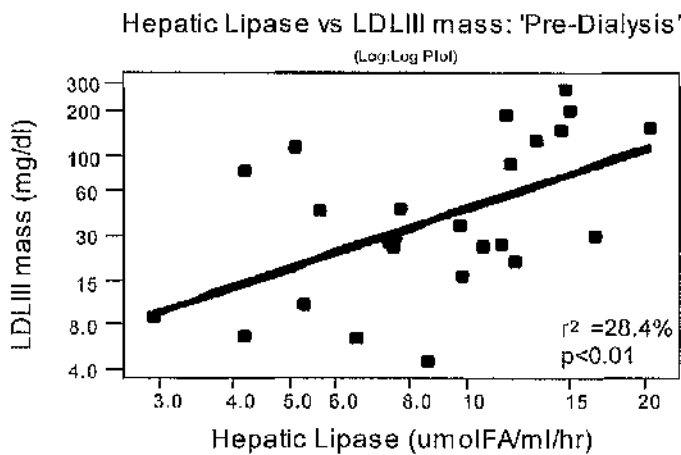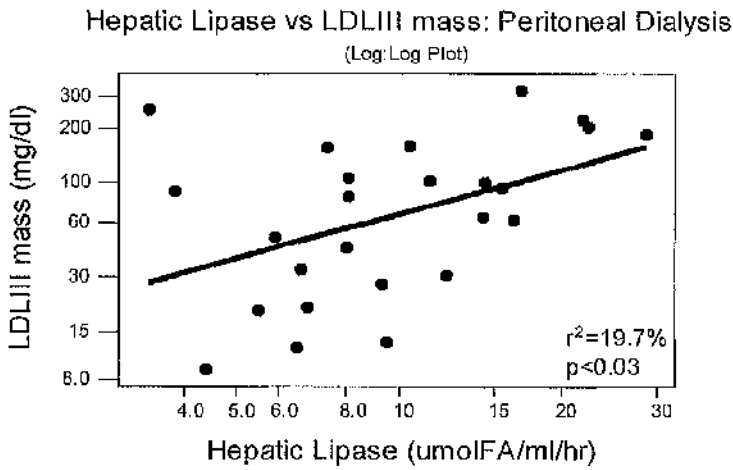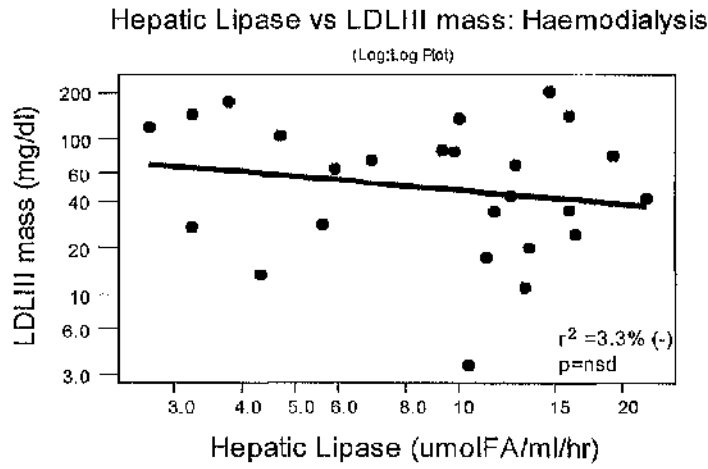
DISCUSSION

Although two previous studies have shown that the LDL size in patients with end-stage renal failure (ESRF) is smaller than control populations [160,161], the extent to which small dense LDL is formed and the precise metabolic origin of this species in uraemic patients, has not been assessed. This study demonstrates that atherogenic small dense LDL is present in greater quantities in patients with ESRF. This increase in concentration of small dense LDL has occurred due to a shift in pattern of LDL towards smaller denser particles, with a corresponding reduction in the larger lighter LDLII both in terms of concentration and as a percentage of total LDL.

The increased concentration of LDLIII is seen despite the total concentration of LDL being slightly lower in ESRF. The shift in particle size was seen across the patient groups with patients treated with peritoneal dialysis (PD), haemodialysis (HD) and pre-dialysis patients all having a higher percentage of LDLIII and decreased proportion of larger lighter LDLII. The increase concentration of LDLIII however was only significant in the PD group as both the pre-dialysis and particularly the haemodialysis patients had a lower concentration of total LDL. There was however, marked heterogeneity in the concentration of LDLIII present in each population. Patients whose LDLIII concentration is greater than 100mg/dl has been shown to have a seven fold increase in cardiovascular risk in non-uraemic populations [141]. In the patients studied, LDLIII concentration was greater than 100mg/dl in 32% of the patients compared with 2.5% of controls. 40% of PD patients and 28% of both HD and pre-dialysis patients had an LDLIII concentration >100mg/dl. The percentage of patients with ESRF and atherogenic levels of LDLIII may also be underestimated in this study as previous authors have estimated that 50% of patients on renal

replacement therapy have triglyceride levels >2.25mmol/l [115], and we found fasting triglycerides to be >2.25mmol/l in only 30% (PD 44% HD 16%). This may in part be due to the exclusion of patients with diabetes.

The results obtained by density gradient ultracentrifugation are supported by analysis of the LDL compositions. These confirm that the LDL in uraemic patients is triglyceride rich and cholesterol deplete compared with controls patients, a finding noted in previous studies of lipoprotein metabolism in ESRF [114,222] and characteristic of the composition of small dense LDL [56]. The abnormal LDL composition was seen in all three of the treatment groups, supporting the finding of a shift to smaller denser particles in PD, HD and pre-dialysis patients.

The two most important factors determining formation of small dense LDL in non-uraemic patients are plasma triglycerides and hepatic lipase. Thus to form atherogenic levels of small dense LDL in non-uraemic patients requires a plasma triglyceride >1.5mmol and hepatic lipase activity >15umolFA/ml/hr (seen in males and post-menopausal females) [56]. As stated previously, the current model for the formation of small dense LDL suggests that in the presence of hypertriglyceridaemia excess $VLDL_1$ is present, which takes part in neutral lipid exchange with LDL via cholesterol ester transport protein (CETP). Triglyceride is passed from the $VLDL_1$ to LDL in exchange cholesteryl ester which is transferred to VLDL, creating a triglyceride rich LDL particle which is hydrolysed by hepatic lipase, shrinking the particle and forming small dense LDL [56]. In keeping with the results of the studies in patients with glomerular disease and diabetes, the data from this study concurs with this hypothesis. Hypertriglyceridaemia is present, $VLDL_1$ concentration is increased, is cholesterol

enriched and triglyceride deplete. LDL is cholesterol deplete and triglyceride enriched and hepatic lipase levels correlate with LDLIII concentration. Moreover previous authors have shown that CETP activity is increased in patients treated with PD [223]. It is therefore likely that the mechanisms underlying small dense LDL formation in uraemia are similar those proposed for non-uraemic populations.

Further support for this hypothesis is seen when examining the correlation between LDLIII concentration, triglycerides and hepatic lipase in the uraemic patients. The strong correlation between LDLIII concentration and plasma triglycerides in the patients confirms the importance of triglycerides in LDLIII formation in uraemia (a correlation that was seen in all three treatment groups), however this data also demonstrates the importance of reduced hepatic lipase (HL) activity in this population, with HL activity an independent determinant of LDLIII concentration on multivariate analysis. As a result there are differences in the triglyceride threshold at which atherogenic levels of LDLIII are formed. The univariate correlation between hepatic lipase is strongest in the groups with the highest triglycerides (PD & PreD i.e. where HL levels would be rate limiting) and not present in HD patients (the group with the lowest triglycerides, therefore the low triglyceride rather than HL is rate limiting). A plasma triglyceride of >1.5mmol in males or post-menopausal females is recognised as a level at which atherogenic levels of LDLIII are formed in normal populations (i.e. LDLIII concentration >100mg/dl) [56]. In this study however only 45% of patients with a plasma triglyceride >1.5mmol had a LDLIII concentration >100mg/dl compared with 73% of patients with a plasma triglyceride >2.0mmol/l. This suggests that in uraemia due to the rate limiting effect of the lower levels of hepatic lipase, patients require a higher triglyceride to form atherogenic levels of LDLIII.

The basic lipoprotein results obtained were typical for this population, however not only were differences found between the dialysis modalities, in the concentration of LDLIII, but there were also marked differences in other aspects of the lipid and lipoprotein profiles. Patients on PD had far higher total and LDL cholesterol (a well recognised finding) [115,118], with similar triglycerides, VLDL-C, HDL-C and lipase activity. PD patients also had raised $VLDL_2$ and total LDL but not $VLDL_1$ when compared with those treated with HD. $VLDL_1$ and $VLDL_2$ are generally under independent metabolic control, $VLDL_1$ is associated with raised triglycerides [144] and tends to be overproduced in patients with insulin resistance [145] and following carbohydrate loading [146]. Production of $VLDL_2$ however is increased in patients with raised total and LDL cholesterol [56] and metabolic studies have shown that $VLDL_2$ production is also increased in proteinuria [171]. The dyslipidaemia pattern seen in the patients on peritoneal dialysis is therefore associated with excess $VLDL_2$, and raised LDL rather than raised triglycerides from increased $VLDL_1$. This would make carbohydrate loading from peritoneal dialysate dextrose absorption less likely to be responsible for difference in dyslipidaemia between these populations and favour the >5g/day peritoneal albumin loss that has been shown to occur in CAPD [224], as the most likely aetiology.

The incidence of cardiovascular disease in ESRF far exceeds that in comparable populations and accounts for 40-50% of reported causes of death amongst patients on renal replacement therapy [9]. In addition to dyslipidaemia, hypertension, fluid overload, left ventricular hypertrophy, hyperhomocystinaemia, diabetes mellitus and smoking all contribute to this increased risk [225]. Hypertriglyceridaemia has been

shown to be an independent risk factor for cardiovascular disease in patients on RRT [134] whilst in the pre-dialysis population total cholesterol, triglycerides and LDL-C are increased in patients with cardiovascular disease compared to those without cardiovascular disease [8]. Triglycerides are physiologically linked to small dense LDL and low HDL, a combination known as the atherogenic lipoprotein phenotype [140], and it is likely that the increased small dense LDL observed in this study, contributes to the atherogenic risk of hypertriglyceridaemia in ESRF.

Despite the high morbidity and mortality from cardiovascular disease in renal failure, nephrologists have until recently, been reluctant to embark on aggressive lipid lowering therapy in uraemic patients . This is partly due to the general finding of normal cholesterol and LDL-C levels in uraemia and partly due to the lack of outcome evidence following lipid lowering therapy in patients with impaired renal function [226]. The finding of atherogenic levels of LDLIII in the context of normal plasma LDL-C highlights the need to look beyond the basic assessment of plasma levels of total and LDL-C when assessing the cardiovascular risk posed by dyslipidaemia in this population. The physiological link and strong correlation between fasting triglycerides and LDLIII concentration, can allow us to use triglycerides as a surrogate marker for LDLIII. Thus in our study using triglyceride of >2mmol/l to identify patients with LDLIII concentration >100mg/dl had a sensitivity of 86% and a specificity of 79%.

In conclusion, atherogenic levels of LDLIII are formed in patients with end-stage renal failure. However, there is marked heterogeneity in LDLIII levels in this population, with a strong association between plasma triglyceride and LDLIII concentration, highlighting the physiological role of triglycerides in LDLIII formation and its

possible use as a surrogate marker. Clearly further studies in uraemic patients are required to assess the risk posed by small dense LDL in this population and to assess the role of lipid lowering agents in a population who have dyslipidaemia with the atherogenic lipoprotein phenotype rather than hyperlipidaemia. Furthermore as nephrologists become more adept at slowing progression of renal failure and postponing dialysis, the qualitative change in LDL before ESRF highlights the need to address the cardiovascular risk from dyslipidaemia before the commencement of renal replacement therapy.

CHAPTER 9 -

General Discussion - The Atherogenic Lipoprotein Phenotype in Renal Disease

This project has examined the metabolism of triglyceride rich lipoproteins in patients with chronic renal disease. We have concentrated on patients with nephrotic range proteinuria, a subset of patients who have an increased risk of coronary artery disease and a high risk of progression to end-stage renal failure. In this final chapter, we will review three main aspects of the studies:

1. The origins and consequences of impaired metabolism of triglyceride-rich lipoproteins in renal disease, including the effect of hepatic lipase and disease state on the formation of small dense LDL in patients with chronic renal dysfunction.

2. The potential influence of the Atherogenic Lipoprotein Phenotype on coronary risk in chronic renal disease

3. Treatment of dyslipidaemia in chronic renal disease

ORIGINS AND CONSEQUENCES OF IMPAIRED METABOLISM OF TRIGLYCERIDE-RICH LIPOPROTEINS

Chapters 3 and 4 outline the possible origins and ultimate consequences of the impaired clearance of triglyceride rich lipoproteins in patients with proteinuria. This is summarised in fig 9.1. The data presented suggests that in this population, the VLDL$_1$ particles produced are smaller and relatively enriched in free cholesterol compared with the control population. These particles are also deficient in

apolipoprotein CII, CIII and E, a deficiency that is present despite seeming to have adequate plasma levels of apolipoprotein CII and CIII. As a result, lipolysis by lipoprotein lipase becomes less efficient and the smaller apolipoprotein deficient particles are less readily taken up by hepatic apoE receptors. The net effect is an increase in $VLDL_1$, excess plasma triglyceride and increased concentrations of partially metabolised lipoprotein remnant particles. In this population, increased plasma concentration of $VLDL_2$ is also present, an increase that is in part, related to impaired clearance of the particles [171]. In this context, it is likely that similar apolipoprotein defects are present on $VLDL_2$ contributing to the impaired clearance.

A similar increase in $VLDL_1$ was identified in the studies involving patients with diabetic nephropathy (chapter 7) and end-stage renal failure (chapter 8). In patients with type 2 diabetes, the plasma concentration of $VLDL_1$ is generally increased [145], due to an increase in production of triglyceride rich lipoproteins. However the microalbuminuric and diabetic control patients in this study did not possess excess $VLDL_1$. It is likely that this resulted from the good glycaemic control in all the diabetic patients studied. We suggest therefore that the excess $VLDL_1$ present in the proteinuric diabetic patients may have a similar origin to that found in the patients whose proteinuria resulted from glomerular disease. In the study involving patients with end-stage renal failure (ESRF - chapter 8), a clear reduction of in-vitro lipoprotein lipase activity was detected. Therefore, in this case, impaired activity of the lipase enzyme will be responsible for the excess $VLDL_1$.

Although the origin of the excess $VLDL_1$ may differ depending upon the population studied, the consequences of the excess triglyceride rich lipoproteins are probably

similar. In patients with excess triglyceride rich lipoproteins, $VLDL_1$ takes part in neutral lipid exchange via cholesteryl ester transfer protein (CETP). Thus triglyceride is passed to LDL in exchange for cholesteryl ester which is passed to VLDL (fig 9.1). The triglyceride in LDL is subsequently hydrolysed by hepatic lipase, shrinking the particle and forming small dense LDL.

Hepatic lipase activity is thought to be the second main factor (after plasma triglyceride) regulating production of small dense LDL [56]. The importance of hepatic lipase is illustrated by the presence of very low levels of small dense LDL in pre-menopausal women, a finding related to the low levels of hepatic lipase present in this population [144]. The influence of low hepatic lipase activity is further demonstrated in this project. In the patients with glomerular disease and proteinuria, normal hepatic lipase activity was detected. Thus the enzyme was not rate limiting and no relationship was identified between hepatic lipase activity and plasma LDLIII concentration. A different picture was seen in the patients with end-stage renal failure, where hepatic lipase levels were reduced. Here, hepatic lipase activity was rate limiting and independently related to plasma LDLIII concentration. It has been proposed that to form atherogenic levels of LDLIII, a plasma triglyceride >1.5mmol/l and hepatic lipase activity >15umolFA/ml/hr is required [56]. As a result of the reduced lipase activity in end-stage renal failure, this triglyceride threshold was increased to 2mmol/l. Post-heparin lipase activity was not studied in the diabetic patients (due to the problem of giving intravenous heparin to patients with diabetic retinopathy), however we have inferred that part of the excess LDLIII in the diabetic patients resulted from patients with increased body mass index having increased activity of hepatic lipase.

Therefore, in this project, we have outlined abnormalities in VLDL₁ which may account for the increased plasma concentration of triglyceride rich lipoproteins in proteinuric disease, the main consequences of which is the production of atherogenic lipoproteins. Although the deficiency of apolipoproteins on the VLDL particle can explain the delayed clearance of the particle, a number of factors need to be explored further. The high surface ratio of free cholesterol : phospholipid, is likely to inhibit post-secretory incorporation of apoC into VLDL, resulting in delayed lipolysis. However it has yet to be established why this high surface ratio is present. Data regarding production of free cholesterol (FC) production in this population is conflicting [188,189], and the high surface ratio could also occur in the context of normal FC production and incorporation, into a smaller VLDL₁ particle. Moreover, the cause of the apparently smaller VLDL₁ particles remains to be explained. This may be linked to the apparent plasma apoE deficiency, which also requires further investigation. ApoE is larger than both apoCII and apoCIII, and given that albumin is found in the urine (which is larger than all three apolipoproteins), it is certainly feasible for both apoC and apoE to be lost. However the plasma concentration of the apoC proteins did not seem to be reduced.

As a result we propose to continue our study of triglyceride rich lipoproteins in this population, in particular looking at:

1. Changes in the composition and size of VLDL particles

2. Fatty acid production from VLDL. In order to assess the ability of lipases to hydrolyse VLDL particles from patients with proteinuria

3. The transfer of apolipoproteins from VLDL to HDL, to explore further the effects

of changes in the surface components of VLDL.

4. Post-prandial apolipoprotein composition of chylomicrons, as clearance of chylomicrons is normal in patients with proteinuria, but the mechanisms by which these triglyceride rich particles are cleared are similar to those seen in VLDL metabolism.

Fig 9.1: METABOLISM OF APO-B CONTAINING LIPOPROTEINS IN NEPHROTIC RANGE PROTEINURIA.
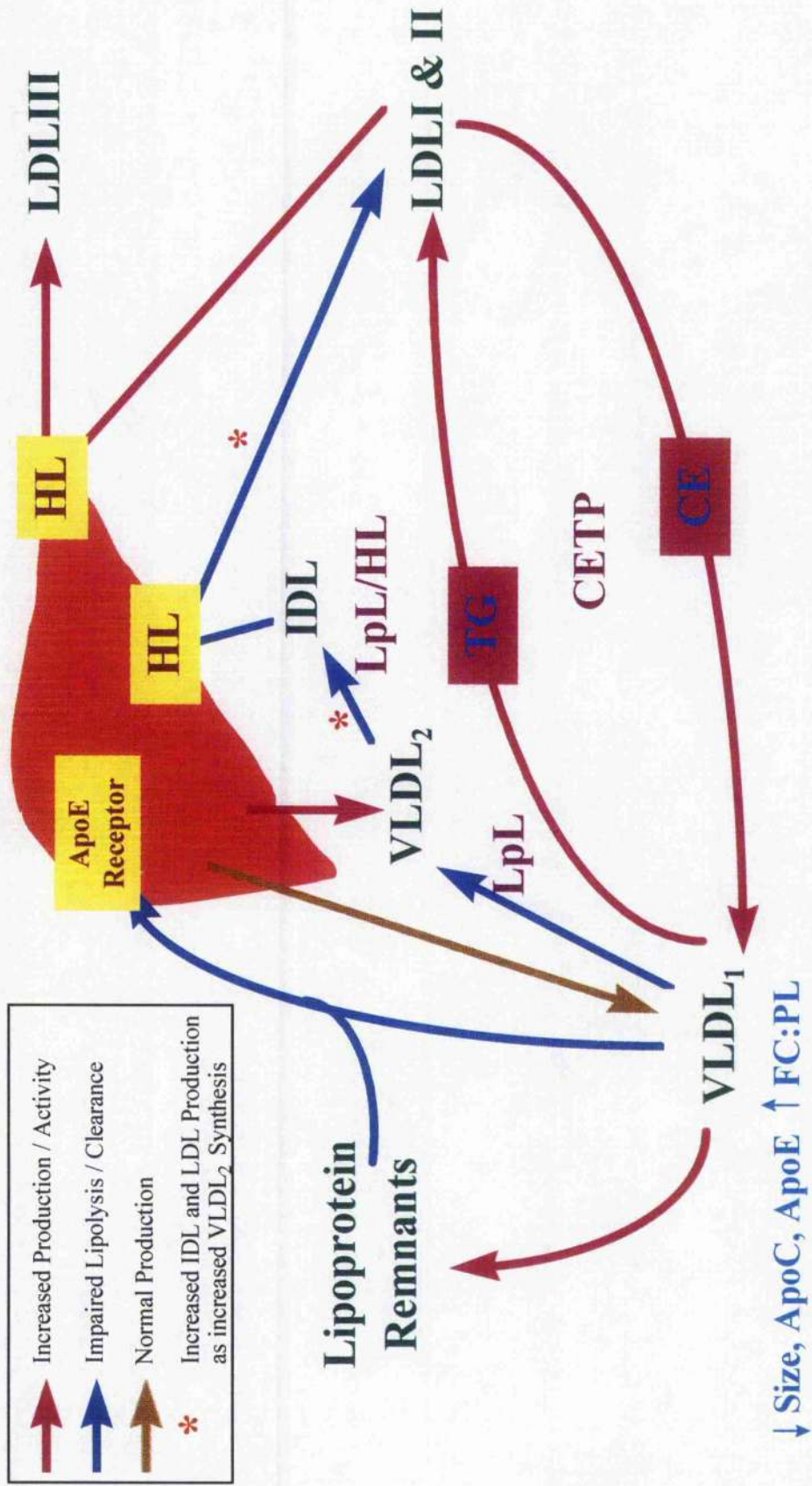
THE ATHEROGENIC LIPOPROTEIN AND CORONARY HEART DISEASE
IN CHRONIC RENAL DISEASE

It is now clearly established that proteinuria is associated with increases in both cardiovascular disease and coronary heart disease, with a 2-5 fold increase in the risk of MI or coronary death [3,7]. Furthermore this increased risk is independent of other cardiovascular risk factors including hypertension and plasma cholesterol [3,7]. To date, there is no data confirming the link between plasma triglyceride and coronary heart disease in patients with proteinuria, however the atherogenic lipoprotein phenotype is amongst the most common lipid disorders found in normal populations with coronary heart disease [227], and the presence of an atherogenic lipoprotein phenotype is associated with a 3-7 fold increase in the risk of coronary heart disease regardless of the level of LDL-C [227]. In the patients with proteinuria and glomerular disease that were studied in chapter 3, 74% possessed a plasma LDLIII concentration >100mg/dl, with the median remnant lipoprotein cholesterol level in the patients with proteinuria increased 2-3 fold, and a 5 fold increase in remnant lipoprotein triglyceride concentration. Even the patients with progressive renal failure (chapter 6), who had lower creatinine clearance when studied (and therefore reduced hepatic lipase activity) had a high prevalence of atherogenic levels of LDLIII, with 57% possessing a plasma LDLIII concentration >100mg/dl. Therefore there seems little doubt that the atherogenic lipoprotein phenotype is extremely common in this population. It is difficult to imagine that in patients with persistent heavy proteinuria, that this dyslipidaemia is not going to have a profound effect on the incidence of coronary heart disease.

In type 2 diabetes, a clear link exists between raised plasma triglyceride and coronary

heart disease [124], indicating a probable link between the atherogenic lipoprotein phenotype and coronary heart disease in this population. Type 2 diabetic patients with proteinuria have a 2-3 fold increase in death from cardiovascular disease compared with type 2 diabetic patients without proteinuria, an increase that is independent of systolic blood pressure and smoking [6]. 75% of the patients studied with type 2 diabetes and proteinuria (chapter 7) possessed a plasma LDLIII concentration >100mg/dl. It is highly likely that this excess small dense LDL contributes to the increased incidence of vascular events that has been demonstrated in this population.

Finally, data in chapter 8 demonstrated that despite reduced hepatic lipase activity, non-diabetic patients with end-stage renal failure (ESRF) had an increased prevalence of the atherogenic lipoprotein phenotype. A plasma triglyceride of 2mmol/l proved to be an accurate marker of a plasma concentration of LDLIII >100mg/dl. Cohort data has shown plasma triglyceride to be an independent predictor of cardiovascular disease in ESRF [134], whilst cross-sectional data has suggested HDL-C to be an independent predictor of coronary heart disease in haemodialysis patients [135]. Therefore a clear link has been established between the atherogenic lipoprotein phenotype and vascular disease in ESRF.

Therefore across the differing groups of patients studied, the importance of the atherogenic lipoprotein phenotype in contributing to coronary heart disease has been outlined. Clearly this is not the only lipid abnormality present in many of these patients. Apart from increases in plasma cholesterol, IDL was increased in both proteinuric and ESRF patients. Increased plasma concentration of IDL is now recognised to contribute to atherosclerosis [228] and has been linked to increased

vascular disease in ESRF [229]. This further 'vascular risk factor' simply serves to highlight the inadequacy of simple measurements of non fasting cholesterol in a population where premature cardiac death is so frequently found.

TREATMENT OF DYSLIPIDAEMIA IN CHRONIC RENAL DISEASE

The rationale behind using pharmacological treatment to ameliorate dyslipidaemia in chronic renal disease is firstly to reduce the incidence of coronary heart disease (CHD) and secondly to try and slow progression of chronic renal failure. To date there is no data to show that lipid lowering treatment with either a statin or a fibrate will reduce cardiovascular morbidity or mortality in a population with either proteinuric renal disease or advanced renal failure, as all the major lipid lowering trials have excluded these patients. There is however, extensive evidence to support the use of statins in particular, but also fibrates, in reducing morbidity and mortality from CHD in 'normal' populations. In the context of secondary prevention, the 4S study showed that in a population with a mean plasma cholesterol of 5.5-8mmol/l, only 11 patients required treatment for 6 years with simvastatin to prevent one myocardial infarction (NNT = number needed to treat) [65]. This has been extended with two further studies in patients with a plasma cholesterol within the normal range demonstrating that treatment with 40mg of pravastatin for 5 or 6 years, the NNT to prevent the combined end points of non-fatal myocardial infarction (MI) or CHD death are 33 and 27 respectively [66,67]. Only the CARE study had a significant number of adverse events [66], an adverse finding not confirmed in the LIPID study, despite recruiting twice the number of patients and using virtually identical drug therapy to CARE [67]. Similar reductions in cardiac events have recently been demonstrated with the use of fibrates,

with gemfibrozil providing a 22% reduction in cardiac events in men with CHD and a HDL-C <1mmol/l (NNT=23 to prevent one death from CHD or non-fatal MI) [191]

The data in primary prevention is now equally extensive, with both statins and fibrates demonstrating a reduction in cardiac events in large scale prospective studies [64,68,70]. The first of these studies was the Helsinki Heart Study which demonstrated a 34% reduction in cardiac end-points after 5 years treatment with gemfibrozil (NNT=71) [70]. Within this study, the highest absolute cardiac risk was present in the sub-group with triglycerides >2.3 and HDL-C <1.06 (i.e. the patients who would have an excess of small dense LDL) [72]. The WOSCOPS study, using pravastatin 40mg for 5 years, demonstrated a 29% reduction in non-fatal MI or CHD death (NNT=40) [64]. Finally the AFCAPS/TEXCAPS study in a population with plasma cholesterol of 4.5-7 and HDL-C <1.2 demonstrated a 37% reduction in a broader end-point of fatal or non-fatal MI, unstable angina or sudden cardiac death (NNT=50), with the number needed to treat decreasing to approximately 40 if the HDL-C was <1.0mmol/l [68]. This wealth of data serves to illustrate the extent to which both statins and fibrates can reduce coronary artery disease in 'normal' populations. What therefore can we conclude about the role of these agents in renal disease ?

Patients with nephrotic range proteinuria have profound dyslipidaemia. It has been estimated that 89% of patients have a fasting plasma cholesterol >6.5mmol/l [104], with reports of 67% [104] and 71% [106] of patients having a plasma triglyceride greater than 2mmol/l and 2.3mmol/l respectively. 74% of the patients studied in chapter 3 possessed atherogenic levels of LDLIII, with increased plasma

concentrations of both remnant lipoproteins and IDL also present. In patients with proteinuria, statins have been shown to successfully reduce plasma cholesterol, triglyceride, apoB and LDLIII, but not remnant lipoproteins (chapter 5). The effects of statins are achieved by reducing cholesterol synthesis and up-regulating the LDL receptor, but also by increased removal of LDL precursors and decreased VLDL production [230]. In chapter 5, we found that in patients with proteinuria, fibrates reduce plasma cholesterol, with a more profound effect on triglyceride rich lipoproteins and both LDLIII and remnant lipoproteins. This is primarily achieved by reducing apoCIII synthesis, thus promoting efficient metabolism of triglyceride rich lipoproteins [152]. It is clear that the ability of these agents to improve dyslipidaemia is similar in both renal and non-renal disease.

The second aim of lipid lowering therapy is that of slowing progression of renal disease. The most extensive clinical data examining the role of dyslipidaemia in slowing progression of renal disease is in diabetic nephropathy where both cholesterol and triglyceride are independently associated with increased risk of renal failure [149,150,201]. In proteinuric glomerular disease there is increasing evidence, supported by the data in chapter 6, that apoB containing lipoproteins contribute to renal failure progression [151,202]. However there are no large scale studies that have addressed the question of whether lipid lowering therapy will slow the progression of renal failure. Multicentre trials are clearly required to resolve this issue.

Given the efficacy of both statins and fibrates in improving lipid profiles in proteinuric renal disease and their proven benefit in reducing cardiac disease in non-renal populations, the main resistance to their use in proteinuria is likely to be related

to a lack of data surrounding drug toxicity in this population. The large scale studies using statins are remarkable in their lack of significant side effects, and therefore to obviate the likely large reduction in cardiac disease in patients with proteinuria, extensive and unexpected adverse effects would have to be seen following their use in this population. There was concern regarding the safety of the older fibrate preparations, however adverse events were not significant in either the Helsinki Heart Study [70] or the recently published Veterans Affairs HIT study [191]. In this context it is significant that meta-analysis of lipid lowering therapies in patients with renal disease has not demonstrated an excess of adverse drug reactions [231]. The data presented in chapter 5 did show an increase in serum creatinine following fenofibrate treatment, an increase that has been noted in other studies, however further investigation did not show a decrease in creatinine clearance, and a reduction in GFR has not been demonstrated in patients with renal dysfunction, despite an increase in serum creatinine [197]. It seems likely that the effect of fenofibrate on serum creatinine results from an increase in creatinine production from muscle. It is noteworthy that previous dose response studies have noted that increases in serum creatinine are transient despite continued therapy [198].

Therefore in patients with chronic renal dysfunction, the two main questions are:

1. Who should we be aiming to treat ?

2. At what aspect of dyslipidaemia should we be targeting our treatment ?

There seems no doubt that treatment decisions should be tailored to individual patients. However within this a distinction should be made between primary and secondary prevention, no matter what the underlying renal pathology. A raised plasma

cholesterol should be treated, but the data presented in this study strongly suggests that simply measuring plasma cholesterol is inadequate in order to fully assess the cardiovascular risk posed by the dyslipidaemia. Full fasting B quantification of lipids and lipoproteins should be analysed and close attention should be made to the plasma triglyceride and HDL-C measurements. In patients with nephrotic range proteinuria or type 2 diabetics with nephropathy, a plasma triglyceride >1.5mmol/l warrants closer attention, whilst in ESRF consideration should be made to treating a plasma triglyceride >2mmol/l. Given the greater amount of data regarding coronary protection and safety, statins seem the logical first choice in renal disease, however the lipid profiles are likely to need aggressive treatment. It is worth noting that despite good lipid lowering in chapter 5, cerivastatin in a dose of 200microg failed to reduce LDLIII mass to <100mg/dl in any of the 10 patients who had an initial plasma LDLIII concentration >100mg/dl and failed to provide a reduction in lipoprotein remnants. Carefully monitored lowering of cholesterol and triglyceride with a combination of statin and fibrate may be required in this population in order to provide maximum cardiovascular risk reduction.

CONCLUSION

Heavy proteinuria is associated with increased risk of vascular disease. It is evident that simply looking at the plasma cholesterol and LDL-C does not allow full assessment of the cardiovascular risk posed by dyslipidaemia in this population. In addition to hypercholesterolaemia, raised plasma triglyceride is well recognised in nephrotic range proteinuria and is consequent upon delayed clearance of triglyceride rich lipoproteins. This results in production of small dense LDL and lipoprotein

remnants. However excess production of LDLIII is not restricted to patients with nephrotic range proteinuria, with atherogenic levels of LDLIII prevalent in both diabetic nephropathy and ESRF. Thus the atherogenic lipoprotein phenotype is a common finding that is not necessarily expressed as an increase in LDL, but is nevertheless highly atherogenic. Despite significant lowering of lipids, lipoproteins and reductions in the concentration of LDLIII, atherogenic levels of small dense LDL remain following lipid lowering therapy in proteinuric renal disease. Increased remnant lipoproteins are also a component of the phenotype that responds to fibrate but not to statin therapy. The differing relationships between LDLIII reduction and (i) triglyceride reduction following fenofibrate and (ii) LDL-C reduction following cerivastatin suggest a potential role for combined therapy to achieve greater reduction in atherogenic lipoproteins. Cardiovascular protection in patients with chronic renal disease should be aimed at not only reducing total cholesterol but also at reducing concentrations of small dense LDL and remnant lipoproteins.

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