Structure, function and atherogenicity of apolipoprotein B containing lipoproteins

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Abstract

Focal deposition of lipids in the form of lipoproteins is an important step in the pathogenesis of the early, fatty streak and the later advanced atherosclerotic plaque. In this thesis, two mechanisms potentially leading to lipid accumulation are examined, namely the interaction of arterial wall proteoglycans with lipoproteins and LDL oxidative modification.

In study 1, chondroitin sulphate proteoglycan (CS-PG) was dissociatively extracted and purified from human aorta in guanidine-HCl. This was incubated with LDL and the extent of complex formation was determined by measuring turbidity at 600nm. The interaction of total LDL (d 1.019-1.063g/ml) was examined in 59 angiographically positive coronary heart disease patients. The extent of APG-LDL complex formation was positively correlated with the percentage of small, dense LDL-III within total LDL and with plasma triglyceride level, while it was inversely related to the percentage of the large buoyant LDL-I within total LDL and plasma HDL cholesterol. In the second study the reactivity of apoB containing lipoproteins towards APG was examined in three groups of subjects whose LDL subfraction profile consisted of mainly LDL-I, LDL-II or LDL-III. Two fractions from each lipoprotein class were isolated by cumulative density gradient centrifugation (VLDL1 Sf 60-400, VLDL2 S_f 20-60, IDL1 S_f 16-20, IDL2 S_f 12-16, LDLA S_f 8-12 and LDLB S_f 0-8. A similar pattern of reactivity was found in all subjects in which IDL2 and LDLA were the most reactive species towards APG followed by LDLB and IDL1 and the least reactivity was found in the VLDL fractions. The magnitude of the reactivity of the lipoproteins was highest in the CHD group of patients with a predominance of small, dense LDL, intermediate in the group who had an LDL-II dominated subfraction profile and lowest in the group with the mainly high level of LDL-I. Lipid lowering treatment with ciprofibrate (100mg/day for 8 weeks) decreased the reactivity of all apoB containing lipoproteins towards APG, this was attributed to the triglyceride lowering effect of the drug.

Neutral carbohydrate and sialic acid concentration were determined in all the apoB containing lipoproteins in study 4. A higher concentration of neutral

carbohydrate, as measured by the phenol sulphuric acid method, was found in LDL-I compared with the small, dense LDL-III subfraction. Sialic acid concentration was highest in VLDL1 and lowest in LDLB in all subjects. Neither the neutral carbohydrate nor sialic acid correlated significantly with the extent of APG-Lipoprotein complex formation. This was confirmed by subfractionation of LDL into 3 subfractions by affinity chromatography on Con-A on the basis of the high mannose structure of apoB. An unbound, a weakly bound and a strongly bound fraction were obtained. Each had similar binding reactivity towards APG. Finally a series of LDL modifications were performed to explore the mechanism of APG-LDL interaction. Neuraminidase treatment of LDL increased its reactivity towards APG by 76% and addition of GM3 ganglioside from plasma decreased it by 12%. Charge modification by cyclohexandione treatment and carbamylation blocked the interaction as did reductive methylation which alters the apoB protein structure. LDL incubation with apoE2 and apoE3 also blocked its binding with APG while apoCIII addition had no effect. These findings suggested that although APG-LDL interaction is charge related, conformation of apoB is critical in controlling the interaction process.

The second major study was determination of the effect of Evening Primrose Oil (EPO) on plasma lipid levels and LDL subfraction profile and LDL susceptibility to oxidative modification in a group of non-insulin dependant diabetics (NIDDM). At a dose of 500mg/day no significant effect was found on lipid parameters. However the extent of in-vitro copper catalysed LDL oxidation measured by fluorescence at 430 was significantly increased after EPO treatment and was positively related to the percentage of small dense LDL within total LDL and plasma triglyceride concentration.

In conclusion the main findings from the studies provide evidence that the heterogeneity of apoB containing lipoproteins as seen in an atherogenic lipoprotein phenotype (ALP) is an important determinant of the atherogenic properties of the lipoprotein. The potential atherogenicity of small dense LDL is linked to its enhanced oxidative modification and its preferential association with APG. These can be modulated pharmacologically.

Contents

Chapter 3 APG-LDL interaction

Chapter 4 Interaction of very low density, intermediate density and low density lipoproteins with arterial wall proteoglycans

Chapter 5 Lipoprotein sialic acid and neutral carbohydrate content and APG binding

Chapter 6 Mechanisms related to APG-Lipoprotein interaction

Chapter 7 Effect of Evening Primrose Oil (EPO) supplementation on plasma lipids, LDL subfraction profile and LDL susceptibility to oxidative modification in NIDDM

Chapter 8 Conclusions

List of Figures

List of Tables

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Author's Declaration

The work presented in this thesis was performed solely by myself under the supervision of Professor CJ Packard.

Sialic acid determination and Ganglioside studies were carried out in close collaboration with Dr John Millar of the Department of Pathological Biochemistry, Glasgow Royal Infirmary

Vian Amber, April 1997

Dedication

To my loving husband Goran and children, Lazia and Arie

To my parents

Chapter 1

Introduction...

1. 1 Impact of CHD on population

Atherosclerosis is the underlying cause of most cardiovascular disease and cerebrovascular disorders in western populations. Coronary heart disease (CHD) is the largest single cause of death in the UK accounting for approximately 26% of all deaths in England in 1991 (OPCS 1990, HMSO 1992) with even a higher rate in Scotland, Wales and Northern Ireland. It accounts for 2.5% of the total National Health Service expenditure. In the United States over half a million people die every year from this cause. Many contributory or risk factors associated with higher rates of CHD have been identified. Until the middle of the 20th century age was considered to be the major determinant of atherosclerosis. However, the absence of the disease at autopsy in many individuals of all age groups is clear evidence that atherosclerosis should be regarded as a disease and not an inevitable consequence of ageing, even though the risk of CHD mortality increases with age. The macroscopic changes of atherosclerosis was first recognised by Leonardo da Vinci (1452-1519) (Keelê 1952) and it was Marchand who coined the term "atherosclerosis" (Aschoff 1924). Several theories were proposed to explain the pathogenesis of atherosclerosis, The response to injury by Virchow (1856) and "the thrombogenic" theory by Rokitansky (1804-1878), both of which recognised lipid accumulation to be involved in the process of atherosclerosis development.

The abundance of cholesterol in human atherosclerotic lesions was well known after the turn of the century (Windaus 1910). Anitschkow propounded the "lipid hypothesis" theory and presented the first evidence associating dietary cholesterol with the development of atherosclerosis (Anitsckow & Chalatow 1913). Following a series of key studies indicated the importance of cholesterol as a predictor of risk (Steiner & Domanski 1943, Gertler *et al* 1950). Another striking observation was the occurrence of coronary artery occlusions in males more than in females (Master *et al* 1939). Framingham study and other prospective studies established the three major risk factors as high serum cholesterol, hypertension and smoking (Dawber *et al* 1959, Dawber 1980). This was further confirmed by extensive data from the Pooling project (Pooling project research group 1978), which added diabetes mellitus, and the MRFIT study (Stamler *et al* 1986). The high incidence of CHD in affluent societies indicates that diet and life style, e.g. unhealthy eating, drinking, smoking and lack of exercise (McKeown 1979) are of critical importance in the causation of CHD. It was first pointed out by Keys that variation in the frequency of CHD between countries, could be attributed to differences in the dietary fat intake (Keys 1957, 1970). In support of this, a higher incidence of CHD was found among Japanese migrants in California who adopted the US diet compared to Japanese in Japan on a traditional fish and rice diet (Keys *et al* 1957). It was also shown that the type of fat ingested was important in determining serum cholesterol (Kinsill *et al* 1952). Diets rich in vegetable oil were shown to lower serum cholesterol level (Kinsill *et al* 1952). A number of intervention trails have demonstrated that lowering blood cholesterol on a population basis leads to lowering of the incidence of ischemic heart disease (LRCP 1984a, 1984b, Frick *et al* 1987).

The incidence of CHD varies and changes constantly in different countries all over the world. Eastern European countries together with Scotland and Northern Ireland lead the international league table of CHD mortality rates (Marmot 1988). Japan has the lowest rate due to low blood cholesterol level in their population. It is noteworthy that a low CHD rate exists in Japan despite high rates of hypertension and smoking.

1.2 Risk factors for CHD

One of the goals in the management of CHD is the identification of patients at risk. This requires assessment of the different factors associated with the disease process. The aetiology of CHD is multifactorial and risk factors can be classified as modifiable or non-modifiable (Table 1.1).

Table 1. 1

Factors associated with increased risk in coronary heart disease

Modifiable	***Non-modifiable***
Hyperlipidemia: ■ Cholesterol ■ Triglyceride ■ Lipoproteins	Family history
Smoking	Diabetes
Hypertension	Age
Obesity	Gender (male)
Lack of physical activity	

During the last 15-20 years CHD mortality rates have fallen in England by 20% (HMSO1992), in Scotland by 10% (Marmot 1988) and in the USA CHD rates have halved in the last 20 years due to improved risk factor status (Stamler and Stamler 1984). In Japan the dramatic fall in hypertension seams to be the major cause behind the large change in CHD rates (Ushima *et al* 1987). In addition, understanding how non-modifiable risk factors influence the occurrence of the disease may suggest other preventive strategies. The greater occurrence of coronary artery occlusion in males than females below the age of 40 (MaGill & Stern 1979) and the decrease in the sex difference with increasing age is suggestive of a relationship to hormonal changes. Evidence from uncontrolled trials indicates that oestrogen replacement therapy in postmenopausal women reduces the incidence of CHD (Barrett- Connor & Bush 1991).

Since CHD has a multifactorial aetiology the individual impact of risk factors must be gauged by multivariate analysis. Further, in the process of managing individuals, the effect of coexisting risk factors should be taken in to account.

For example, a hypertensive man who has a mild elevated cholesterol carries a greater risk than a male with a normal or low blood pressure with a high cholesterol concentration due to the multiplicative effect of risk factors.

The initial observations of the association between high serum cholesterol level and increase risk of CHD was made on the basis of total cholesterol. With the development of analytical centrifugation (Lindgren *et al* 1951, Gofman *et al* 1950) and paper electrophoresis (Less & Hatch 1963), it was shown that the distribution of cholesterol among lipoproteins, which are complex molecules responsible for the transport of lipids around the body in plasma, also predicted risk. There follows a description of the structure, heterogeneity and metabolism of lipoproteins, particularly of apoB100 containing lipoproteins. Finally, the role of a specific lipid and lipoprotein abnormalities in the pathogenesis of atherosclerosis is discussed.

1.3 Lipoprotein structure

Five main classes of lipoproteins, chylomicrons (CM), very low density lipoprotein (VLDL), intermediate density lipoprotein (IDL), low density lipoprotein (LDL) and high density lipoprotein (HDL), are defined based on their hydrated density (g/ml), electrophoretic mobility, particle size and flotation rate (Mills *et al* 1984). The last property is determined in the analytical centrifuge at a background density of 1.063 or 1.21 g/ml. Flotation rates are given in Svedberg units (Sf), one unit Sf = 10^{-13} cm/s/dyne/g. The physicochemical properties of apoB100 containing lipoproteins are illustrated in Table 1.2.

While all lipoproteins have the same basic pseudomicellar structure each class has a distinct composition, function and metabolic behaviour. They all contain a core of neutral lipid consisting cholesteryl esters (CE) and triglyceride (TG) and a surface shell of polar lipid (unesterified cholesterol and phospholipids) and protein (apolipoprotein). The latter are required for the synthesis and secretion of specific lipoproteins and interact with specific cell surface receptors that are responsible for the removal of the lipoprotein from plasma.

Table 1.2

Physicochemical properties of apoB_{100} containing lipoproteins.

Properties	*VLDL*	*IDL*	*LDL*
Density range g/ml	0.94-1.006	1.006-1.019	1.019-1.063
Flotation rate Sf	60-400	12-20	0-12
Particle diameter nm	30-80	25-35	18-27
EP-mobility	Pre β	slow Pre β	β
Main apolipoproteins	B_{100}, C, E	B_{100}, C, E	B
Chemical composition			
% FC	7	9	8
% EC	12	29	42
% TG	55	23	6
% PL	18	19	22

VLDL, very low density lipoprotein; IDL intermediate density lipoprotein; LDL, low density lipoprotein; Ep mobility, electrophoretic mobility; %FC, %EC, %TG, %PL, percentages of free cholesterol, esterified cholesterol, triglyceride, and phospholipid within the lipoprotein respectively; C/P ratio;, cholesterol to protein ratio.

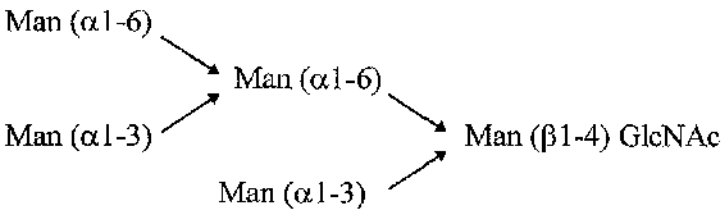
Among the four major categories (apoA, apoB, apoC, and apoE) of these apolipoproteins, apoB_{100} has the highest molecular weight (550 kDa), with 4536 amino acid residues (Yang *et al* 1986, Chen *et al* 1986). It is important in maintaining the structural integrity of the lipoprotein, and serves as a ligand, through its positively charged arginine and lysine aminoacid residues, for the cell surface LDL receptor (Brown & Goldstein 1986, Shepherd *et al* 1979, Brown *et al* 1975).

ApoB is a glycoprotein containing 5-9 % carbohydrate, consisting of galactose, mannose, glucosamine and sialic acid (Ehenholm *et al* 1972, Marshall & Kummerow 1962, Swaminathan & Aladjem 1976, Vauhkonen *et al* 1985). Mannose residues constitute the highest percentage, 37% of the total monosaccharides amounting up to 10% of the total high mannose oligosaccharides in serum (Vauhkonen *et al* 1985) (Fig 1.1). The presence of terminal mannopyranosyl and glucopyranosyl or sterically related residues, on

the carbohydrate moiety of LDL, gives it the property of binding avidly to the sugar lectin "Concanavalin-A" (Con-A) (McConathy & Alaupovic 1974). Con-A is a phytohaemagglutinin isolated from Jack beans (Fig 2.5, chapter 2) which specifically binds α-D mannopyranosyl and α-D glucopyranosyl .

Figure 1.1

Mannose Structure of apoB from human LDL



$ApoB_{100}$ is synthesised mainly by the liver and intestine and occurs in VLDL, IDL and LDL. $ApoB_{48}$, which is half the size of $apoB_{100}$, is produced in the intestine by editing of the mRNA transmitted from the apoB gene and occur only in chylomicrons and their remnants. ApoA (AI, AII) are synthesised in the liver and intestine and are carried on CM and HDL. Finally apoE, present in three isoforms (apoE2, apoE3, and apoE4) and apoC (CI, CII and CIII) are synthesised in the liver and are found in CM, VLDL, IDL and HDL. ApoE is another ligand for the receptor-mediated uptake of cholesterol in tissues (Mahley 1988) while apoCII and CIII are regulators of lipoprotein lipase activity.

1.4 Lipoprotein heterogeneity

Heterogeneity can be readily demonstrated in the major lipoprotein classes by high resolution techniques such as density gradient ultracentrifugation which subfractionate lipoproteins into relatively discrete components. Investigation of this phenomenon has improved our knowledge of the role of lipoproteins in the pathophysiology of CHD. It was first suggested by Gofman *et al* that the occurrence of atherosclerosis was more predicted by the existence of specific

lipoprotein subclasses than by total blood cholesterol (Gofman *et al* 1950). Evidence suggests that lipoprotein heterogeneity may have a genetic basis and be influenced by environmental factors (Gaw *et al* 1990).

1.5 Very low density lipoprotein (VLDL)

VLDL particles (30-100nm particle diameter) are apoB containing triglyceride rich particles isolated from plasma at a density of 1.006 g/ml (Table 1.2). VLDL consists of heterogeneous particles (Streja *et al* 1977) whose size and lipid composition may vary in response to changes in nutritional status (Hamilton 1983). They can be isolated from plasma by cumulative flotation density gradient centrifugation into a large VLDL1 and a small VLDL2 fraction (Lindgren *et al* 1972). VLDL are synthesised in the liver and are mainly responsible for the transport of endogenous triglyceride in plasma. A small proportion of VLDL may be also synthesised in the small intestine to act as a vehicle for the reabsorption of the endogenous cholesterol and fatty acids of biliary origin. VLDL also contain carbohydrate associated with its lipid as well as its protein components. The function of the carbohydrate moiety has not been fully illustrated. However, it may have a significant influence on VLDL and LDL metabolism and their subfraction profile, since LDL is produced by delipidation of VLDL (discussed below).

1.6 Intermediate density lipoprotein (IDL)

These particles are formed during the conversion cascade of VLDL to LDL (discussed below), hence the term IDL. Sometimes they are called VLDL remnants. They are smaller in size than VLDL (25-35nm) (Table 1.2) can be isolated at a density 1.006-1.019 g/ml (Herbert *et al* 1978) and a flotation rate of S_f 12-20. They contain apoB100, apoC and apoE and show a slow pre B mobility on agarose gel electrophoresis. They have been shown to consist of two subpopulations (IDL1&IDL2) on gradient gel electrophoresis (Musliner *et al* 1986).

1.7 LDL structure

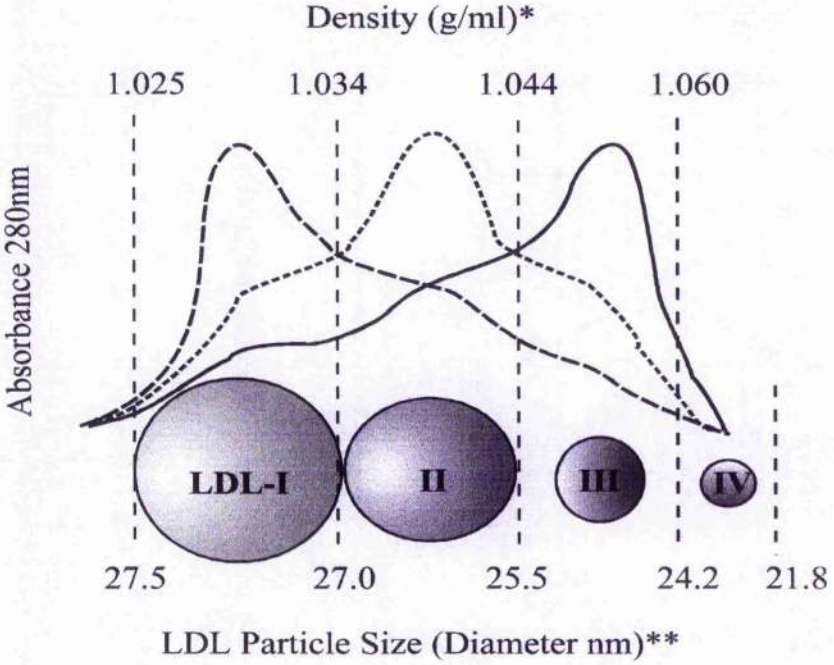
LDL is the main cholesterol carrying lipoprotein in plasma. The physicochemical properties of which are shown in Table 1.2. The lipid core consists mainly of cholesteryl ester and little triglyceride. Its major protein component is apoB_{100} It is produced either directly by the liver (Gaw *et al* 1993, 1995) or indirectly by delipidation of VLDL through IDL (Nicholls & Lewis 1980) (Discussed below). LDL consists of a heterogeneous population of particles, distinct in size and floatation rate which are distributed over a density range of 1.019-1.063 g/ml. LDL heterogeneity was not described until the early eighties where Burke and Krauss were among the first to provide evidence for the existence of distinct LDL subclasses that can be identified by ultracentrifugal and gel electrophoretic techniques (Krauss & Burke 1982). These subclasses are present in both normal and hyperlipidemic patients (Austin *et al* 1988). Based on non-denaturing gradient gel electrophoresis performed using 2-16% polyacrylamide gel, two distinct LDL subclass phenotypes denoted subclass pattern "A" and "B" have been described (Austin *et al* 1988). LDL subclass pattern A was characterised by large buoyant LDL particles with a peak diameter of more than 255°A and LDL subclass pattern B had small dense LDL particles with the peak diameter of less than 255°A. ApoB is reported to be the result of a single dominant gene which is expressed in 15- 25% of population (Austin & Krauss 1986). The regulation and formation of these LDL subclass patterns are governed by compounding influences of environmental and genetic factors. With the development of a new centrifugation procedure Griffin *et al* were able to isolate three LDL subfractions directly from plasma analogous to those seen on gradient gel electrophoresis (Griffin *et al* 1990) (Figure 1.2) In this procedure, a buoyant LDL-I (d= 1.025-1.034 g/ml) (particle diameter =27-27.5nm), an intermediate LDL-II (d= 1.035-1.044 g/ml) (particle diameter =25.5-27nm) that represent the main LDL subclass in terms of lipoprotein mass in adult males and a small dense LDL-III particle (d= 1.044-1.060 g/ml) (particle diameter =24.25.5nm) were isolated. The last is depleted in cholesteryl ester and relatively enriched in protein (Griffin *et al* 1990). A smaller particle

(LDL-IV) with a smaller particle diameter <24 nm is seen in hypertriglyceridemic patients (Musliner & Krauss 1988, Griffin *et al* 1990, Griffin 1995) (Fig 1.2).

A profile predominated by LDL-I (phenotype A) is mainly present in young healthy subjects and premenopausal females. High LDL-III levels (phenotype B) are found in males, post menopausal female, subjects with hypertriglyceridemia and coronary artery disease (CAD) positive patients (Griffin *et al* 1990, Austin *et al* 1990, Watson *et al* 1994).

Figure 1.2

LDL subfractions isolated by density gradient centrifugation.



**LDL subclass density profile obtained by density gradient ultracentrifugation and representative of a typical normal healthy female (-.-.-), male (- -) and coronary artery disease patient (____) **LDL: particle size as determined by 2-16% gradient gel electrophoresis.* *(Griffin et al 1990)*

1.8 Lipoprotein metabolism

1.8.1 Exogenous lipid transport

Daily each individual takes approximately 120g of fat in the form of cholesterol and triglyceride. Both lipids are hydrolysed if necessary by the action of pancreatic enzymes, mixed with phospholipid cholesterol and bile acids from bile in the intestinal lumen prior to being absorbed mainly in the duodenum and upper jejunum (Danielson & Sjovall 1975). All the glyceride and about half of the cholesterol which is reesterified by the action of acyl coenzyme A cholesterol acyl transferase (ACAT) (Norum *et al* 1983), (the other half is lost in the faeces) are packaged within intestinal enterocytes into large TG-rich chylomicron particles to be secreted into the blood stream via the thorasic duct. Chylomicrons contain apoA1 $apoB_{48}$. The latter has been shown to represent the N-terminal half of $apoB_{100}$ and therefore lacks the receptor binding domain (Marcel *et al* 1982, Hospattaker *et al* 1986) which is a prerequisite for the uptake of apoB100 containing lipoproteins by the liver and cells. Once in plasma, chylomicron particles acquire apoC and apoE from HDL (Havel *et al* 1973) (Fig 1.3). Both of these apolipoproteins are required for subsequent processing of chylomicrons by lipoprotein lipase, the activity of which is modulated by apoCII and apoCIII (Ginsberg *et al* 1986).

Endothelium bound lipoprotein lipase in adipose tissue and striated muscle hydrolyses the chylomicron triglyceride core into fatty acids and glycerol. Redundant phospholipid and protein from the surface of the particle are shed to HDL (Shaefer *et al* 1978) and the process result in the production of CM remnants. Thus, postprandially two lipoprotein species appear CM and CM remnants. The cholesteryl ester content of the latter is obtained by transfer of the lipid from LDL and HDL in exchange for triglyceride (Nicholl & Smith 1965) facilitated by cholesteryl ester transfer protein (CETP). CM remnants are recognised and taken up through specific apoE receptors by the hepatocytes, thereby delivering dietary cholesterol into the liver (Sherrill *et al* 1980, Mahley *et al* 1981).

No circulating daughter particles are formed from the chylomicron remnants therefore, their contained lipid is used in the synthesis and secretion of VLDL (Fig 1.3).

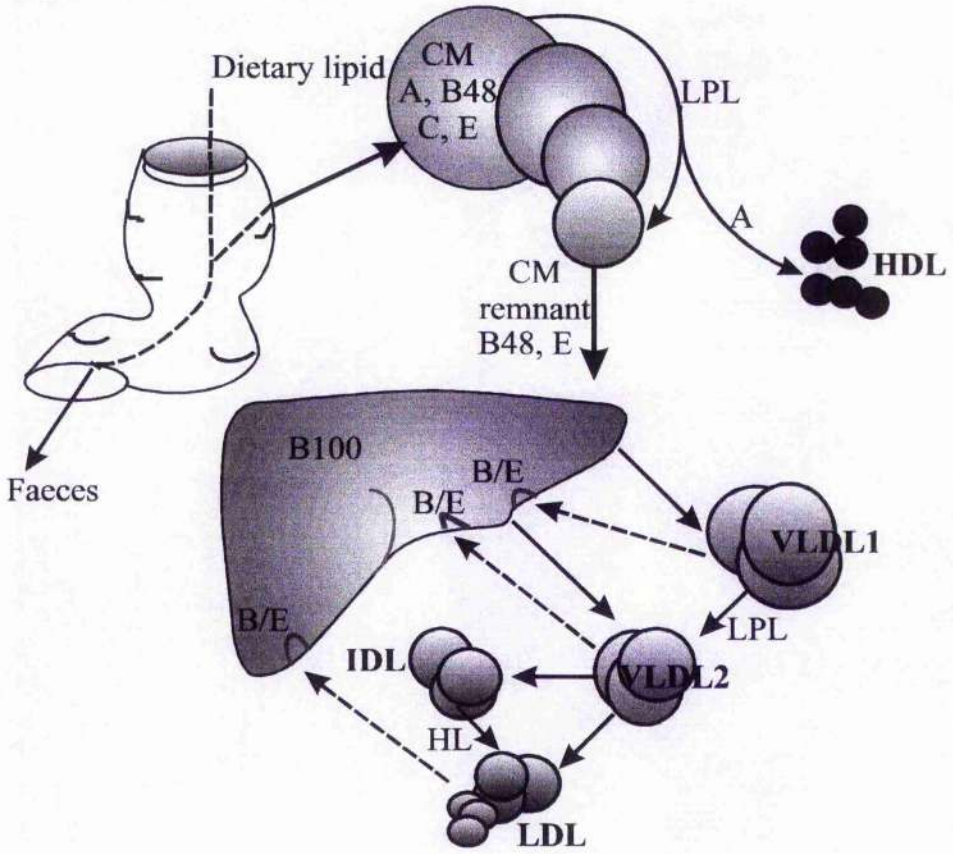
1.8.2 Endogenous lipid transport

The liver acquire exogenous cholesterol and TG, by the chylomicron pathway but can also synthesise phospholipid and TG either from fatty acids released from adipose tissue or following the generation of fatty acids from small molecular weight precursors (Barter *et al* 1972). Similarly cholesterol is produced by liver cells by a metabolic pathway where key pacemaker enzyme is 3-hydroxy 3-methylgluteryl Coenzyme A reductase (HMG-Co A reductase). A significant proportion of intrahepatic lipid exported in association with newly synthesised apoB_{100} particles, which undergo glycosylation before being secreted (Hamilton 1983). The rest are stored in the cytoplasm or secreted in the bile.

Nascent VLDL particles are heterogeneous encompassing a size range of 40-70nm. Their apoB100 is bound with such high affinity that it remains with a single lipoprotein particle from secretion to ultimate endocytosis. In plasma, these particles gain more apoE and apoC from HDL, required for their delipidation cascade. They contain a higher phospholipid and TG with less esterified cholesterol than the plasma counterpart (Hamilton *et al* 1991). Large TG-rich VLDL enter a metabolic cascade similar to that of CM. They under go lipolysis by the action of lipoprotein lipase a process which requires apoCII as a cofactor (Nilsson-ehle *et al* 1980). The rate of hydrolysis of triglycerides is also regulated by the inhibitory effect of apoCIII. During LDL metabolism CE are acquired from other lipoproteins such as HDL, in exchange for TG, by the process of neutral lipid exchange mechanism under the influence of CETP. Delipidation leads to the generation of VLDL remnants and IDL particles (Fig 1.3). In normal individuals, most of the TG depleted remnant particles, rich in apoE, are endocytosed by the liver by LDL receptor mediated mechanisms (Shepherd & Packard 1987). The remainder, are further delipidated to LDL by the hepatic lipase activity (Nicholl & Lewis 1980) (Fig 1.3).

Figure 1.3

Lipoprotein metabolism



Schematic diagram of lipoprotein transport pathways showing the interrelationship between exogenous and endogenous lipid metabolism. LPL; lipoprotein lipase; HL, hepatic lipase; A, apoprotein A; B; apoprotein B; C, apoprotein C

It is known that the affinity of lipoprotein lipase is greater for large, TG-rich particles than for smaller remnants, whereas hepatic lipase, which has both triglyceride lipase and phospholipase activities, seems to favour smaller VLDL and IDL particles. Therefore, the absence of lipoprotein lipase results in accumulation of particles of flotation rate greater than Sf 100 that contain both apoB100 and apoB48 (Behr *et al* 1981, Goldberg *et al* 1982). While, antibody induced inhibition of hepatic lipase in cynomolgus monkeys (Goldberg *et al*

1982) causes the accumulation of smaller VLDL and IDL, and LDL becomes relatively enriched in triglyceride (Demant *et al* 1988). In conditions that lead to hypertriglyceridaemia, such as impaired clearance of postprandial lipoproteins, obesity and insulin resistance, failure of hormone sensitive lipase suppression is believed to promote the release of fatty acids from adipose tissue into the circulation (Frayn 1993) and thereby increase production of endogenous VLDL1 (Fisher *et al* 1993, James & Pometta 1991). Both CM and VLDL1 are removed from plasma by a common saturable pathway regulated by LPL (Bjorkegren *et al* 1996). The preferential affinity of LPL for CM increase the residence time of VLDL in plasma during the postprandial phase and so increases its availability for neutral lipid exchange by CETP. This involves the transfer of TG from both CM and TG-rich VLDL1 to LDL in exchange for CE, generating a TG-rich LDL. The latter undergo further reduction in size by the activity of increased hepatic lipase (Karpe *et al* 1993, Watson *et al* 1994, Tan *et al* 1995) resulting in the production of small dense LDL (Karpe *et al* 1993).

Since VLDL is precursor to LDL formation, heterogeneity in VLDL particles may account for the production of different LDL subclass patterns. It is not known which particular structural features regulate metabolic heterogeneity in LDL and LDL protein composition and conformation is likely to be important. The high mannose glycosylation of apoB synthesised in the liver might also account for differences in the carbohydrate structure of LDL synthesised by direct or indirect mechanisms as mentioned above.

1.9 LDL subfraction profile in relation to plasma lipids and lipoproteins

Large buoyant LDL-I (subclass pattern A), shows a strong correlation with the concentration of large HDL2 and HDL cholesterol, and an inverse relationship correlated with the plasma triglyceride, VLDL and small dense LDL-III (McNamara *et al* 1992, Williams *et al* 1990, Watson *et al* 1994, Griffin *et al* 1995). In contrast, small dense LDL-III (subclass pattern B) shows a strong positive association with plasma triglyceride (Austin *et al* 1990, Griffin *et al* 1994) and an inverse association with HDL cholesterol. Plasma triglyceride level has been shown to be the most important determinant factor in the

distribution of these LDL subclass patterns (Campos *et al* 1992, McNamara *et al* 1992, Coresh *et al* 1993, Griffin *et al* 1994) independent of age, sex, diabetes, high density lipoprotein (HDL) and LDL cholesterol level in plasma. A further key factor determining LDL size is hepatic lipase activity. It is higher in male than female subjects (Tikkanen & Nikkila 1987, Karpe *et al* 1993, Watson *et al* 1994) and its activity related to LDL-III concentration (Tan *et al* 1995). Further, hepatic lipase deficiency has been shown to be associated with the accumulation of large LDL-I (Auwerx *et al* 1989), LDL-III is believed to be generated by transfer of triglyceride into LDL-II and subsequent lipolysis of the particle by hepatic lipase (McNamara *et al* 1987, Zambon *et al* 1993) which has been shown to be responsible for 30% of the variation (Watson *et al* 1994).

1.10 Plasma lipids and lipoproteins and relative risk of CHD

1.10.1 Cholesterol as a coronary risk factor

For many years cholesterol and its esters, phospholipids and fatty acids have been known to be prominent components of atheromatous lesions. The cholesterol hypothesis states that raised blood cholesterol is directly related to CHD and that lowering cholesterol by diet and drugs reduces the risk of CHD (LRCP 1984a, 1984b). Epidemiological studies (NCEP 1991, Davis *et al* 1990) suggest that for every 1% increase in total cholesterol the risk of CHD rises by 2%. This has been supported by the results of clinical trials (LRCP 1984a ;1984b), which have shown that for every 1% reduction in cholesterol levels, risk decreases by 2%. Several intervention trials have attributed the reduction in CHD event rate in treated subjects to the elevation in HDL cholesterol as well as the decrease in LDL and total cholesterol (LRCP 1984a, 1984b, Frick *et al* 1987). This has led to the formulation of recommendations for the treatment of hyperlipidaemia. However, lower blood cholesterol levels are associated in epidemiological studies with higher rates of cancer (Hiatt & fireman 1986, Isles et al 1989) and stroke (Iso *et al* 1989) and some have suggested that the existence of those relationships should caution against aggressive reduction in cholesterol levels. However, recent data on cholesterol lowering in the West of Scotland (WOSCOPS) (Shepherd *et al* 1995) and the 4S study (Scandanavian

Simvastatin Survival study 1994) have clearly shown that the lowering of plasma cholesterol levels brings benefit in terms of reduction of coronary disease without an increase in morbidity and mortality related to non cardiovascular factors.

1.10.2 Triglyceride as coronary risk factor

The vast majority of trials have focused on the significance of serum cholesterol as a risk predictor. This has diverted attention from the other major lipid "triglyceride" (TG) circulating in plasma. Results from most case control and prospective studies have shown in univariate analysis (Castelli *et al* 1977, Simons 1986, Barrett-Connor & Khaw 1987) an association between triglyceride and CHD. The relationship is lost when other risk factors are taken into account, notably HDL cholesterol concentration with which triglyceride level is strongly, inversely related (Castelli *et al* 1977, Castelli 1994, Griffin *et al* 1994). The reason behind this might be the existence of a metabolic link between abnormalities of TG and HDL, so that they cannot be treated as independent covariates. Fasting levels of plasma triglyceride level may not be the best index of risk. Most of the lipid is found in bloodstream during the postprandial period and postprandial lipaemia has been shown to be enhanced in patients with CHD (Patsch *et al* 1992, Groot *et al* 1992). Zilversmit suggested that atherosclerosis is a postprandial phenomenon (Zilversmit 1979). Postprandial increases in TG are associated mainly with chylomicrons and their remnants. CM remnant particles in postprandial lipaemia have been shown in cell culture studies to promote rapid accumulation of cholesterol within arterial cells (Ellsworth *et al* 1986). While in-vivo an increased plasma concentration of chylomicron remnant particles as determined by measuring $apoB_{48}$: $apoB_{100}$ ratio in the density ($d<1.006$) fraction of postprandial plasma has been demonstrated in those with CHD.

There is evidence that TGRL particles may be atherogenic *per se*, but also that elevation in plasma triglyceride might exert an indirect influence on CHD risk, by promoting structural changes in LDL and HDL in particular by perturbing the subfraction distribution within these density classes (Fisher *et al* 1993,

James & Pometta 1991). This is reflected in the dyslipidemic syndrome termed atherogenic lipoprotein phenotype (ALP) with its lipid and lipoprotein abnormalities (Austin *et al* 1990) as discussed in section 1.10.4.

1.10.3 LDL subclass pattern, LDL carbohydrate and relative risk of CHD

There is now incontrovertible evidence to link plasma low density lipoprotein cholesterol level to the development of coronary heart disease . However not all LDL may be equally atherogenic. A predominance of small dense LDL has been consistently associated with an increased risk of CHD and may be directly involved in the atherogenic process via a number of. Mechanisms. A size profile in which small dense LDL is predominant is associated with a 3-fold increase in risk of CHD (Austin *et al* 1988). Griffin *et al* (Griffin *et al* 1994) demonstrated an even greater relative risk (7-fold) with a plasma small dense LDL >100mg/dl.

LDL kinetic studies have provided evidence for the existence of two metabolic pools with the lipoproteins, one with "fast" clearance and another which is cleared more slowly from plasma (Caslake *et al* 1992). Small dense LDL appears to arise in subjects with slowly catabolised LDL. Thus, the atherogenicity of small dense LDL could be partly attributed to its prolonged residence time in circulation. This increases the opportunity for infiltration into the arterial wall and trapping by cellular and extracellular components in the arterial intima-media (Camejo *et al* 1990). Furthermore, small dense LDL has also been shown to be more susceptible to oxidative modification in-vitro (de Graaf *et al* 1991) which would promote its internalisation into cells involved in the plaque formation.

Earlier work has proposed that the carbohydrate moiety of apoB may be linked to the atherogenicity of the lipoprotein (Swaminathan & Aladjem 1976).

A strong relationship has been demonstrated between LDL subclass pattern and both the quality and the quantity of carbohydrate on the lipoprotein (LaBelle & Krauss 1990). In this study, the lipid and lipoprotein ($apoB_{100}$) components of LDL from subjects with LDL subclass pattern A were shown to contain more carbohydrate compared to LDL from pattern B subjects (LaBelle & Krauss 1990). The variation in the lipid carbohydrate per particle may be explained by

the difference in the total lipid mass between large and small dense LDL but this would not apply to variation in the carbohydrate associated with the LDL protein. Different LDL subclasses also differed in their sialic acid content (LaBelle & Krauss 1990), where less sialic acid was associated with the glycolipid, rather than the glycoprotein in LDL from subjects with pattern B. LDL from CHD patients has been shown to have lower sialic acid content compared to LDL from normal volunteers (Tertov *et al* 1993). This sialic acid poor (SAP-LDL) was shown to induce 2-4 fold increase in intracellular lipid accumulation in cultured cells compared to the sialic acid rich (SAR-LDL) LDL from healthy volunteers (Tertov *et al* 1993). Desialation in-vivo of LDL from patients with atherosclerosis was suggested to confer potentially atherogenic properties on this lipoprotein (Orekhov *et al* 1989, 1991). All of these were supported by the finding that in-vitro desialation of LDL by treatment with neuraminidase and LDL glycation in diabetic patients increased the lipoproteins capacity to induce intracellular lipid accumulation (LaBelle & Krauss 1990, Sobenin *et al* 1991). In addition , increased reactivity of LDL from survivors of myocardial infarction towards arterial wall proteoglycans (APG) (Linden *et al* 1989) was suggested to be due to the presence of small dense LDL fraction with a lower sialic acid content and a higher isoelectric point (Camejo *et al* 1985b). Sialic acid are negatively charged residues, its presence reduces the net positive charge on the lipoprotein particle thus render the particle less susceptible to interact with the negatively charged APG, thereby increasing potential atherogenicity of the lipoprotein. This is supported by the increase in APG-LDL interaction after desialation of LDL by neuraminidase treatment (Camejo et al 1985b). The role of carbohydrate and sialic acid content of LDL subfractions and apoB containing lipoproteins in relation to its interaction with arterial wall proteoglycan binding is examined in this thesis.

1.10.4 ALP , insulin resistance, obesity and CHD risk

The dyslipidemic syndrome of atherogenic lipoprotein phenotype (ALP) is a term which describes abnormalities in plasma lipoproteins characterised by a predominance of small dense LDL (phenotype B), moderately raised plasma

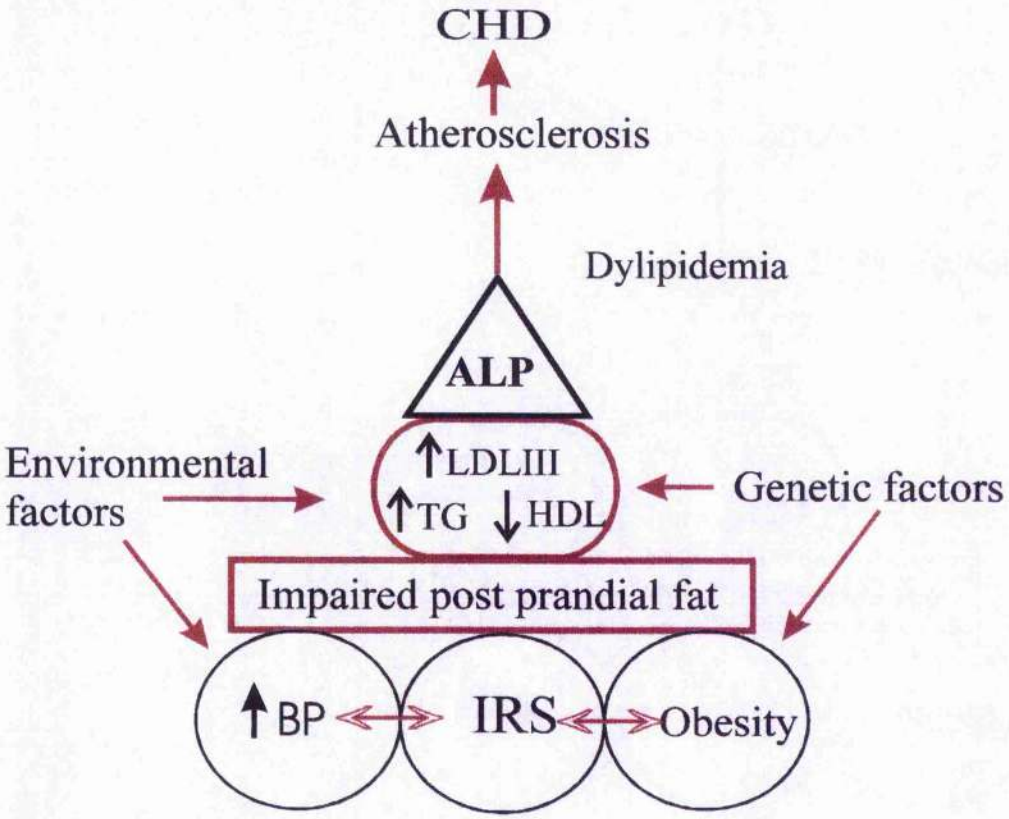
triglyceride level (circulating as TG-rich very low density lipoprotein) and low HDL cholesterol (Austin *et al* 1990). ALP is associated with 3-7 fold increase risk of CHD. It is a recognised feature of the insulin resistance syndrome (Reaven 1988, Reaven *et al* 1993a) and associated with enhanced postprandial lipaemia. It is believed that these metabolic disorders confer increased risk of CHD through their link with the ALP (Fig 1.4).

There is evidence that insulin resistance exerts its effect as a CHD risk by underlying a cluster of several risk factors such as ALP, abnormalities in clotting mechanisms (increases in fibrinogen and plasminogen activator inhibitor-1), hypertension, central obesity and smoking (Reaven 1988, Laws & Reaven 1993, Despres & Marette 1994, Stern 1995, Davidson 1995). High insulin levels and insulin resistance have been shown to be associated with high total and VLDL triglyceride (through failure of insulin to suppress the activity of hormone sensitive lipase and activate lipoprotein lipase in adipose tissue (Frayn 1993) low HDL cholesterol (Haffner *et al* 1992, Mykänen *et al* 1994), small dense LDL (Reaven *et al* 1993a, Stewart *et al* 1993, Austin & Selby 1995) and postprandial lipaemia (Taskinen 1995). These metabolic disturbances appear to be consequences of the prevailing hypertriglyceridaemia which itself may be caused by insulin promoting VLDL secretion from the liver.

In the past two decades the role of obesity in the causation of CHD has been investigated (Hubert *et al* 1983, Hartz *et al* 1984, Manson *et al* 1987; 1992, Grobbe *et al* 1990, Kissebah & Krakower 1994, Willet *et al* 1995). Although increase in body mass index (BMI) has been shown to be associated with increase risk of CHD (Willett *et al* 1995, Rimm *et al* 1995), the distribution of adiposity in the body estimated by the waist to hip ratio (WHR) was suggested to be a better predictor of CHD risk in both men (Rimm *et al* 1995) and women (Prineas *et al* 1993, Folsom *et al* 1993). A recent refinement has been the demonstration that an increase in visceral fat area is an even better predictor of CHD risk in patients with similar BMI (Nakamura *et al* 1994).

Figure 1. 4

Insulin resistance syndrome and related disorders associated with increased CHD risk through an ALP.



Hypertension and obesity are associated with the syndrome of insulin resistance and all are influenced both by genetic and environmental factors. BP, blood pressure, IRS insulin resistance syndrome.

Obesity appears to be an independent risk factor leading to a number of metabolic alterations that contribute to an increased cholesterol level, but more frequently its major effect is to raise plasma TG level and lower HDL cholesterol (Walton *et al* 1995). It also promotes increase risk of CHD by acting through other risk factors. For example it promotes insulin resistance (Bjortrop 1990, Fukioka *et al* 1991) and hypertension (Garrison *et al* 1996). Decrease in visceral fat has been reported by Kanai *et al* (Kanai *et al* 1996) to be accompanied by a significant fall in blood pressure and the amount of visceral

fat was correlated strongly with blood pressure (Matsuzawa *et al* 1995). This is believed to be through an increase in the sympathetic nerve activity which leads to renal sodium retention and hypertension (Reaven *et al* 1996, Macdonald 1995) in individuals with appropriate genetic predisposition and environmental exposure. Similar mechanism links the association between hypertension and insulin resistance (Swislocki *et al* 1989).

1.11 Pathogenesis of atherosclerosis

Abnormal lipoprotein patterns, mainly of apoB containing lipoproteins have been consistently associated with an increased relative risk of CHD. These patterns of which familial hypercholesterolaemia, familial combined hyperlipidaemia and ALP examples are produced as a result of genetic and environmental factors which affect the synthesis, processing or catabolism of lipoprotein particles. The underlying disease mechanism in CHD is atherosclerosis a term which comes from the Greek "athere" meaning porridge and "Scler" meaning hard.

Initiation of atherosclerotic lesions is dependant on focal accumulation of lipids, mainly cholesterol in the form of cholesteryl ester, and lipoproteins (mainly apoB containing lipoproteins, such as VLDL, IDL LDL and lp(a)) in the arterial intima-media. The presence of these substances causes the migration of blood monocytes to lesion site. These cells ingest the lipid/lipoprotein and retain the cholesterol. If lipid uptake is excessive the monocyte becomes a resident macrophage. Lipid and macrophage recruitment leads to formation of fatty streak. The earliest atherosclerotic lesions. Further pathological changes in which the artery wall is damaged, endothelium though replace the surface of a fatty streak leads to the involvement of the blood haemostatic system. High fibrinogen and white cell counts all appear to accelerate the atherosclerotic process (Getz *et al* 1969). Elevated levels of serum cholesterol (mainly in LDL) promote lesion development through its association with increased intimal influx of lipoproteins (Nordestgaart *et al* 1992, Nordestgaart & Nielsen 1994), through vascular damage, promotion of hypercoagulability and enhanced platelet reactivity (Badimon *et al* 1991). Evidence in support of the direct link

between lesion development and plasma apoB containing lipoproteins is their existence and isolation from atherosclerotic plaque (Mawhinney *et al* 1978, Hoff & Bond 1983, Rapp *et al* 1994) both in human and animal models.

For lipoprotein particles to cause atherosclerosis they need to cross the single-cell- thick membrane, the vascular endothelium to be retained in the intimal subendothelial space. Subsequent modification, internalisation and degradation by cellular and extracellular components of the arterial wall leads to cholesterol deposition and plaque formation. Lipoprotein retention can be influenced either by factors related to the constituents of the arterial wall such as: the vascular endothelial barrier, cellular and extracellular components of the intima-media or structure, function, and modification of different lipoproteins. It is this early step in the formation of lesions that is the principal topic of this thesis.

1.11.1 Endothelial dysfunction and lipoprotein influx into the arterial wall

The contributory role of vascular endothelium in vascular physiology and lesion development has now been well studied. Although endothelial denudation and injury, described in the response to injury hypothesis (Ross *et al* 1977) play an important role mainly in restenosis after balloon injury, it is now clear that atheroma develops in areas with intact endothelial layer (Katsuda *et al* 1992, Dicorleto & Soyombo 1993, Stary *et al* 1994). Nevertheless, changes in the endothelial function such as increased permeability, which can occur as a result of cholesterol load in hypercholesterolaemia (Nielsen *et al* 1992, Fry *et al* 1993, Herrmann *et al* 1994, Wu *et al* 1995) and shear stress induced endothelial alterations (Gibson et al 1993) have been implicated in lesion development. Increased endothelial permeability facilitate the influx of lipoproteins into the subendothelial space. The size of the particle, then, is of critical value to determine which lipoprotein crosses more. Therefore, LDL and particularly small dense LDL are more prone to cross the endothelial barrier (Nordestgaard *et al* 1992). This is followed by enhanced retention of apoB containing lipoproteins in the arterial intima (Falcone *et al* 1984, Schwenke & Carew 1989a, 1989b, Spring & Hoff

1989, Schwenke & Clair 1992). The rate of lipoprotein entry into the arterial wall is not different in lesion susceptible versus unsusceptible sites (Schwenke & Carew 1989b), it exceeds the rate of intimal lipoprotein accumulation (Carew *et al* 1984). Retention, rather than enhanced influx of lipoproteins is a critical pathological event in lesion development.

Endothelial dysfunction has also been shown to cause the release of platelet derived factors which enhance smooth muscle cell proliferation and promote extracellular proteoglycan synthesis leading to lipoprotein entrapment and retention. Proliferating smooth muscle cells can also act as macrophages undertaking lipoprotein internalisation and degradation. Vascular cell adhesion molecule-1 has been shown to be expressed by dysfunctioning endothelial cells. These molecules promote intimal recruitment of human monocyte derived macrophages from plasma leading to foam cell formation (Williams & Tabas 1995). On the other hand, endothelial cell layer may be as important in preventing apoB containing lipoproteins from leaving the intima as permitting their influx into the arterial wall (Nordestgaart 1996).

1.11.2 Arterial wall proteoglycan (APG) binding

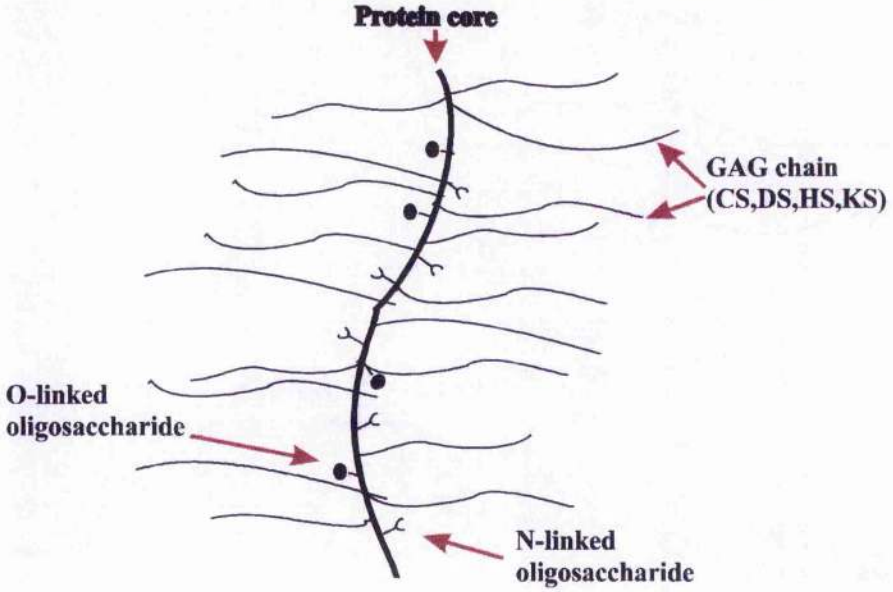
The most directly relevant functional changes related to entrapment and retention of lipoproteins in intima-media, leading to lesion development, is believed to be altered extracellular matrix proteoglycan structure (Mahley & Hawksworth 1965, Wight *et al* 1983, Hoff & Wagner 1986, Camejo *et al* 1990, 1993, Cardoso & Mourão 1994). Selectivity in proteoglycan binding of different lipoproteins is also critical in lipoprotein entrapment e.g LDL binds to a greater extent than VLDL while HDL (Camejo *et al* 1980b).does not bind at all to APG (Camejo *et al* 1993).

Proteoglycans are a group of complex macromolecules present in all living tissue. They consist of a protein core to which glycosaminoglycan (GAG) chains are covalently attached through O and N-linked glycosidic linkages (Fig 1.5). APG constitute only 2-5% of the vascular tissue, yet it has been demonstrated to play an important role in influencing viscoelasticity,

permeability, lipid metabolism, hemostasis and thrombosis (Williams & Tabas 1995, Olivercrona *et al* 1993, Bourin & Lindahl 1993, Wight 1989).

Figure 1.5

A schematic structure of the proposed proteoglycan monomer



The molecule consists of a central protein core to which GAG chains are covalently attached through O and N-linked oligosaccharides. Usualy one type of GAG chain is associated with a single protein core. CS,DS,HS, and KS are chondroitin sulphate, dermatan sulphate, heparan sulphate and keratan sulphate GAG respectively.

Four main families, on the basis of their attached GAG chain, are identified in blood vessels (Table 1.3). Chondroitin sulphate rich proteoglycan (CSPG) are synthesised mainly by arterial smooth muscle cells and also by differentiated arterial wall blood derived cells, such as human monocyte derived macrophages. They have been shown to be responsible for the entrapment of apoB containing lipoproteins mainly LDL (Camejo *et al* 1988, Srinivasan *et al* 1986, Wagner *et al* 1989) and lp(a) (Bihari-Varga *et al* 1988) in the arterial intima-media. Synthesis of CSPG is regulated by different growth factors , such as platelet derived growth factor (PDGF), transforming growth factor β1 (TGF-β1) and interleukin I (IL-I) (Schönherr *et al* 1991, 1993). Heparan sulphate

proteoglycans (HSPG) are synthesised by endothelial cells which also produce dermatan sulphate proteoglycan (DSPG) after modulation of the cell to a migratory state (Wight 1989). These proteoglycans form complexes with LDL through proteins (e.g lipoprotein lipase and apoE) which act as bridging between the lipoprotein and APG (Mulder *et al* 1993, Williams & Tabas 1995). A subset of these proteoglycans may be protective against atherosclerosis by inactivating clotting enzymes (Bourin & Lindahl 1993, Marcum & Rosenberg 1987).

Table 1.3

The four main types of proteoglycan of vascular tissue

proteoglycan	***common name***	***P core (kDa)***	***GAG type***	***Synthesis***
Chondroitin-S	Versican	263	CS	SMC, macrophage
dermatan-S	Decorin, Biglycan	36-38	DS	EC
Heparan-S	Perlican	467	HS	EC
Keratan-S	Lumican	35	KS	

CS,DS,HS, and KS are chondroitin sulphate, dermatan sulphate, heparan sulphate and keratan sulphate GAG respectively. SMC, smooth muscle cells; EC endothelial cells.

Accumulation of proteoglycans in the arterial wall is a characteristic feature of atherosclerosis (Camejo 1982, Berenson *et al* 1984, Wight 1989). Alteration in APG structure has been shown to occur in atherosclerosis, hypertension, diabetes and restenosis (Williams & Tabas 1995). Changes in the nature of proteoglycans such as increased GAG chain length and degree of sulphation increase proteoglycan interaction with lipoproteins (Wagner *et al* 1989, Alves & Mouroa 1988, Sambandam *et al* 199, Camejo *et al* 1993, Cardoso & Mouroa 1994). However, lipoprotein heterogeneity may influence the interaction process to a greater extent.

Since, proteoglycans, mainly CSPG, have been implicated in the pathogenesis of atherosclerosis through interaction specifically with apoB containing

lipoproteins such as VLDL, LDL lp(a) (Vijayagopal *et al* 1981, Camejo *et al* 1985a, 1988, 1989, Bihari-Varga *et al* 1988) but not HDL (Camejo *et al* 1980b), many studies have been focusing on the factors determining this interaction process. The interaction between proteoglycans and lipoproteins can take many forms and depend on several parameters. It has been suggested to be charge related, between the negative charges on APG and positively charged aminoacid sequences on apoB (Camejo *et al* 1988, Olsson *et al* 1993). Lipoprotein particle size (Wagner *et al* 1989), cholesteryl ester enrichment (Avila et al 1978) and low sialic acid content (Camejo *et al* 1985b) have also been shown to be critical in the binding process. Therefore, structure, particularly carbohydrate content which can modulate the net charge of the particle, may be an important determinant of APG-Lipoprotein interaction. Small dense LDL has many features which favour binding and may be the most vulnerable candidate among the apoB containing lipoproteins for APG mediated lipoprotein retention (Fig 1.6).

Other extracellular matrix structures, such as collagen (Jimi *et al* 1994), fibronectin (Labat-Robert & Bihari-Varga 1990) may also play a role in lipoprotein retention.

1.11.3 LDL oxidation

Another process proposed to be central to atherogenesis is lipoprotein oxidative modification by cells generated free radicals (Parathasarathy *et al* 1989, Hurt-Camejo *et al* 1992, Camejo *et al* 1991) within the arterial wall.

It was Goldstein and Brown who first proposed that modification of LDL was a prerequisite for macrophage uptake by acetyl LDL receptor (Goldstein *et al* 1979). A series of studies then followed demonstrating that oxidative modification of LDL, produced by a variety of techniques, led to enhanced uptake by macrophages and cholesteryl ester accumulation (Steinberg *et al* 1989, Steinberg & Witztum 1990). Oxidation of LDL can be induced by incubation with cells (endothelial, smooth muscle cells and macrophages) (Parathasarathy *et al* 1989). These cells can generate free radicals which

mediate the oxidative modification process. Oxidation of LDL also occur by interaction with proteoglycans (Camejo *et al* 1991 , Williams & Tabas 1995) and heavy metal ions such as copper (Cominacini et al 1991, de Graaf *et al* 1991, Tribble *et al* 1995). The latter has been used in-vitro to reproduce the modification process that occur in-vivo and has been shown to produce physical changes in LDL which are remarkably similar to that of oxidised LDL isolated from coronary lesions. The mechanism of LDL oxidation appears to involve lipoxygenase and cycloxygenase systems (Steinberg *et al* 1989) and occurs in different stages. The initial stage (lag phase) involves the consumption of endogenous antioxidants, such as vitamin E, carotinoids and ubiqunol-10. This is followed by a rapid peroxidation of unsaturated fatty acid side chains called the propagation phase (Cominacini *et al* 1991). A variety of reactive aldyhydes generated from lipid peroxidation form covalent bonds with lysine amino groups of apoB, thus producing a negatively charged apolipoprotein. As a consequence of this new epitopes are formed within the modified LDL that are not recognised by the LDL receptor. This leads to preferential uptake of the lipoprotein by scavenger pathways (Parathasarathy *et al* 1992) and ultimately deposition of LDL cholesteryl ester in coronary plaque (Steinberg *et al* 1989). In addition oxidised LDL has been shown to have a number of potentially atherogenic properties. These include cytotoxicity affecting endothelial cells, promotion of expression of cell adhesion molecules on endothelial cell surface, chemotaxis of monocytes (Parathasarathy *et al* 1992). All these together demonstrate the important role of LDL oxidation in promoting atherosclerotic lesion progression from fatty streaks to advances plaque formation.

The extent of lipid peroxidation has been determined by various measures of antioxidants, lipid peroxides, reactive aldehydes, such as thiobarbituric acid reactive substances (TBARS) as well as fluorescence spectroscopy. The latter has been used as a convenient method for detecting fluorophores generated from the oxidatively modified protein moiety and has been validated by direct comparison with other measures of oxidation (Cominacini *et al* 1991).

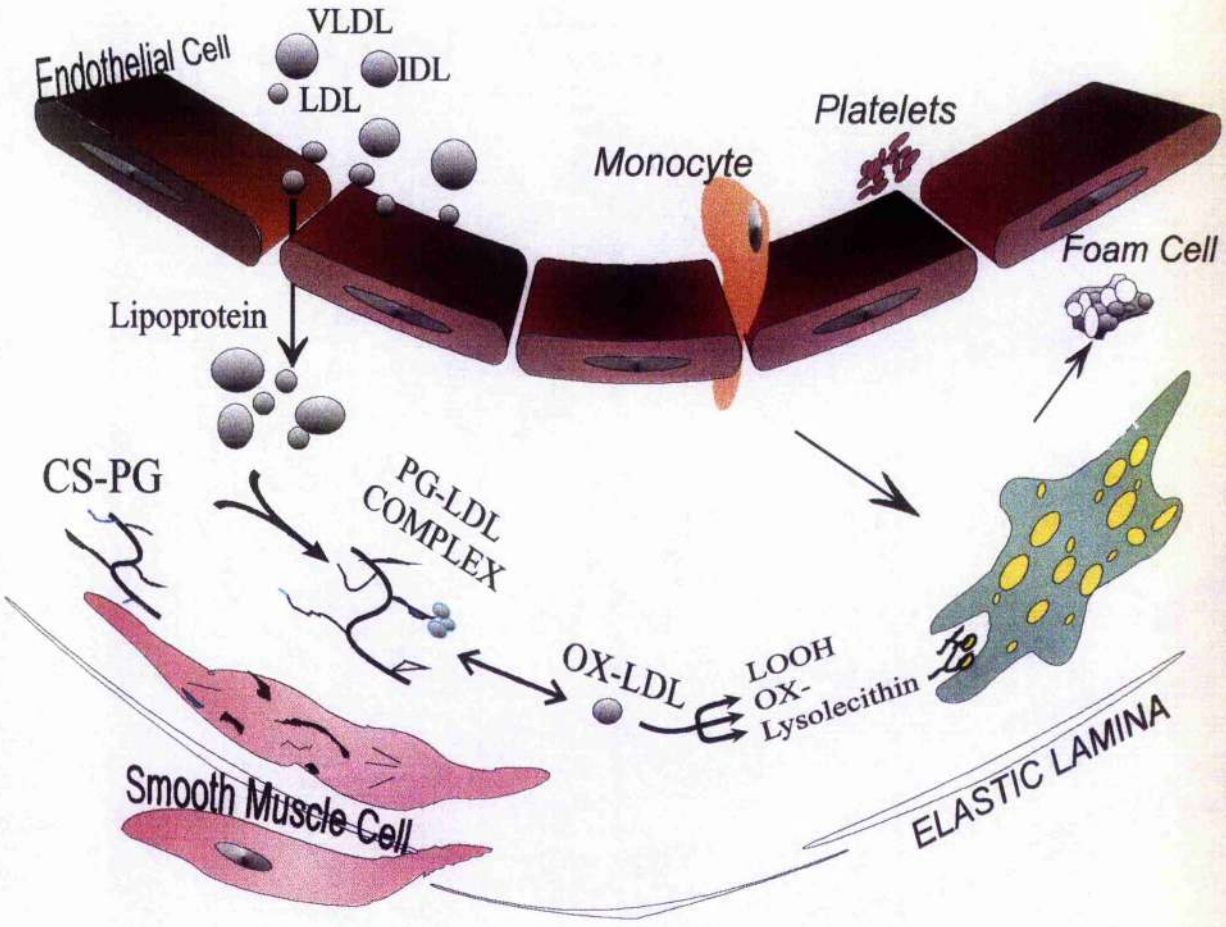


Figure 1.6

Pathophysiology of atherosclerosis: an increase concentration of small dense LDL, such as seen in an ALP, enhances the influx of these particles into the arterial wall by increasing endothelial permeability. Owing to their small size these particles cross the endothelial barrier more readily. Once in the arterial subendothelial space, they bind with a greater affinity to CSPG. This promotes oxidative modification of the LDL particles on the one hand, and on the other hand these complexes are rapidly taken up by macrophages via both LDL and scavenger receptors leading to foam cell formation

1.11.4 *Determinants of LDL oxidation*

Susceptibility to LDL oxidation varies among individuals and is enhanced in various forms of dyslipidemia. Increased LDL oxidation has been reported in association with elevated serum cholesterol (Maggi *et al* 1994) (Type IIa

hyperlipoproteinemia (Cominacini *et al* 1993) and familial hypercholesterolemia (Lavy *et al* 1991)). Antioxidant levels such as alpha-tocopherol, the most important antioxidant in LDL, and the modulatory effects of factors such as polyunsaturated fatty acid (PUFA) content in LDL might be responsible for variation in oxidation in different individuals (Tribble 1995). PUFA are known to promote oxidation and a low ratio of alpha-tocopherol:PUFA in hyperlipidemic individuals has been attributed to LDL enrichment with arachidonic acid (Lavy *et al* 1991, Cominacini *et al* 1993). In addition, in-vitro LDL oxidation studies have shown that LDL enrichment with linoleic acid (Reaven *et al* 1994) or arachidonic acid (Lavy *et al* 1991) leads to increased oxidative susceptibility.

A predominance of small dense LDL is a component of the dyslipidemic syndrome of an ALP (Austin *et al* 1990). The latter is associated with a 3-fold increase in risk of CHD (Austin *et al* 1990) and is a characteristic feature of the insulin resistance syndrome (Reaven 1988, Reaven *et al* 1993a). Therefore, it is reasonable to postulate that the increase CHD risk in these patients may be attributed to enhanced susceptibility of their small dense LDL to oxidation. Another lipid abnormality which characterises both ALP and diabetics with insulin resistance is low plasma HDL level (Uusitupa *et al* 1986). This also contributes to the enhanced LDL oxidation by associated lower serum paroxygenase enzyme activity (Mackness *et al* 1991, 1993). The same group of investigators has attributed the enhanced LDL oxidation in diabetics to increase glycation of apo B seen in these patients (Tames *et al* 1992). This was based on the knowledge that glycated LDL show enhanced susceptibility to oxidation (Hunt *et al* 1990).

Since a predisposition of small dense LDL is associated wih 3-7 fold increase in CHD risk and is suggested to have a greater binding reactivity to APG, it would be reasonable to postulate that, an increase concentration of small dense LDL, such as seen in an ALP, enhances the influx of these particles into the arterial wall (Nordestgaart & Nielsen 1994) by increasing endothelial permeability (Henry *et al* 1995). Owing to their small size these particles cross the endothelial barrier more readily. Once in the arterial subendothelial space, they bind with a greater affinity to CSPG (Hurt camejo *et al* 1990, Wagner *et al*

1989). This promotes oxidative modification of the LDL particles (Camejo *et al* 1991) on the one hand, and on the other hand these complexes are rapidly taken up by macrophages via both LDL and scavenger receptors (Hurt *et al* 1990, Hurt-Camejo *et al* 1992, Camejo *et al* 1993, Williams & Tabas 1995) leading to foam cell formation. In turn, this leads to the secretion of platelet derived growth factor (PDGF) and transforming growth factor β1 (TGF β1) (Schönherr *et al* 1991, 1993) which stimulate smooth muscle cell to synthesise more CSPG that trap more LDL and so on (Fig 1.6)

1.12 General aims and objectives

It is evident from the above discussion that heterogeneity and structure of lipoproteins mainly those of apoB containing lipoproteins determine their fate and potential atherogenicity. An extensive body of work has already been performed to elucidate the pathophysiology of atherosclerosis through examining the interaction of lipoproteins with arterial wall proteoglycans and oxidative modification studies which are thought to be prerequisite in lesion development. Lipid abnormalities particularly those related to an ALP and the role in modulating the structure and function of apoB containing lipoproteins are tested in this thesis. The present work has two aims. Firstly, to examine the interaction of apoB containing lipoproteins with CS-PG dissociatively extracted from human aorta. A series of studies are designed to achieve this. We hypothesise that small dense LDL-III might be more reactive towards APG. Therefore the objective of study 1 is to determine the association between a predominance of small dense LDL within total LDL and APG binding reactivity. This hypothesis together with the knowledge that small dense LDL is a component of the atherogenic lipoprotein phenotype, in addition to the wide variation in PG-LDL reactivity between different individuals, prompted us to seek abnormalities within the other apoB containing lipoproteins (VLDL, IDL and LDL). This is aimed in study 2 to determine the relative reactivity of different apoB containing lipoproteins across S_f 0-400 spectrum with the

postulation that this might be affected by pharmacological modulation of the LDL subfraction profile. Since LDL subfractions have been shown to differ in their neutral carbohydrate and sialic acid content, the question which arises next is whether this structural variability is related to LDL ability to interact with APG and thus possibly revealing the mechanisms related to this interaction process. Study 3 and 4 are set to possibly answer these questions.

Interestingly, LDL oxidation is believed to be a prerequisite step in lesion development Therefore the second major aim of this work is to evaluate the susceptibility of LDL subfractions to oxidative modification in a group of NIDDMs and examine the effect of dietary supplementation with Evening Primrose Oil (EPO) on lipid and lipoproteins and LDL oxidation in these patients.

Chapter 2

Materials and Methods....

2.1 Study outline

The mechanisms linking apolipoprotein B containing lipoproteins with atherogenesis may be explained by several theories. Two of which are considered to be most critical, interaction of lipoproteins with arterial wall proteoglycans and LDL oxidative modification, were studied.

First, the interaction of chondroitin sulphate-rich fraction arterial wall proteoglycan, extracted and purified from human aorta, with low density lipoprotein and triglyceride rich (VLDL and IDL) apoB containing lipoproteins was examined in-vitro. Determinants and mechanisms related to this interaction process with respect to structure and function of the lipoprotein particles were studied.

In-vitro APG-Lipoprotein binding assay was established and was used to examine the extent of apoB containing lipoproteins reactivity towards APG.

Second, the effect of Evening Primrose Oil (EPO), containing linoleic acid and gamma linolenic acid (PUFA), which has been shown to have a lipid lowering effect, on LDL subfraction profile and oxidative modification was studied.
The extent of LDL oxidative modification, in-vitro, was determined by measuring the relative fluorescence unit/hr at 430nm (excitation 350) after incubation with a heavy metal (copper).

2.2 Materials

All reagents used were of analytical grade and names and addresses of all suppliers are shown in the appendix together with the manufacturers or suppliers of all hardware and software used in this book.

2.3 Proteoglycan-Lipoprotein interaction

The interaction between human chondroitin sulphate-rich arterial wall proteoglycan with total LDL (native or modified) and apoB containing lipoprotein subfractions was studied using an in-vitro binding assay (Anber *et al* 1996).

2.3.1 Isolation of total LDL

After 12h fast, 50ml of blood was obtained from the subject by venepuncture and collected in K_2EDTA (final concentration 1mg/ml) as an anticoagulant. Plasma was harvested at 4°C by low speed centrifugation (3000rpm).
To 4ml plasma 0.32ml, 1.182 g/ml density solution was added and overlaid with 1.68ml , 1.019g/ml density solution to give a total volume of 6ml. This was centrifuged in a fixed angle Beckman *Ti* 50.4 rotor in L7-55 Beckman ultracentrifuge at 35,000rpm for 24h (15°C). VLDL and IDL (d=1.006-1.019g/ml) were removed from the top 2ml.

To the remaining 4ml 1.47ml of 1.182 g/ml density solution was added and overlaid with 0.53ml of 1.063g/ml density solution to give a final volume of 6ml. Centrifugation was performed as above and total LDL (d=1.019-1.063g/ml) was removed in the top 2ml.

The isolated lipoprotein was dialysed against appropriate buffer solutions for the different studies.

2.3.2 Isolation of apoB containing lipoproteins

After 12hr fast, 50ml of blood was obtained from the subject by venepuncture and collected in K_2EDTA (final concentration 1mg/ml) as an anticoagulant. Plasma was harvested at 4°C by low speed centrifugation (3000rpm).

From this plasma apoB containing lipoproteins were fractionated into large VLDL1 (S_f 60-400), small VLDL2 (S_f 20-60), large IDL1 (S_f 16-20), small IDL2 (S_f 12-16), large LDLA (S_f 8-12) and small LDLB (S_f 0-8) by a modification of the cumulative density gradient centrifugation technique described by Lindgren *et al* (Lindgren *et al* 1972).

Density solutions

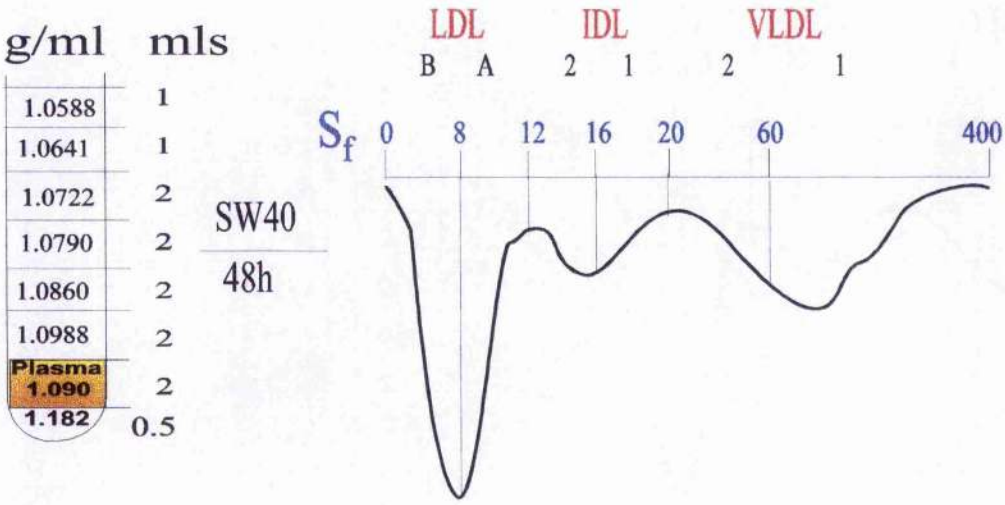
The density gradient was prepared from 1.006 g/ml and 1.182 g/ml NaBr density solutions, containing EDTA, as follows and were checked with a digital densitometer (DMA 35 Paar Scientific Ltd):

Density (g/ml)	Density 1.006g/ml	Density 1.182 g/ml
1.0988	25 ml	27.89 ml
1.0860	25 ml	20.83 ml
1.0790	25 ml	17.72 ml
1.0722	75 ml	50.05 ml
1.0641	25 ml	12.31 ml
1.0588	25 ml	10.73 ml

The density of 2 ml plasma was increased to d = 1.118 g/ml by addition of 0.341 g NaCl. This was laid over 0.5 ml d = 1.182 g/ml in a coated (with polyvinylalcohol) Beckman polyallomer tube and overlaid with a discontinuous NaBr gradient from the above density solutions (Fig 2.1) using an AAII pump (Technicon Ltd).

Figure 2.1

Isolation of plasma lipoprotein fractions by cumulative density gradient centrifugation



Centrifugation was performed in a Beckman SW 40 swinging bucket rotor in a Beckman L-5 centrifuge over 48h at 23°C. Lipoprotein subfractions were removed by a fine tipped pipette from the top of the tube after deceleration without brake. The following centrifugation conditions were applied: 1h 38min at 39,000 rpm for VLDL1 (remove in a volume of 1ml and replace with 1.0 ml of d =1.0588 g/ml), centrifugation was resumed at 18,500 rpm 15h 46min after which VLDL2 was recovered from the top 0.5ml of the gradient which was replaced with 0.5 ml d =1.0588 g/ml. IDL1 and IDL2 were collected each in 0.5 ml after centrifugation for 1h 15min and 1h 22min at 39,000 rpm respectively. following centrifugation at 39000rpm 2h 9min LDLA was

removed from the top 0.5ml and finally LDLB was isolated from the top 0.5 ml after further centrifugation at 40000rpm for 17 h.

2.3.3 Extraction and purification of human arterial wall proteoglycans (APG).

Human arterial wall proteoglycan was extracted in 4 mol/l guanidine hydrochloride and purified by isopycnic density gradient ultracentrifugation in $CsCl_2$ as described before (Ogema *et al* 1979, Salisbury and Wagner 1981, Wagner *et al* 1986).

Buffer solutions

- Transport buffer = Tris-HCl 5.0 mmol/l pH 5.8, containing 0.15 mol/l NaCl, 0.01 mol/l EDTA, 0.01% (w/v) NaN_3, and 0.01 mol/l ∈-amino caproic acid (as protease inhibitors.

  = 0.788 g Tris-HCl + 8.76 g NaCl + 3.722 g EDTA + 0.1 g NaN_3 + 1.312 g ∈-amino caproic acid in 1litre of distilled water.

- Extraction buffer = 4 mol/l guanidine -HCl pH 5.8, containing 0.05mol/l Na acetate, 0.01 mol/l EDTA, 0.1mol/l ∈-amino caproic acid and 0.005 benzamidine- HCl.

  =

- Dialysis buffer = 5.0mmol/l Tris-HCl buffer pH 7.2, containing 6.0mmol/l KCl, 4.0mmol/l $CaCl_2$, 1.0mmol/l $MgCl_2$, 0.1mol/l ∈-amino caproic acid and 0.005 benzamidine- HCl (as protease inhibitors).

  = 0.788 g Tris-HCl + 0.4473 g KCl + 0.444 g $CaCL_2$ + 0.2033 g $MgCl_2$ + 0.656 g ∈-amino caproic acid and 0.783 g Benzamidine-HCl in 1 litre of distilled water.

- Binding buffer = dialysis buffer without the protease inhibitors.

2.3.3a Aortic tissue selection and handling

An aorta (Thoracic and abdominal segments) from 70-year old deceased female, <24h post mortem, was obtained from Department of Pathology, Glasgow Royal Infirmary, the cause of death was aortic valve stenosis and pulmonary embolism with no history of MI. The tissue was placed in 5.0 mmol/l Tris-HCl buffer pH 5.8, containing 0.15 mol/l NaCl, 0.01 mol/l EDTA, 0.01% (w/v) NaN_3, and 0.01 mol/l ∈-amino caproic acid (as protease inhibitors) and transported to the laboratory on ice.

The aorta extending from the aortic arch down to the common iliac artery was cleaned of adventitial tissue, opened longitudinally, examined and any visible, heavily atheromatous and calcified plaques were excised. The remaining tissue, which appeared normal, was cut into small cubes and weight for extraction. Handling of the tissue was performed on ice at all times.

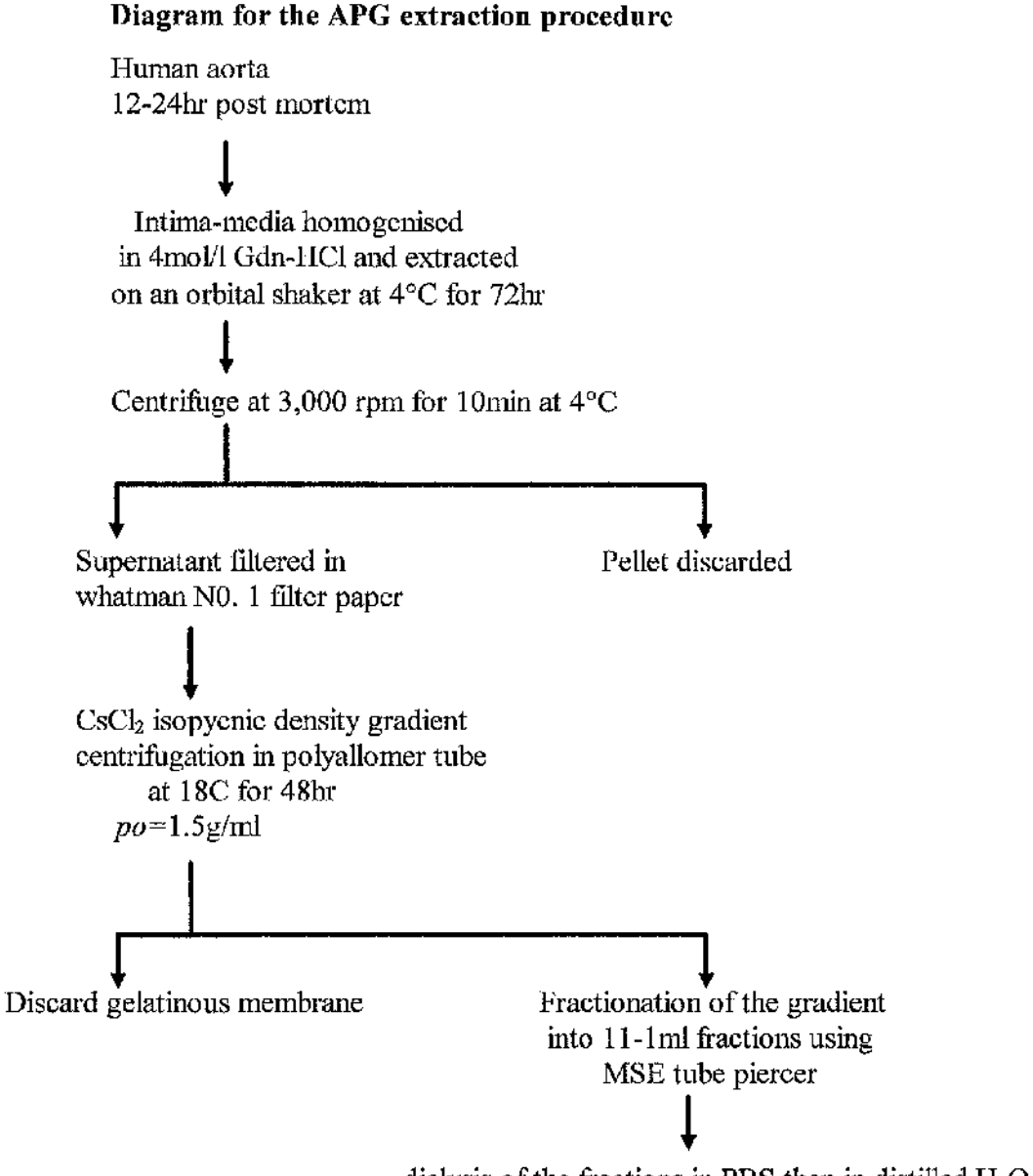
2.3.3b Extraction of APG from aortic tissue

The cleaned material (approximately 20 mg of aortic tissue) was homogenised in quickfit flasks (Bibby Science Products) using an ultra-Turrax homogeniser (Janke and Kunkle), and the resulting homogenate extracted with 15 volumes of 4.0 mol/l guanidine hydrochloride solution containing 0.05 mol/l sodium acetate pH 5.8, 0.01 mol/l EDTA, in the presence of 0.1 mol/l ∈-amino caproic acid and 0.05 mol/l benzamidine-HCl (as protease inhibitors). The extract was mixed on an orbital shaker for 48 h at 4°C and then cell debris was removed by low speed centrifugation (3,000 rpm) for 10 min at 4°C (Fig 2.2).

The supernatant containing a crude proteoglycan preparation was filtered through Whatman No.1 filter paper and further purified by isopycnic gradient centrifugation.

Figure 2.2

Diagram for the APG extraction procedure



2.3.3c Purification and fractionation of APC

The density of the extract was adjusted to 1.5 g/ml by the addition of 0.59 g $CsCl_2$ per gram of supernatant. 1ml of which was placed in each polyallomer tube with caps. Centrifugation was performed in a fixed angle *Ti* 60 rotor with Delrin adapters for 48 hr at 18°C (40,000 rpm).

After centrifugation a thick 3-4 mm gelatinous membrane covering the top of the tube was removed by a fine tipped forceps composed mainly of protein and collagen.

The tube content were fractionated by downward displacement using an MSE tube piercer (Fisons) and the density of each fraction was measured by a digital densitometer (DMA 35 Paar Scientific Ltd).

All fractions were dialysed initially against PBS and then against distilled H_2O at 4°C using (spectra pore dialysis tubing) for 72 h and lyophilised. The freeze dried fractions were stored at -70°C for further characterisation and binding assay.

Calculation of the density gradient using Beckman polyallomer centrifuge (16 × 76 mm, 13.5 ml) tubes in Beckman *Ti* 60 rotor with Delrin adapters (303307) as follows (Fig 2.3):

Maximum speed for this is 40,000 rpm.

$$r'_{max} = r_{max} - [(d_1-d_2)/2] - [t - ((d_1-d_2)/2)] \sin\theta$$

$$= 89.9 - [(25.65-16.51)/2] - [12.7 - ((25.65 - 16.51)/2)] \sin 23.5$$

$$r'_{max} = 82.09 \text{ mm}$$

$$r'_{min} = r_{max} - (d_1/2) - (t - d_1/2 + L) \sin\theta - (d_2/2) \cos\theta$$

$$= 89.9 - 12.825 - (12.7 - 12.825 + 71.42)\ 0.399 - 8.225 \times 0.91$$

$$r'_{min} = 41.085 \text{ mm}$$

r_{max} = the distance in millimetres from the axis of rotation to the farthest part of the tube cavity (= 89.9mm for 60 *Ti*)

r_{min} = the distance in millimetres from the axis of rotation to the nearest part of the tube cavity (=36.9 for 60 *Ti*)

r_{av} = the distance in millimetres from the axis of rotation to the middle part of the tube cavity (=63.4 for 60 *Ti*)

d_1 = outside diameter of the adapter

d_2 = inside diameter of the adapter

L = adapter cavity length

t = thickness of the adapter bottom

θ = tube of the rotor being used

$$re = \sqrt{[r'_{max}{}^2 + (r'_{max} - r'_{min}) + r'_{min}{}^2]/3}$$

$$= \sqrt{[\,82.09^2 + (82.09 \times 41.085) + 41.085^2]\,/\,3}$$

re = 62.71 mm

dp at r $= (r\text{-}re)\ dp/dr + P°$

$dp/dr = \omega^2\ r\ /\ \beta(p)$

ω = 2Π rpm /60

= 0.10472 rpm

$dp/dr = (0.10472rpm)^2\ re\ /\ \beta(p)$

$= (0.010966 \times 160{,}000\ 000 \times 6.271)/\ 1.245 \times 10^9$

= 0.088 g/ml cm

density at r_{max} $= (r_{max} - re)\ 0.88 + p°$

= (8.209 -6.271) 0.088 + 1.5

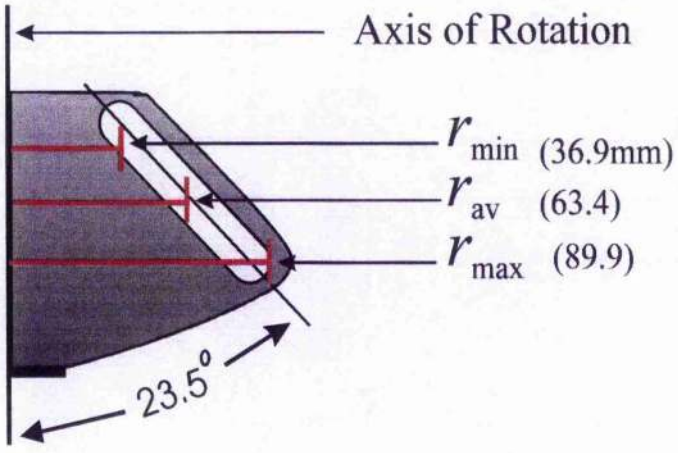
= 1.67 g/ml

density at r_{min} $= p° - (re\text{-}r_{min})\ 0.088$

= 1.5 - (6.271 - 4.1085) 0.088

= 1.3097 g/ml

Figure 2.3
Schematic diagram of Beckman type 60 *Ti* fixed angle rotor



r_{max}, the distance in millilitres from the axis of rotation to the farthest part of the tube cavity (= 89.9mm), r_{min}, the distance in millimetres from the axis of rotation to the nearest part of the tube cavity (=36.9mm), r_{av}, the distance in millimetres from the axis of rotation to the middle part of the tube cavity (= 63.4mm)

The amount of $CsCl_2$ used for a given density was calculated as follows:

wt in grams per 100 g of solution = 137.48 - [138.11 ($1/p°$ at 25°C) w

wt in g = 0.59 g/ g solution

$p°$ = starting density

2.3.3e *Alcian Blue colorimetric assay for chondroitin sulphate determination*

The freeze dried fractions were redissolved in a 50 mmol/l sodium acetate buffer pH 5.8 containing 0.2 mol/l $MgCl_2$ and 0.002% (w/v) NaN_3. Proteoglycans were quantified by chondroitin sulphate as measured by an alcian blue microassay based on the method described by Hinnie and Serafini-Fracassini (Hinnie & Serafini-Fracassini 1986) and protein determinations by the modified procedure of Lowry *et al* (Lowry et al 1951).

Solutions:

1. 200mmol/l MgCl2 hexahydrate (40.66 g/l)
   50 mmol/l Na acetate trihydrate (6.804 g/l)
   0.002% Na azide (0.02 g/l)
   adjust pH to 5.8 with HCl

2. 2% SDS (electrophoresis quality) (20.00 g/l)
   50 mmol/l Na acetate trihydrate (6.804 g/l)
   adjust pH to 5.8 with HCl

Standards:

chondroitin sulphate A (Sigma cat. No. C-0914) is dried overnight at 4°C in a dessicater. 50 mg of the dried chondroitin sulphate was dissolved in 100 ml of solution 1 to give a concentration of 0.5 mg/ml and standard solutions are made up at the following concentrations by dilution in solution 1:

chondroitin sulphate (µl)	solution 1 (µl)	concentration (mg/ml)
125	875	0.063
250	750	0.125
375	625	0.188
500	500	0.250
625	375	0.313
750	250	0.375
875	125	0.438
1000	-	0.500

Assay procedure:

Day 1. Prepare a 0.05% solution of Alcian Blue 8GX in solution 1 (12.5 mg in 25 ml for one microwell plate). Stir overnight to ensure maximal dissolving of dye.

Day 2.

- spin dye solution at 3,000 rpm for 15 min and discard pellet.
- add 10µl of standard, blank and sample to duplicate wells of the microwell plate (Nunc Cat. No. 269620). Use solution 1 as blank.
  The plate format is defined in program 24 ("ALCIAN") on Dynatech MR5000.
- add 200 µl of dye solution to each well and mix on Varishaker (Dynatech) (setting 3.5) for 2 h.
- spin plate at 3000 rpm for 15 min.
- discard supernatant (one flick with plate inverted do not reinvert the plate till dry), wash pellet in 200 µl absolute ethanol and mix on Varishaker setting 3.5 for 2min.
- spin plate at 3000 rpm for 15 min.
- discard supernatant, wash pellet in 200 µl absolute ethanol and mix on Varishaker setting 3.5 for 2min.
- spin plate at 3000 rpm in for 15 min.
- discard second ethanol supernatant and allow ethanol residue to evaporate.
- dissolve pellet in 200 µl of solution 2 and mix on Varishaker setting 3.5 for 30 min.

- burst any bubbles on the surface and read plate on Dynatech MR 5000 at 630nm using program 24 ("ALCIAN") which will draw a calibration curve and calculate the unknown samples.

2.3.3f Characterisation of glycosaminoglycan (GAG) chains

Further characterisation of the GAG components of the proteoglycan was performed by Mr Martin Langly, Department of Basic Dental Sciences, Dental School, University of Wales, with thanks, using SDS-PAGE as described by Waddington *et al* (Waddington *et al* 1989).

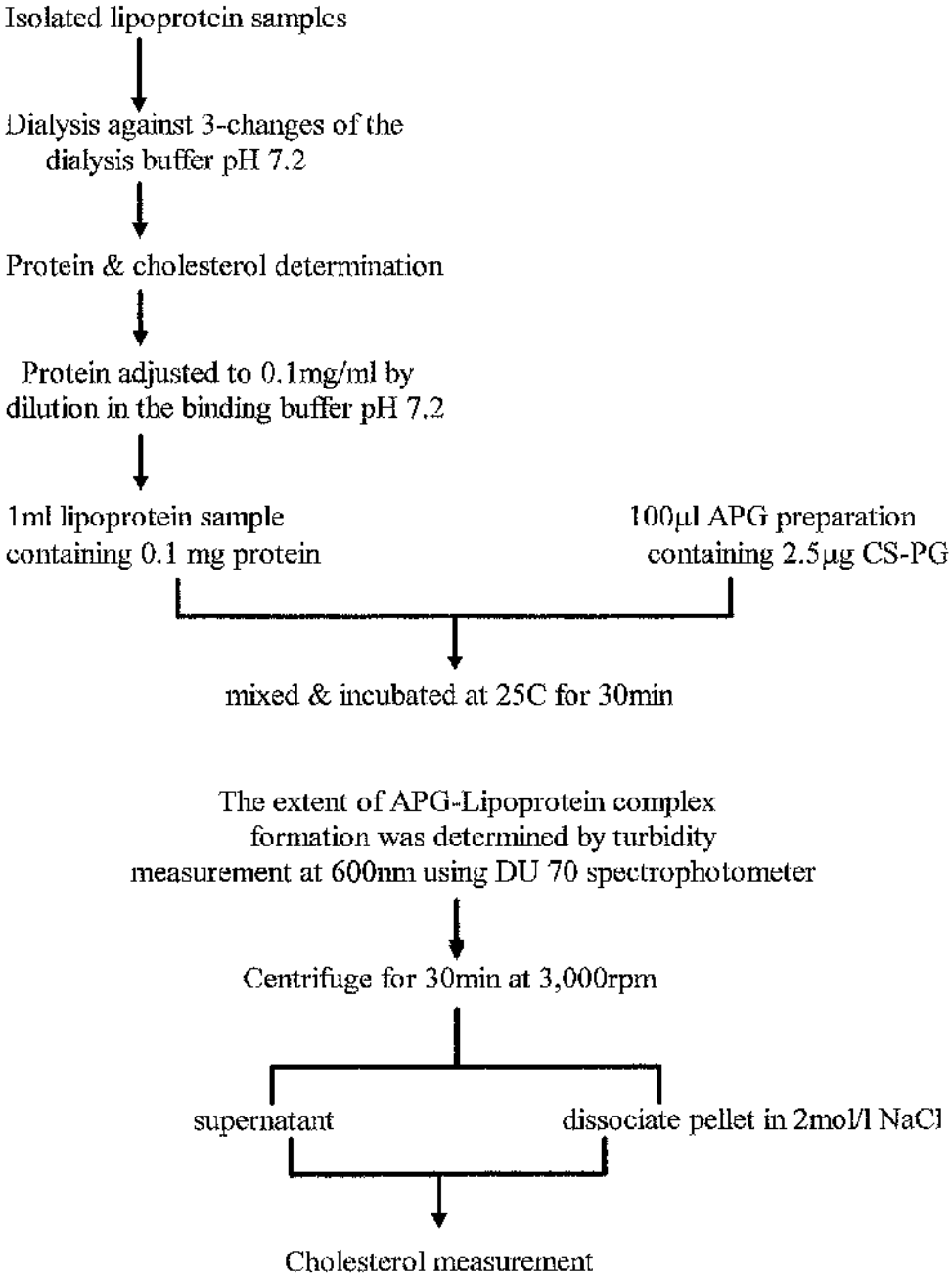
2.3.4 Lipoprotein - APG in-vitro binding assay

The assay was established (Anber *et al* 1996) and used in all the studies (Fig 2.4). The amount of chondroitin sulphate in the freeze dried material was measured by alcian blue assay (section 2.3.3e). The chondroitin sulphate concentration in the preparation was then adjusted to 2.5µg/100µl (This concentration was selected after mixing 0.1mg/ml LDL with different concentration of the chondroitin sulphate-rich APG, chapter 3) and used in all the assays. Total LDL samples isolated by sequential gradient ultracentrifugation (section2.3.1), all lipoprotein subfractions (VLDL1-LDL3) isolated by cumulative density gradient centrifugation (section 2.3.2) and modified LDL samples (section 2.5), were dialysed at 4°C for 1 h against the dialysis buffer of 5.0 mmol/l Tris-HCl buffer pH 7.2, containing 6.0 mmol/l KCl, 4.0 mmol/l $CaCl_2$ and 1.0mmol/l $MgCl_2$ (section 2.3.3) using spectrapore dialysis tubing. The buffer was changed and dialysis continued for 3 h and then

following another change, overnight. The protein concentration of dialysed samples was measured by a modification of the procedure of lowry *et al* (lowry *et al* 1951) and adjusted to 0.1 mg/ml by dilution in the binding buffer (section 2.3.3). Diluted lipoprotein samples were divided into two 1 ml aliquots, one of which served as a control, and placed in plastic LP3 (Sarstedt). A standard amount (100 µl) of APG solution containing 2.5 µg chondroitin sulphate was mixed with the test sample, but not with the control and both tubes were vortexed and incubated at 25°C for 30 minutes. Following incubation, turbidity was measured by absorbance at 600nm (visible wavelength) using a Beckman DU70 spectrophotometer. The samples were centrifuged at 3,000 rpm for 30 min to separate the lipoprotein-APG complex from the unreacted lipoprotein in the supernatant. The pellet was dissociated with 0.2 ml of 2.0 mol/l NaCl buffer and cholesterol was measure in this fraction, the control tube and the supernatant by a modification of the lipid research clinic protocol (LRCP 1975).

Figure 2.4

Schematic diagram of the APG-Lipoprotein binding assay



2.3.5 Data analysis

The extent of complex formation was determined both by absorbance at 600 nm, having subtracted the control tube as a blank, and by the percentage of precipitated cholesterol by measuring the cholesterol content of the precipitate divided by the total cholesterol added to the incubation mixture. Repeated analysis using the same LDL preparation showed an intraassay coefficient of variation of <3% and an interassay coefficient of variation of <6% (chapter 3, Fig 3.2).

2. 4 Determination of LDL carbohydrate

The heterogeneity of human plasma total and LDL subfractions with respect to the carbohydrate content of the lipoprotein particle was investigated by fractionation of total LDL by affinity chromatography on Concanavalin-A, using the method described by McConathy & Alaopovic (McConathy & Alaopovic1974). Neutral sugars and sialic acid content of the total and LDL subfractions were determined using phenol sulphuric acid (Beeley 1985, LaBelle & Krauss 1990) and resorcinol-hydrochloric acid (Svennerholm 1957) assays respectively.

2.4.1 Separation of LDL subfractions by affinity chromatography on Concanavalin-A.

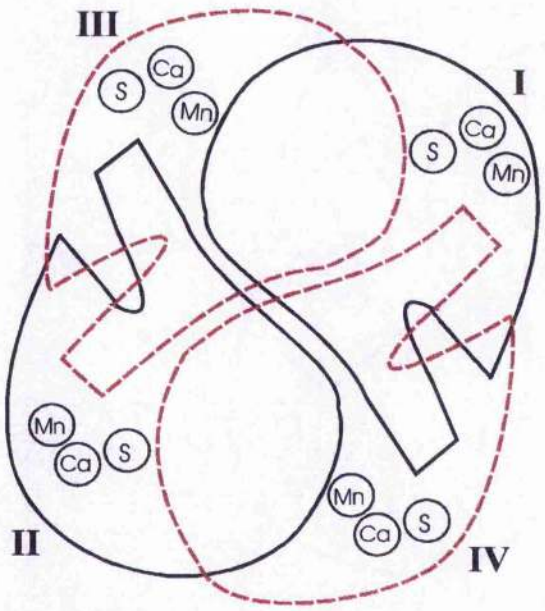
Con-A, is a plant lectin purified from Jack bean. It binds molecules which contain α-D-mannopyranosyl, α-D-glucopyranosyl and sterically related residues. The binding sugar requires the C-3, C-4, and C-5 hydroxyl groups for the reaction with Con-A (Goldstein *et al* 1965) (Fig 2.5).

Con-A-Sepharose is concanavalin A coupled to Sepharose 4B by cyanogen bromide method. The concentration of coupled lectin is approximately 8mg/ml gel and the binding capacity for porcine thyroglobulin (MW 670,000) is

approximately 8.5 mg/ml gel. The high capacity is due to the macroporous structure of the matrix. The binding of substances with Con-A requires the presence of both Mn^{2+} and Ca^{2+} and the active protein-metal ion complex is stable at neutral pH in the absence of free metal ions.

Figure 2.5

Schematic diagram of the Con-A tetramer



Con-A is a tetramer of identical subunits at physiological pH. One binding site for Calcium, Manganese and specific saccharide units are indicated by Ca, Mn and S respectively.

Con-A- Sepharose was supplied by Pharmacia pharmaceuticals as a suspension of 100ml sedimented gel in acetate buffer solution (0.1 mol/l, pH 6.0) containing 1mol/l NaCl, 1mmol/l $CaCl_2$, 1mmol/l $MgCl_2$, 1mmol/l $MnCl_2$ and thimerosal (0.02%) (as a preservative). The gel should be stored at 4-8°C.

Buffer solutions

- Equilibrating buffer = Tris-HCl 0.02 mol/l pH 7.2 containing 0.5 mol/l NaCl, 1mmol/l $CaCL_2$, 1mmol/l $MgCl_2$ and 1mmol/l $MnCl_2$.
  = 3.152 g Tris-HCl + 29.22 g NaCl + 0.111 g $CaCL_2$ + 0.2033 g $MgCl_2$ + 0.1979 g $MnCl_2$ were dissolved in 1litre of distilled water and the pH was adjusted to 7.2.

- Eluting buffer 1 (α-DG) = 0.2 mol/l α-D-methylglucopyranoside (19.42 g Methyl- glucopyranoside in 500 ml of the Tris-HCl equilibrating buffer)

- Eluting buffer 2 (α-DM) = 0.3 mol/l α-D-methylmanopyranoside (29.13 g Methyl- manopyranoside in 500 ml of the Tris-HCl buffer)

- Regeneration buffer 1(R1) = 0.1 mol/l Tris-HCl pH 4.5, containing 0.5 mol/l NaCl.
  = 7.88 g Tris-HCl + 29.22 g NaCl.

- Regeneration buffer 2 (R2)= 0.1 mol/l CH_3COONa pH 8.5, containing 0.5 mmol/l NaCl and 1mmol/l $CaCl_2$.
  = 8.203 g CH_3COONa + 29.22 g NaCl + 0.111 g $CaCl_2$ in 1 litre of distilled water.

8 ml of Concanavalin-A gel was washed with 10 volume of the equilibrating buffer. It was loaded on the column with a Bio-Rad pump at a flow rate of 0.1/min and then equilibrated with 20-column volumes of equilibrating buffer.

Total LDL was desalted using 1.0 × 10 cm columns of Sephadex G-25 (PD10 column, Pharmacia). 2.5ml LDL was loaded on to the column and 3.0ml was eluted with the equilibrating buffer, pH 7.2 and was used immediately.

1mg of LDL protein, as measured by a modification of Lowry et al (Lowry *et al* 1951) was applied to the column and fractions were collected under the following conditions:

Sample volume = 3ml

Protein = 1mg LDL protein

Flow rate = 0.1ml/min

Fraction size collected = 0.5 ml/ Tube (collect peak and non-peak)

The elution timing and pattern was as follows:

Function	Time (min)	Valve	buffer (volume in ml)
1	0	A	Tris-HCl (32)
2	320	B	Sample (1mg/3ml)
3	350	A	Tris-HCl (32)
4	670	C	0.2 α-DG (32)
5	990	D	0.3 α-DM (32)
6	1310	E	R1 (32)
7	1630	A	R2 (32)
8	1950	B	R1 (32)
9	END		
1	0	A	R2 (32)
2	320	B	R1 (32)
3	640	A	R2 (32
4	960	B	Tris-HCl (32)

NB: The last four functions was started again as the Bio-Rad system only has nine functions.

After sample application the unbound fractions were eluted using the equilibrating buffer. The elution of the bound substance was performed in two stages: the weakly bound fraction was eluted by 0.2 mol/l α-DG. This was followed by the elution of the strongly bound fraction by 0.3 mol/l α-DM. The

Con-A column was regenerated between different samples by regeneration buffer 1 and 2 alternately three times each. The affinity chromatography procedure was performed using Bio-Rad Econosystem with an automatic 5 valve controller (A, B,C,D and E) to switch between buffers. Protein was determined on collected fractions by optical density measurement at 280nm (UV wave length) using DU-70 spectrophotometer.

2.4.2 Labelling of total LDL

Radiolabelling of isolated total LDL (d=1.019-1.063 g/ml) was carried out by iodine monochloride method of MacFarlane (1958) as modified by Shepherd, Bedford and Morgan (1976).

The following iodination mixtures were used:
Two 1.0ml aliquots of total LDL (d=1.019-1.063g/ml) (protein concentration =1.6mg/ml) were mixed separately with 0.5ml of 1.0mol glycine, pH 10 and 2.0mCi of Na [^{125}I].

An appropriate volume of iodine monochloride solution (25mmol in 1mol NaCl) was added to yield an ICl:protein ratio of approximately 20mol:500,000 Da of protein, and mixed gently. This procedure introduces an iodine atom into tyrosine residues at a level of less than 10mol iodine /molecule of LDL protein.

Bound and free radio-iodide were separated by passing the two iodination mixtures over separate 1.0 × 10 cm columns of Sephadex G-25 (PD10 column, Pharmacia). The eluting buffer used was 0.15 NaCl containing 0.01% Na_2EDTA (pH 8.1).

The radio activity concentration (μCi/ml) was determined by counting 10μl aliquots of ^{125}I-LDL and comparing with ^{125}I simulated standards.

2.4.3 Separation of ^{125}I-LDL subfractions by Concanavalin-A in a tube assay.

2 ml Con-A washes with 10 volume equilibrating buffer (section 2.3.1) and left to settle. The excess supernatant buffer was removed by a fine tipped pipette. 100µl I^{125}-LDL, containing 0.16mg protein, was diluted in equilibrating buffer (final volume =2ml). Radio activity was determined as counts per minute (cpm) on a gamma counter (Canberra Packard). The 2ml I^{125}-LDL sample in buffer was mixed with the Con-A gel and radio active counts was measured again. This was done to compare the effect of Con-A on count measurement. For clarity the next steps are illustrated in diagram as follow:

1. 2ml Con-A gel +2ml I^{125}-LDL (0.16 mg protein)

   ↓ counts measured in cpm

2. Mixed on a roller mixer for 1hr at room temperature

   ↓ Stand to settle

3. Remove supernatant with a fine tipped pipette

   ↓

4. Measure counts on both , supernatant and remaining Con-A on a gamma counter (cpm)

   ↓

5. Add 2ml 0.2 α-DG and repeat steps2-4

   ↓

6. Add 2ml 0.3 α-DM and repeat steps 2-4

2.4.4 Data analysis

The amount of radioactivity, in cpm, of the unbound, eluted by equilibrating buffer, the weakly bound, eluted by 0.2 α-DG and the strongly bound, eluted by 0.3 α-DM, fractions was measured directly on the supernatant as well as by

subtracting the cpm on the remaining Con-A from the previous measurement in each step.

The percentage of the unbound, the weakly bound and the strongly bound fractions were calculated by dividing the amount of radioactivity of each of the fractions by the total multiplied by 100.

2.4.5 Phenol sulphuric acid assay for neutral carbohydrate

This procedure , as by Beeley (1985), is a scaled down version of the method of Dubois et al (1956).

Reagents:

- Concentrated H_2SO_4 (DBH Aristar grade)
- Phenol: aqueous solution of colourless phenol crystals (5% w/v); solution stable for several weeks at room temperature
- Standard D-mannose solution (stoke standard 4 mg/ml) stored at -18°C.

LDL (d=1.019 -1.063 g/ml) and LDL subfractions, LDLI (d = 1.025 - 1.034 g/ml), LDLII (d =1.034 - 1.044 g/ml) and LDLIII (d = 1.044 -1.060) isolated by sequential and density gradient centrifugation respectively, were desalted by gel filtration on Sephadex G-25 columns (NAP 5, Pharmacia). 0.5 ml of the sample was applied and eluted in 1.0 ml with 0.15 mol/l PBS pH 7.1 containing 0.01% EDTA.

preparation of standard curve:

D- mannose stock standard 1:50 (80 µg/ml) was diluted to prepare solutions as follows

D-mannose (µl)	PBS(µl)	µg sugar /tube
500.0	-	40
375.0	125.0	30
250.0	250.0	20
188.0	312.0	15
125.0	375.0	10
62.0	438.0	5
-	500.0	0

Assay procedure:

The reaction was carried out in round - bottomed "Labco" glass boiling tubes. LDL protein concentration was determined by the modification of the procedure of Lowry et al (1951). To a 100 µl of desalted total LDL and 200 µl of LDL subfractions (containing 0.3 - 2.0 mg/ml protein) in a final volume of 0.5 ml in PBS 0.3 ml of the 5% phenol was added and mixed. 2.0 ml of concentrated H_2SO_4 was added rapidly from a fast - flowing glass pipette and mixed immediately. Stand tubes at room temperature for 30 min.

Absorbance was read at 484 nm visual wavelength.
The neutral carbohydrate concentration of the samples were expressed as µg carbohydrate /mg protein.

Warning : Gloves and safety spectacles should be worn during the assay procedure which was carried out in a safety cabinet.

2.4.6 Estimation of the sialic acid in LDL and lipoprotein subfractions

determination of sialic acid was performed using a modification of the resorcinol hydrochloric acid method by Svennerholm (1957).

Reagents:

- N-acetyl neuraminic acid standard (Several N-acetyl neuraminic acid are available from Sigma, basically they are all similar, however the purer the standard the better, 98% pure neuraminic acid from Sigma).
- Stock Resorcinol 0.2% w/v in water (store at 4°C).
- 10 N HCl.
- 0.1 M copper sulphate (add 0.25 g $CuSO_4$ to 10 ml H_2O).
- Butyl Acetate: n-butanol (85:15 v/v)

Procedure:

A stock standard solution of (98% pure) N-acetyl neuraminic acid (Sigma) 32µg/ml was prepared in distilled H_2O.

A working standard curve in the range of 0 - 8 µg /250µl were prepared by taking appropriate volumes of the stock standard (0 - 250 µl). The final volume was adjusted to 250µl with distilled H_2O.

Resorcinol - HCl reagent was prepared by adding 10 ml of stock resorcinol to a mixture of 250 µl of 0.1 M $CuSO_4$ and 80ml of 10 N HCl.

Sample blank was prepared by adding 10 ml of distilled water to 250 µl of 0.1 M $CuSO_4$ and 80ml of 10 N HCl.

250 µl of resorcinol - HCl reagent was added to 250 µl standard, control and samples in glass screwcapped tubes. These were heated for 45 min at 100C using DB3 block heater. After cooling in a water bath at room temperature for 5 min, 400 µl of butylacetate: n- butanol solution was added to each tube. the mixture was vortexed and centrifuged for 5 min at 3,000 rpm at 4°C

Absorbance was measured on the organic layer at 580 nm visual wavelength against a water blank.

2.5 Chemical and enzymatic modification of LDL

Total LDL isolated by preparative sequential gradient ultracentrifugation was modified by various chemical and enzymatic modification to investigate the mechanism of APG-lipoprotein interaction.

CHD modification, Carbamylation and reductive methylation were performed following the technique described by Weisgraber *et al* (Weisgraber *et al* 1978). Neuraminidase treatment of LDL was performed as described by Camejo *et al* (1985b).

2.5.1 CHD Modification of arginine residues

1.0 ml LDL at a protein concentration of 1-2 mg/ml was added to 2.0 ml of freshly prepared 0.15 mol/l 1,2-cyclohexandione (CHD) in 0.2 mol/l borate buffer, pH 8.1 in a 50 ml Universal tube (total volume 10.5 ml).

This mixture was incubated on a roller mixer at 37°C for 2 h.

Modified LDL was separated from unbound by passing the mixture over a 1.0 × 10 cm column of Sephadex G-25 (PD10 column, Pharmacia) and eluted with d = 1.006 g/ml NaCl . The eluted sample was stored at 4°C overnight before dialysis for APG binding.

2.5.2 Carbamylation of lysine residues

1.5 ml of LDL (protein concentration = 1-2 mg/ml) was diluted with 0.75 ml of 0.3 mol/l sodium borate buffer, pH 8.0, to this 50 mg of potassium cyanate was added (20 mg/mg protein).

The mixture was incubated at 37°C for 2 h, after which it was stored at 4°C overnight before dialysis for APG binding.

2.5.3 Reductive methylation of lysine residues

1.5 ml of LDL (protein concentration = 1-2 mg/ml) was diluted with 0.75 ml of 0.3 mol/l sodium borate buffer, pH 8.0, to this 0.5 mg of sodium borohydrate was added.

1.0 µl of 40% formaldehyde was added every 6 min to the sample for 30 min at room temperature.

The reaction was then stopped by passing the mixture over a 1.0 × 10 cm column of Sephadex G-25 (PD10 column, Pharmacia) and eluted with d = 1.006 g/ml NaCl and stored at 4°C overnight before dialysis for APG binding.

2.5.4 Neuraminidase treatment

0.3 ml neuraminidase gel containing 0.136 units neuraminidase type VI-A from Clostridium Perfringens was placed in a screwcap culture tube and washed 3 times with water and 2 times with 1:10 dilution of the incubation sodium acetate buffer, pH 5.2 containing NaCl and EDTA. To this 2.0 ml of LDL containing mg protein was added along with 1.0 ml incubation buffer.

The mixture was incubated on a roller mixer (Danley) at 37°C for 25 h.

Following incubation the tubes were centrifuged for 1 min at 1000 rpm, the supernatant was collected and the pellet was washed with 0.5 ml Saline. After recentrifugation the supernatants were mixed and stored at 4°C overnight before dialysis for the APG binding assay and sialic acid measurement.

The extent of charge modification on the native total, chemically modified and neuraminidase treated LDL samples was tested on agarose gel electrophoresis.

2.5.5 Incubation with ganglioside

A stock solution of appropriate ganglioside 15-25μg/ml (Crude ganglioside from brain, GM3 ganglioside from plasma, GM1 and asialo-GM1 ganglioside) was prepared I in the APG binding. Different dilutions (0-40 mol/mol apoB) were prepared from the appropriate ganglioside stock solution. These were incubated with LDL (final protein concentration of 0.1mg/ml) for 1h at 30°C.

2.5.6 Incubation with apoE2, E3 and apoCIII

A stock solution of the appropriate apoprotein (apoE2, apoE3, apoCIII) was prepared in the APG binding buffer. A range of 0-1mol (apoE2, apoE3, apoCIII)/mol apoB were prepared by taking appropriate volumes of the stock solution. These were incubated with LDL (to give a final LDL protein concentration of 0.1mg/m) for 1h at 30°C.

2. 6 LDL oxidation

Fluorescence can be used to measure the extent of LDL oxidative modification after 24 h or by, determining the rate at which this fluorescence develops to measure the susceptibility of LDL to oxidative modification.
The method used was described by Comanicini *et al* (Comanicini *et al* 1991).

Reagents and solutions

QS : a standard solution of quinine sulphate (59.17 ng/ml in 0.1 N H_2SO_4

PBS buffer : PBS + chloramphenicol 0.1 mg/ml pH 7.3

2 mmol/l $CuSO_4$: 50 mg/100ml H_2O

1.5 mg/ml EDTA

Lipoprotein isolation and desalting

Total LDL and LDL subfractions were prepared for oxidative studies from fresh plasma by sequential and density gradient centrifugation respectively. LDL preparations were dialysed for 1 h at 4°C against PBS buffer, pH 7.3 containing 0.1 mg/ml chlorumphenicol using Spectra/Por dialysis membrane tubing No.2 (molecular cut off 12,000 - 14,000, 10 mm × 50ft). The buffer was changed and dialysis continued for 3 h and then following another change, overnight with no more than six 1 ml samples per 1 litre buffer. All solutions were prepared in deionised water.

After dialysis the protein concentration of the LDL samples were determined by the modified procedure of Lowry et al (1975) and adjusted to 100 μg/ml by dilution in PBS.

Diluted LDL samples were divided into two 2 ml aliquots in LP3 tubes, each containing 200 μg of protein, one of which was incubated in the presence of 10 μl of 2mmol/l $CuSO_4$, final concentration of copper ions was 10 μmol, the other served as a native control.

Incubation was carried out by continuous mixing on an orbital shaker (Danley-Tech Ltd) at 37°C for 24 h in the dark. Oxidation was quenched after 24 h by addition of 45 μl EDTA.

Fluorescence spectroscopy

The fluorimeter (Perkin - Elmer Ls-50) was calibrated by adjustment of the emission slit width to give a deflection of 100 relative units with a standard solution of quinine sulphate (59.17 ng/ml). Qs fluorescence was measured between emission (EM 380 - 550nm) and excitation (Ex. 350nm) with the Ex. peak at 450nm.

Fluorescence emission spectra of both native and oxidised LDL samples were measured between 380 - 550 nm, an excitation of 360 nm and scan speed of 500.

2.6.1 Data analysis

Fluorescence measurement was performed at 0, 1 h, 2 h, 3 h, 4 h, 5 h and 24 h.
The spectra of PBS was recorded and subtracted from the lipoprotein spectra using the "ARITH" function.
In the event of visible lipoprotein aggregation, fluorescence spectra were recorded before and after filtration (using 0.45 μ millipore filter).
Results were expressed in relative units of fluorescence intensity.

2.7 Plasma lipids, proteins and lipoproteins

These were assayed using a variety of chemical, enzymatic, centrifugal and immunoassay techniques as described in detail below.

2.7.1 Plasma lipid assays (β Quantification)

Lipoprotein classes in fresh, whole plasma by a combination of ultracentrifugation and selective precipitation, using standard methodologies (Lipid Research Clinics Program 1975). VLDL was separated as a floating fraction. The infranatant was then treated with heparin /Mn^{2+} (at a final concentration of 1.3 g/ml heparin and 0.092 mol/l Mn^{2+}) to precipitate LDL and leave HDL in solution. The cholesterol content of whole plasma, of the top (VLDL), and bottom (LDL and HDL) fractions and that of HDL were measured as described below.

Five ml of plasma was placed in an Ultra-clear tube (13 x 64 mm) (Beckman Instruments, Fullerton, CA) and overlayered with 2 ml of d 1.006 g/ml solution.

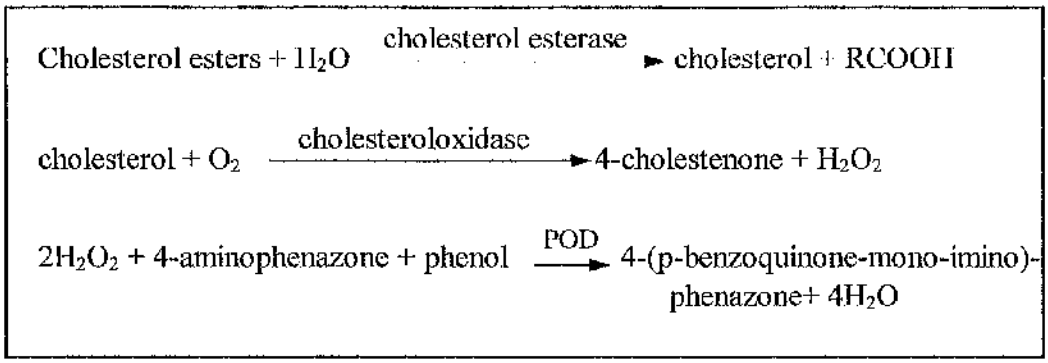
Tubes were capped and centrifuged overnight at 35,000 rpm (4°C) in a Beckman 50.4 rotor, then sliced 25 mm from the top and the supernatant collected into a 3 ml volumetric flask. The contents of the bottom fraction were transferred to a 5.0 ml volumetric flask, the tube washed with saline, the wash added to the flask, and the volume adjusted to 5.0 ml with 0.15M NaCl..

A 1.0 ml aliquot of this bottom fraction was then placed in a Beckman centrifuge tube and 50 µL of precipitating reagent [9.56 g $MnCl_2.4\ H_2O$ + 1.05 g (approximately $5x10^5$ units) heparin sodium salt in 25 ml 0.15M NaCl] were added and mixed. The mixture was kept at 4°C for 15 min then centrifuged at 10,000 rpm for 30 min, and the supernatant separated immediately for cholesterol analysis.

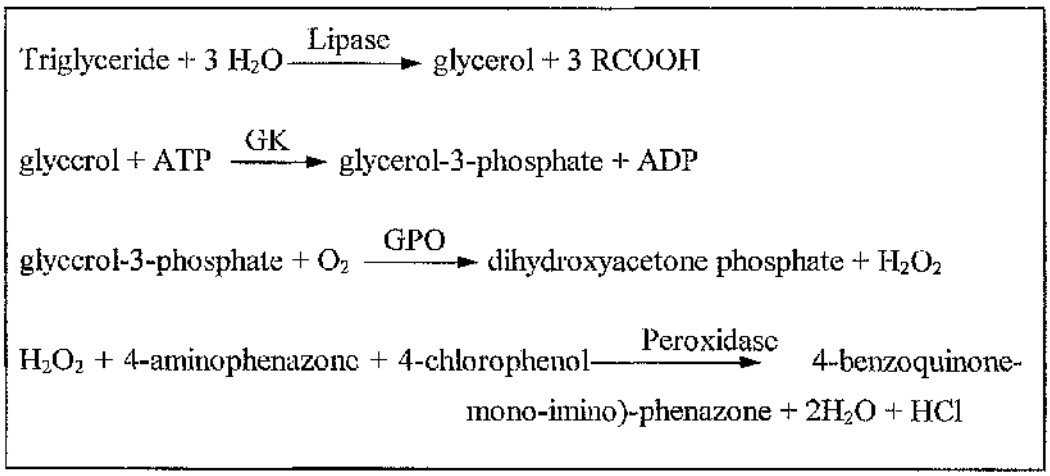
2.7.2 Compositional analysis

Total cholesterol and triglyceride were determined in whole plasma and in lipoprotein preparations by enzymatic colorimetric assays on Hitachi 704 auto-analyser.

Total cholesterol was assayed using the oxidative method (Boerhringer Kit No. 704121), the principal of which is shown below:



Triglyceride was assayed using an enzymatic method the principal of which is shown below:



The resultant colour changes were measured spectrophotometrically at 505 nm. Free (unesterified) cholesterol and phospholipid were determined by enzymatic colorimetric assays on a centrifichem Encore centrifugal analyser (Baker instruments).

Free cholesterol was estimated with Boerhinger Kit No. 310328 and esterified cholesterol was calculated by difference.

Phospholipid was estimated with Boerhinger Kit No. 691844 by an enzymatic colorimetric assay.

2.7.3 Modification of the Lowry Protein assay

Protein measurements were performed by a modification of the procedure of Lowry *et al* (Lowry *et al* 1951).

Reagents and solutions

Stock Reagents:

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

Solution B - 2% NaK Tartrate in H_2O (deionised)

Solution C - 1% $CuSO_4$ in H_2O (deionised)

Folin Ciocalteu reagent (BDH): dilute 1:1 with deionised H_2O

Working reagents:

Biuret reagent : To a 100 ml solution A, 1ml solution B and 1ml solution C was added. To this 1 mg/ml of SDS (sodium dodecyl lauryl sulphate) is added if the sample to be analysed is turbid.

Standards:

A stock standard solution of human serum albumin 1mg/ml stored in aliquots at -20°C.

A working standard curve in the range of 0 - 50 μg were prepared by taking appropriate volumes of the stock standard (0 - 50 μl). The final volume was adjusted to 400μl with deionised H_2O.

Quality control:

Bovine serum albumin 0.15 mg/ml and 0.30 mg/ml stored at -20C.

100 μl will be equivalent to 15 μg and 30 μg respectively. The final volume in the assay was adjusted to 400 μl to deionised water.

Sample preparation

Sample requiring dilution were adjusted to a final volume of 400 μl with deionised water. For VLDL1 and VLDL2 fractions 100 μl sample was used for IDL, 50 μl and for LDL 20 μl sample was used.

Method

2.0 ml biuret reagent was added to 400 μl standard, control and samples. These were vortexed and then allowed to stand for 10min. To this 200 μl of Folin Ciocalteu reagent was added with immediate mixing and allowed to stand for 30 min at room temperature. Optical density was measured by absorbance at 750 nm visual wavelength on a DU 70 densitometer.

2.7.4 Analytical ultracentrifugation of HDL

Plasma concentration of HDL2 and HDL3 were estimated by analytical ultracentrifugation in Beckman L8-70 ultracentrifuge equipped with an ultraviolet scanning attachment, using an AnF rotor with double sector centrepiece. HDL subfraction masses were estimated by Mrs Dorothy Bedford and Mrs Elizabeth Murray (Institute of Biochemistry, Glasgow Royal Infirmary).

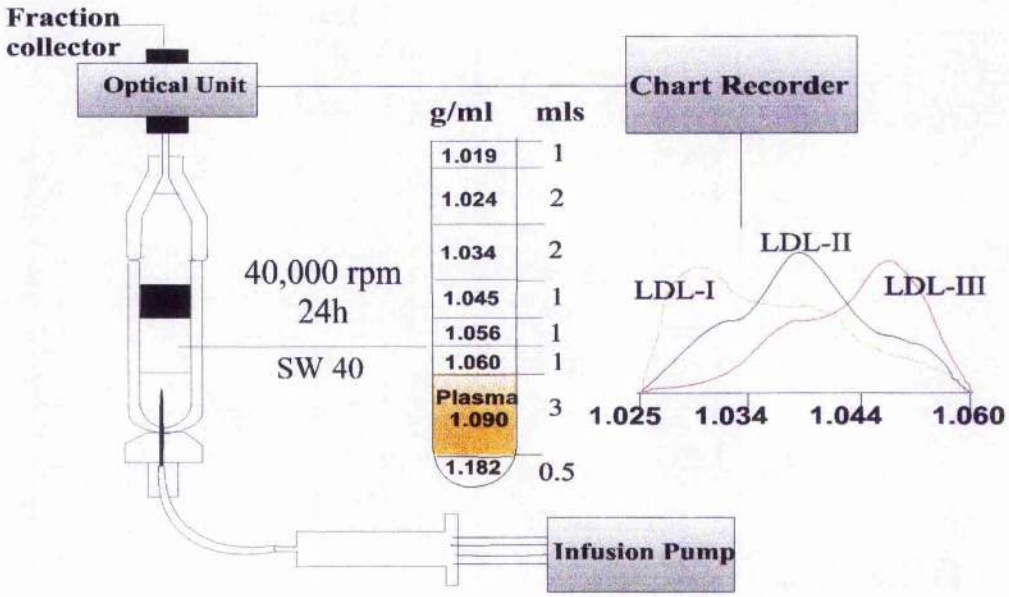
2.7.5 LDL subfraction analysis

The LDL subfraction distribution in all the patients were estimated following the method established by Griffin *et al* (1990) using a non-equilibrium density gradient centrifugation (Fig 2.6).

All density solutions used were prepared from density 1.006 g/ml and 1.182 g/ml and checked with a digital densitometer.

Figure 2.6

Isolation of LDL subfractions using density gradient ultracentrifugation



3 ml fresh plasma was adjusted to a density of 1.09 g/ml by adding 0.25 g KBr and 0.3 ml of density 1.182 g/ml solution. The sample and a 6-step salt gradient were layeredover 0.5 ml of 1.182 g/ml density solution in polyvinyl alcohol coated Beckman polyallomer SW 40 tubes (Fig 2.6) by a peristaltic pump. Centrifugation was carried out at 23°C with slow acceleration at 40,000 rpm for 24 h in a Beckman L8-60 and deceleration with out brake. After centrifugation the LDL subfractions were eluted by upward displacement using a heavy density solution (Maxidens, 1.9 g/ml, Nyegaard) by a constant infusion pump (Sage instruments) at a flow rate of 0.69 ml/min and detected by continuous monitoring by a UV detector (MSE/Fison) at 280nm. Three distinct LDL subfractions, LDLI, LDLII and LDLIII were resolved in all subjects. The individual subfraction areas under the concentration curve were integrated (Data Graphics: Beckman), corrected for differences in extinction coefficient and then expressed as percentage of total LDL. LDLI optical density unit (OD) = 2.63 mg lipoprotein /ml, LDLII OD = 2.94 mg lipoprotein /ml, LDLIII OD = 1.92 mg lipoprotein /ml. Total LDL (d = 1.019 -1.063 g/ml) lipoprotein mass (free cholesterol + triglyceride + cholesteryl ester + phospholipid + protein), determined as above, was distributed on the basis of LDL subfraction concentrations in mg of lipoprotein /dl plasma.

2.7.6 Apolipoprotein E phenotyping

Apolipoprotein E phenotyping was performed on all patients by Mrs Dorothy Bedford (Institute of Biochemistry, Glasgow Royal Infirmary). To detect apoE isoforms, 10 µl of plasma were delipidated, redissolved in 6 mol/l urea containing 0.1 mol/l TRIS pH 10.0, 5% 2-mercaptoethanol and 1% sodium decyl sulphate and applied to a vertical polyacrylamide slab gel of 5% (w/v) acrylamide in 8 mol/l urea containing 1% ampholyte (pH range 4-6). The cathode buffer was 0.2 mol NaOH and anode buffer 0.1 H_3PO_4. Isoelectric focusing was carried out at 3W per gel for 16 h. Transfer by electrophoresis from acrylamide slab gels to nitro-cellulose membranes was performed as described by Towbin *et al* (Towbin *et al* 1979). The transfer buffer contained 0.2 mol/l glycine, 0.025 mol/l TRIS and 20% methanol and the current applied

was 0.4 A for 3 h. The apoE isoforms bound to the membrane were visualised by immunostaining using a monoclonal or polyclonal apoE-specific antibody and an appropriate IgG-binding second antibody linked to horseradish peroxidase.

2.7.7 Lipoprotein (a)

Plasma lipoprotein (a) [Lp(a)] was determined using the commercial kit, Innotest Lp(a) (Innogenetics SA, Belgium). This assay consists of a solid phase mouse monoclonal anti-Lp(a), and a sheep anti-apoB polyclonal second antibody, which is labelled with the enzyme horseradish peroxidase. This label binds to any solid phase antibody/Lp(a) complex because it can bind to the apoB moiety of the Lp(a) complex. Further incubation with enzyme substrate produces a blue colour, which turns yellow when the reaction is stopped with sulphuric acid. The intensity of the colour formed is proportional to the amount of Lp(a) in the original sample.

Chapter 3

APG-LDL interaction...

3.1 Introduction

Focal deposition of lipids in the form of lipoproteins is an important step in the pathogenesis of the early, fatty streak and the later advanced, atherosclerotic plaque. Lipid accumulation occurs both intracellularly and in association with components of the extracellular matrix of the arterial wall (Srinivasan *et al* 1970; 1982; 1986, Bihari-Varga *et al* 1964; 1983, Camejo 1982) consisting of collagen , elastin, microfibrillar protein, laminin and proteoglycans. The last are highly-electronegative glycoproteins, consisting of glycosaminoglycan (GAG) chains covalently attached to a protein core through an O or N- glycosidic linkage (Fig 1.5, chapter 1). These macromolecules influence the viscoelasticity and permeability of the arterial wall intima and media (Berenson *et al* 1984, Wight 1980). There is now general agreement that arterial wall proteoglycans play a key role in the development of atherosclerosis by interacting with plasma lipoproteins (Srinivasan *et al* 1970; 1982; 1986; 1990, Bihari-Varga *et al* 1964; 1983, Camejo 1982, Camejo *et al* 1993, Schwenke & Carew 1989a; 1989b, Berenson *et al* 1984, Wight 1980). It has been shown that intimal pericellular

and extracellular proteoglycans, particularly those rich in chondroitin sulphate (CS) and dermatan sulphate (DS) which are synthesised by smooth muscle cells (Wight 1989, Srinivasan *et al* 1989), bind specifically to apoB containing lipoproteins (Bihari-Varga *et al* 1964, Srinivasan *et al* 1970), VLDL, LDL and lipoprotein (a) but not to HDL (Srinivasan *et al*, 1989;1990, Bihari-Varga *et al* 1988). This binding reaction is believed to modify the structure of the lipoprotein, increase its residence time in the arterial wall and stimulate its uptake by macrophages (Camejo *et al* 1993a, Schwenke & Carew 1989a; 1989b, Srinivasan *et al* 1990, Berenson *et al* 1986), all of which may contribute to foam cell formation. Furthermore, APG-LDL complexes have been isolated from sites of lipoprotein deposition in arterial wall lesions (Srinivasan *et al* 1975; 1984, Mawhinney *et al* 1978, Camejo *et al* 1985a).

The interaction of plasma total LDL with APG, particularly with the CS rich fraction, has been studied both by immuno-histochemical techniques (Hoff & Bond 1983, Lark *et al* 1988) and by in-vitro binding assays (Bihari-Varga *et al* 1964; 1986, Camejo 1982, Srinivasan *et al* 1989, Camejo *et al* 1980, Kempen *et al* 1989, Steele & Wagner 1987, Sambandam *et al* 1991). Total plasma LDL isolated from post - MI patients has been shown to have a greater binding reactivity to APG than that from normal subjects (Linden *et al* 1989). This observation suggests that inter-individual variation in the properties of LDL may influence the interaction. LDL is known to consist of a heterogeneous population of particles which differ in their size, density and metabolic properties (Krauss & Burke 1982). They can be separated by high resolution techniques such as density gradient ultracentrifugation (DGUC) or gradient gel electrophoresis (GGE). The former method distinguishes 3 major subfractions, LDL-I (d =1.019-1.033 g/ml), LDL-II (d =1.033-1.044 g/ml) and LDL-III (d =1.044-1.063 g/ml) in normolipaemic individuals (Griffin *et al* 1990). In case-control studies it has been shown that an LDL profile in which there is a predominance of small dense LDL, determined either by GGE as pattern B (Austin *et al* 1988), or by DGUC as an LDL-III >100mg/dl (Griffin *et al* 1994), is associated with a 3-7 fold increase in risk of myocardial infarction (MI). Predominance of small dense LDL is linked to a pattern of dyslipidaemia in which there is a moderately raised plasma triglyceride level and a low HDL

cholesterol concentration, a profile which has been termed the "atherogenic lipoprotein phenotype" (ALP). This is the commonest lipid disorder seen in patients with coronary heart disease (CHD) (Austin *et al* 1990). In the present study we examined the hypothesis that the perturbed structure of LDL in subjects exhibiting an ALP alters the ability of the lipoprotein to interact with APG and hence contributes significantly to the risk of arterial disease. It was found that the extent of interaction between LDL and APG did vary with plasma lipid levels in general and was enhanced in the presence of an ALP.

3.2 Subjects

Plasma samples were obtained after an overnight fast (12h) from 59 patients (39 men and 20 women) aged 45-73 years who were undergoing diagnostic coronary angiography in the Department of Medical Cardiology, Glasgow Royal Infirmary. Subjects in the fasting state donated 50ml of blood which was collected by venepuncture using K_2EDTA (final concentration 1mg/ml) as anticoagulant. Plasma was harvested at 4°C by low speed centrifugation (3000rpm) and aliquots for lipid, lipoprotein measurements and LDL subfractionation used immediately. Subjects with recent MI (i.e. in the previous 3 to 6 months), renal disease, thyroid disease, diabetes or those taking lipid-lowering medications were excluded, as were those with a plasma cholesterol concentration of >9.0mmol/l and a plasma triglyceride level of >5.0mmol/l. All eligible subjects undergoing angiography were recruited to the study over a 6 month period. The study was approved by the Research Ethics Committee of Glasgow Royal Infirmary and each volunteer gave written informed consent.

3. 3 Statistical analysis

Statistical analysis and manipulations were performed using the PC version of MINITAB Release 10 for Windows (Minitab Inc., PA). All variables were assessed by drawing normality plots and the ones which were not normally distributed were normalised by appropriate transformations. Plasma triglyceride was normalised by log transformation, and LDLI, LDLII, LDLIII

concentrations and HDL cholesterol were normalised by taking their square root. Associations between variables were tested by calculating the Pearson correlation coeffecient. The significance of association between pairs of variables was determined by linear regression.

3.4 Identification and characterisation of proteoglycans

An aorta (Thoracic and abdominal sigments) from 70-year old deceased female, <24h post mortem, was obtained from Department of Pathology, Glasgow Royal Infirmary, the cause of death was aortic valve stenosis and pulmonary embolism with no history of MI. Extraction and purification of proteoglycans was performed as described in chapter 2, section (2.3.3). Eleven fractions (d = 1.38 - 1.63g/ml) were obtained from the $CsCl_2$ gradient following dissociative extraction of proteoglycans from the arterial homogenate with guanidine-HCl (total freeze dried weight = 0.9g) (Table 3.1).

Table 3.1

Chondroitin sulphate (CS), measured by Alcian blue assay, and the protein content of the different APG fractions.

Fraction No.	*1*	*2*	*3*	*4*	*5*	*6*	*7*	*8*	*9*	*10*	*11*
Density g/ml	1.38	1.40	1.42	1.44	1.47	1.49	1.52	1.54	1.57	1.60	1.63
CS µg/mg*	29	30	43	85	78	181	187	195	457	443	459
Protein mg/mg*	0.95	1.00	0.95	0.80	0.70	0.65	0.55	0.50	0.26	0.25	0.24

**µg/mg, mg/mg dry weight*

The three most dense bottom fractions (d>1.57g/ml) contained the highest CS concentration determined by Alcian blue colorimetric assay (63% of the total GAG present) (Hennie & Seraffini 1986 and lowest percentage of protein, while the fractions at the top of the gradient were GAG poor. Fractions 9, 10 and 11 which had similar CS content (Table 3.1) gave an LDL binding reactivity that was 4-8 folds greater than any of the other fractions (Table 3.2).

Table 3.2
The amount of chondroitin sulphate proteoglycan and the LDL binding reactivity of each APG fraction extracted dissociatively with 4mol/l guanidine hydrochloride.

Fraction No.	*1*	*2*	*3*	*4*	*5*	*6*	*7*	*8*	*9*	*10*	*11*
CS/Protein Ratio	<0.1	<0.1	<0.1	0.1	0.1	0.3	0.3	0.4	1.8	1.8	2.0
%CS*	3	3	4	9	8	8	18	20	46	44	50
LDL-Binding reactivity⇑	0.06	0.06	0.07	0.09	0.10	0.16	0.16	0.18	0.54	0.54	0.57

** %CS, the percentage of chondroitin sulphate per weight freeze dried material in each fraction isolated by $CsCl_2$ density gradient ultracentrifugation, measured by Alcian Blue colorimetric assay described by Hennie & Seraffini (1986).*
⇑ LDL-Binding reactivity of each APG fraction measured by absorbance at 600nm.

The gradient contents were divided in to 4 main pools designated "A" (least dense) to "D" (most dense) and the differing proportions of chondroitin sulphate, dermatan sulphate, keratan sulphate and hyaluronic acid was determined (Table 3.3). Heparan sulphate was present in only trace amount in all fractions.

Table 3.3
Glycosaminoglycans and the protein content of the different APG† pools.

APG Pools§	*GAG**					*Protein**
	CS	*DS*	*KS*	*HA*	*Total*	
A	0.03	0.01	nd	0.02	0.06	0.95
B	0.07	0.03	nd	0.05	0.15	0.80
C	0.18	tr[a]	0.08	0.07	0.33	0.58
D	0.45	nd[b]	0.15	0.11	0.71	0.25

** mg/mg dry weight, †APG, arterial proteoglycan; GAG, glycosaminoglycan; CS, chondroitin sulphate; DS, dermatan sulphate; KS, keratan sulphate; HA, hyaluronic acid. [a] tr, traces; [b]nd, not detectable.*
§A, (d, 1.38-1.42g/ml); B, (d, 1.44-1.47g/ml); C, (d, 1.49-1.54g/ml); D, (d, 1.57-1.63g/ml). Heparan Sulphate is present in trace amount in all fractions.

pool "A" was composed of fractions 1-3 (d = 1.38-1.42 g/ml), pool "B" composed of fractions 4-6 (d = 1.44-1.49 g/ml), pool "C" composed of fractions 7 & 8 (d = 1.52-1.54 g/ml) and pool "D" composed of fractions 9-11 (d = 1.57-1.63 g/ml). These fractions were pooled on the basis of their content of CS measured by alcian blue assay, as their LDL complexing activity increased with increasing CS content (Table 3.2, 3.3).

The aminoacid analysis of the pools A-D showed (Table 3.4) that the chondroitin rich sulphate fractions had a different aminoacid composition which was higher in thrionine, serine, leucine and glutamic acid and lower in glycine, cystine, methionine, tyrosine, lysine and arginine, in agreement with the results of other investigators (Kapoor *et al* 1981).

Table 3.4

Amino acid composition of the four different APG pools.

Amino Acid*	***Pool A***	***Pool B***	***Pool C***	***Pool D***
Aspartic acid	107	109	109	125
Thrionine	69	78	93	108
Serine	36	41	49	58
Glutamic acid	89	80	92	96
Proline	100	71	77	73
Glycine	88	110	86	79
Alanine	102	74	74	80
Cystine	8	3	2	2
Valine	38	37	44	42
Methionine	16	7	6	4
Isoleucine	43	106	61	66
Leucine	88	94	95	144
Tyrosine	30	19	14	18
Phenylalanine	38	33	39	35
Lysine	73	65	55	48
Histidine	21	21	27	21
Arginine	54	49	38	36

* *Residues /1000*

Freeze dried material from pool D was stored at -70°C and was reconstituted in the proteoglycan binding buffer pH 7.2 (Section 2.3, chapter 2). It was used in the binding assay because it contained the highest concentration of chondroitin

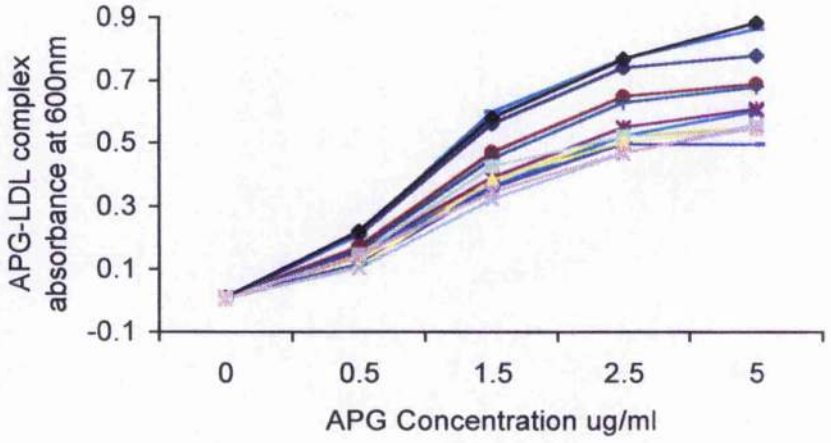
sulphate (CS = 45% of the freeze dried material) and the highest LDL reactivity (Table 3.2, 3.3).

3.5 Turbidity measurement and the amount of cholesterol precipitated by APG.

A standard amount of pool D containing 2.5 μg chondroitin sulphate was selected for the in-vitro binding assay after mixing different concentrations of APG (0, 0.5, 1.5, 2.5 & 5 μg/ml) with a fixed concentration of total LDL protein (0.1mg/ml), measured by the modified procedure of Lowry *et al* (Lowry *et al* 1951). APG-LDL complex formation increased with increasing concentration of APG from 0-2.5 μg/ml thereafter it started to plateau in almost all the LDL samples. Figure 3.1 shows the binding reactivity of total LDL, from 15 subjects with variable lipid profile, with the different concentrations of APG.

Figure 3. 1

The binding interaction of total LDL with different concentration of chondroitin rich APG

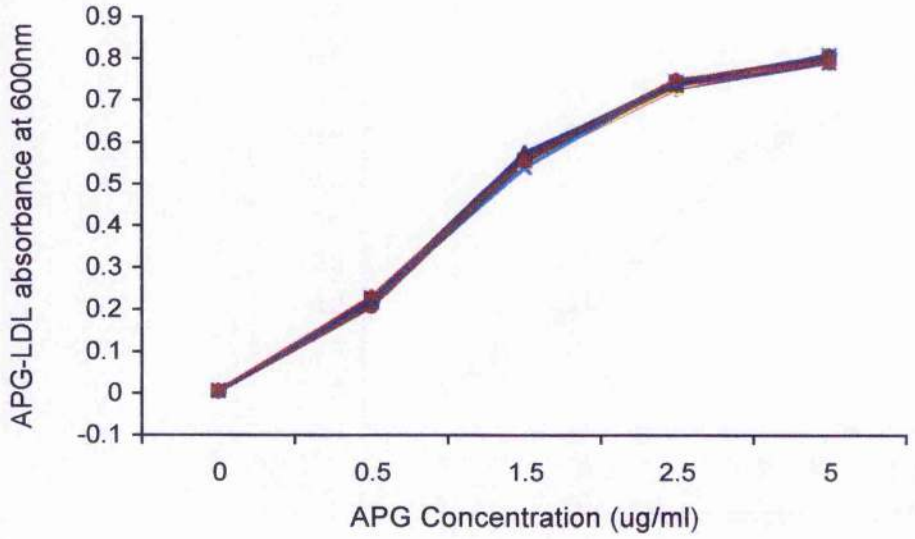


Total LDL protein (0.1 mg/ml) from 15 subjects was mixed with APG (pool D) 0-5μg CS. The extent of APG-LDL complex formation, as measured by absorbance at 600nm visual wave length, increased significantly from 0 to 2.5 μg APG concentration ($p<0.001$). There was no significant differences between complex formation at 2.5 and 5 μg.

The reproducibility of the APG-LDL binding assay was verified with the same sample of LDL. Figure 3.2 show 10 determinations of the binding reactivity of an LDL sample performed in one assay.

Figure 3. 2

The interaction of APG with LDL. 10 determinations of the same LDL sample.



0.1 mg/ml LDL protein was mixed with APG (0-5 μg). CV = 3%, P<0.0001

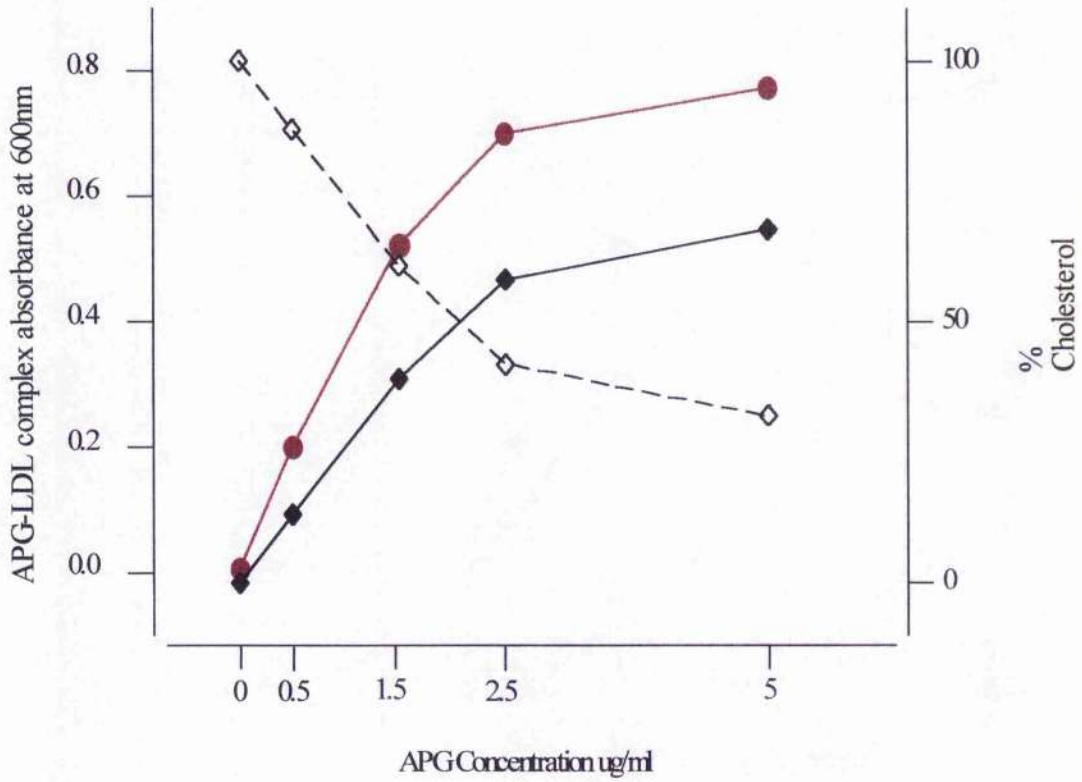
The increase in the turbidity measurement by absorbance at 600nm was associated with a decrease in the percentage of unreacted cholesterol in the supernatant (Fig 3.3) and correlated strongly with the amount of cholesterol precipitated in the pellet ($r = 0.77$, $p<0.0001$). The former was used as a convenient index of LDL reactivity with APG, although the relationships below were also observed with the amount of LDL precipitated.

In order to verify the effect of storage, we performed the binding assay on 20 total LDL samples before and after freezing at -20°C for one week. This resulted in a change in the binding reactivity of the LDL. Most of the samples were found to have a lower reactivity after freezing (Fig 3.4) (Table 3.5). Fresh LDL samples up to 72 hr after isolation stored at 4°C gave the same binding

reactivity in different assays (Table 3.5). Fresh LDL samples were used immediately after isolation for all the in-vitro binding assays.

Figure 3. 3

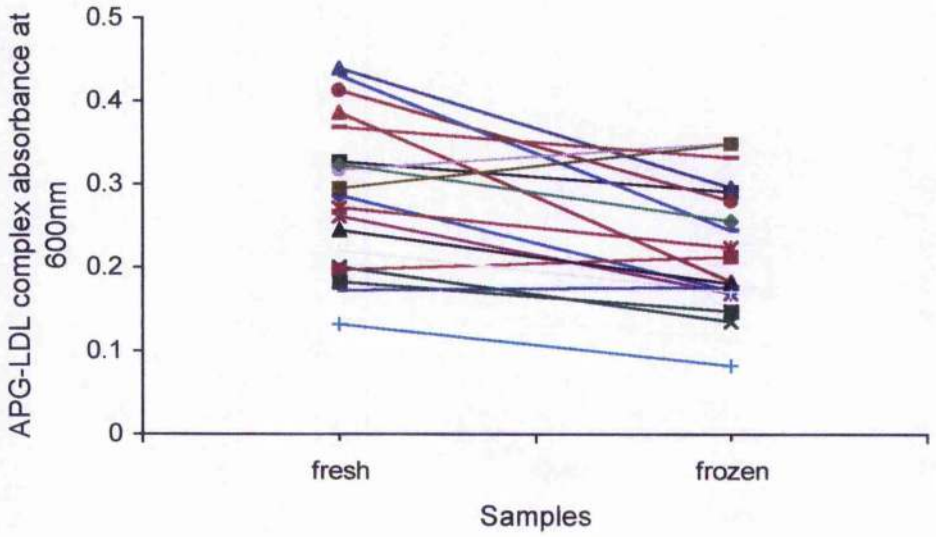
Correlation between turbidity measurement and the percentage of precipitated and unreacted cholesterol in the pellet and the supernatant respectively.



% precipitated cholesterol in the pellet ◆——◆, % unreacted cholesterol in the supernatant ◇·····◇, Turbidity measurement by absorbance at 600nm ●——● . r= 0.77, p<0.0001

Figure 3. 4

The effect of storage by freezing at -20°C for 1 week on the LDL interaction with APG



2.5μg CS-PG was mixed with 0.1 mg/ml LDL protein, from 20 subjects, before (immediately after isolation by sequential gradient ultracentrifugation) and after freezing the same samples for 1 week at -20℃. All determinations were performed in duplicates.

Table 3.5

APG-LDL complex formation, comparison between fresh and stored LDL samples in two different assays

LDL Sample	*Assay 1*	*Assay 2*
Fresh-1	0.23 ± 0.006	0.24 ± 0.004
Fresh- 2	0.46 ± 0.004	0.46 ± 0.002
Fresh- 3	0.55 ± 0.006	0.55 ± 0.005
Frozen	0.29 ± 0.100	0.22 ± 0.100*

** $p<0.01$, values represent mean and standard deviation. Five determinations of fresh LDL samples used within 72 hr after isolation. Duplicates of frozen samples (n=20) were used before and after freezing for 1 week at -20℃.*

3. 6 Lipids and lipoprotein profile

Mean plasma lipid levels and LDL subfraction profile in the subjects are shown in Table 3.6. Plasma triglyceride level was positively correlated with total cholesterol (Fig 3.5), LDL cholesterol (Fig 3.6) and apolipoprotein B concentration within the plasma (Fig 3.7) and negatively correlated with HDL cholesterol (Fig 3.8). Plasma triglyceride was also the strongest and most constant correlate of LDL subfraction concentrations showing a negative correlation with the percentage and concentration of LDL-I (Fig 3.9) and LDL-II (Fig 3.10) and a positive association with small dense LDL-III (Fig 3.11) (Table 3.6) within total LDL.

Table 3.6

The patients lipid and lipoprotein profile. Correlation with Plasma triglyceride and the extent of APG-LDL interaction.

Variable		*Mean*	*r TG**	*r APG-LDL†*
Age		52.5 ± 12.8	0.18	- 0.08
Gender		---	- 0.36**	-0.33**
Total cholesterol	mmol/l	5.6 ± 1.2	0.45*	0.10
VLDL cholesterol	mmol/l	0.6 ± 0.4	0.84*	0.37
LDL cholesterol	mmol/l	3.8 ± 1.1	0.39**	0.18
HDL cholesterol	mmol/l	1.3 ± 0.4	- 0.57*	- 0.37**
plasma triglyceride	mmol/l	1.6 ±	---	0.37**
Apolipoprotein B	mg/dl	1.0 ± 0.4	0.44**	0.33***
LDL-I		19 ± 12.4	- 0.57*	-0.47*
LDL-II		54 ± 14.8	- 0.33**	- 0.19
LDL-III		27 ± 19.4	0.65*	0.48*
HDL/ApoB ratio		1.5 ± 1.1	- 0.53*	-0.41**
Triglyceride/ApoB ratio		1.4 ± 1.1	0.55	0.21

**Plasma triglyceride, †APG-LDL complex determined by turbidity measurement by absorbance at 600nm, Values represent mean ± standard deviation. r, Pearsons correlation coefficient. *p<0.0001, **p<0.001, ***p<0.05*

Figure 3.5

correlation between total cholesterol and plasma triglyceride level

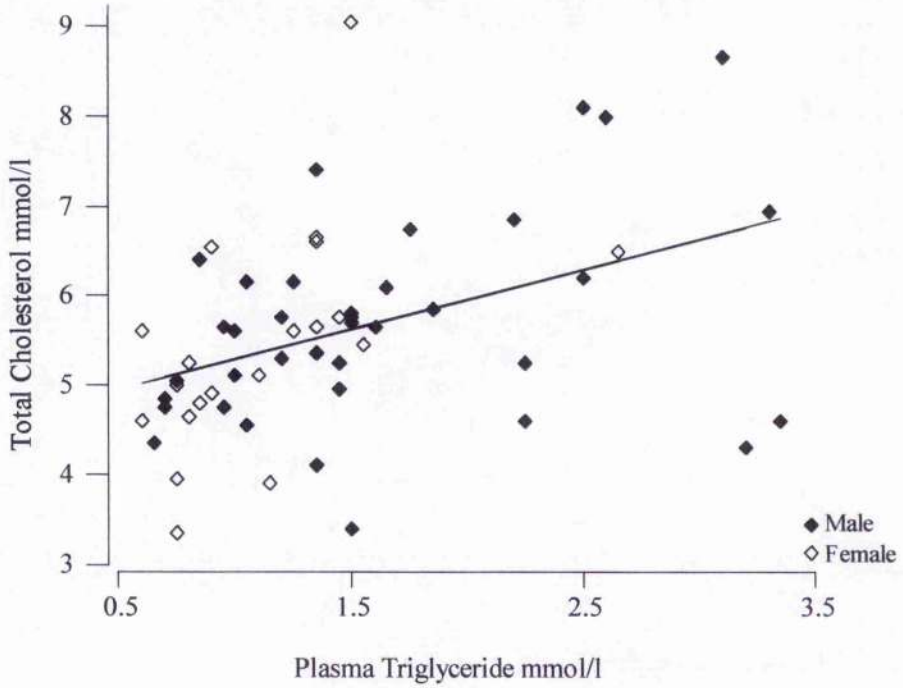


Figure 3.6

Correlation between LDL cholesterol and plasma triglyceride level

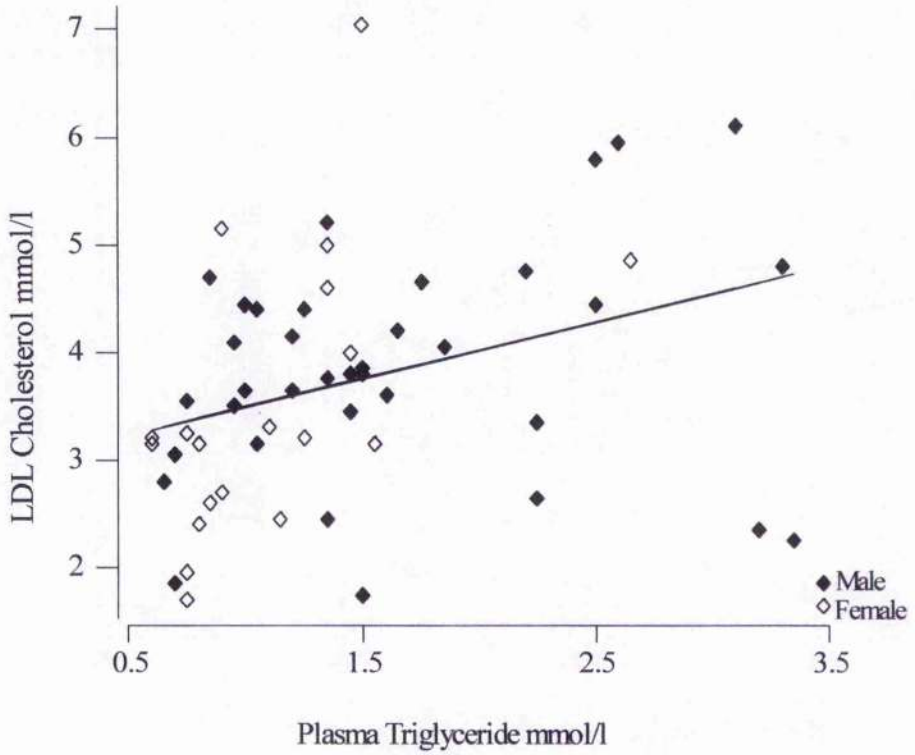


Figure 3.7

Correlation between plasma triglyceride and apoB concentration in plasma

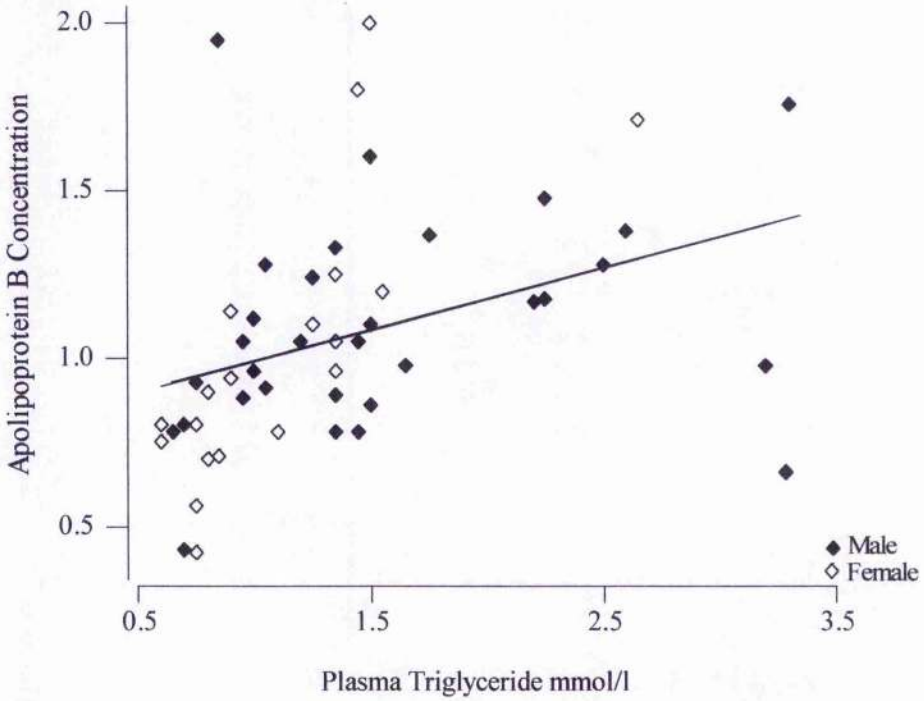


Figure 3.8

Correlation between HDL cholesterol and plasma triglyceride level

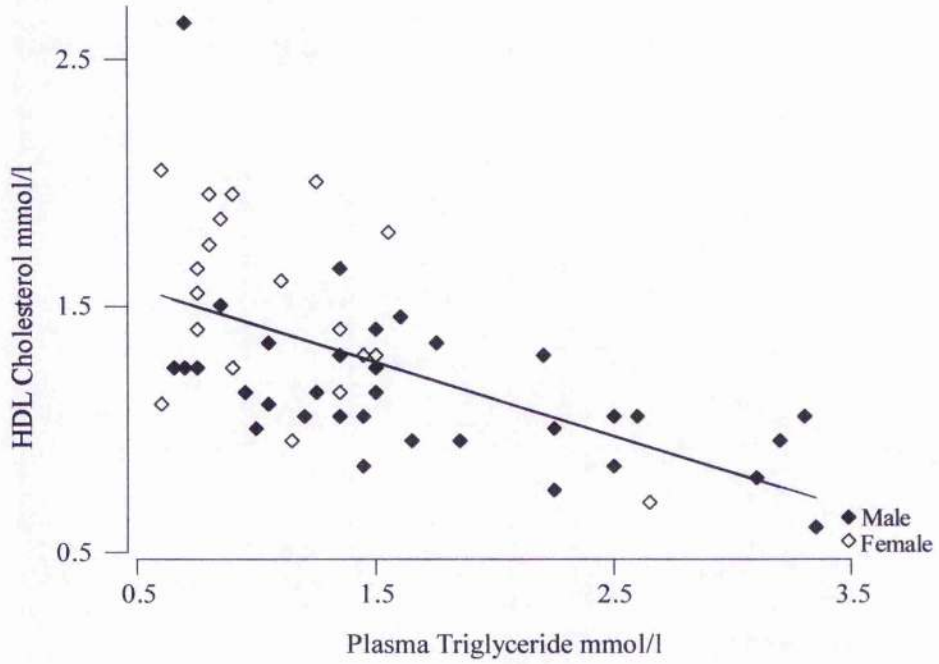


Figure 3.9
Correlation between plasma triglyceride level and the percentage of LDL-I (% LDL-I) within the plasma

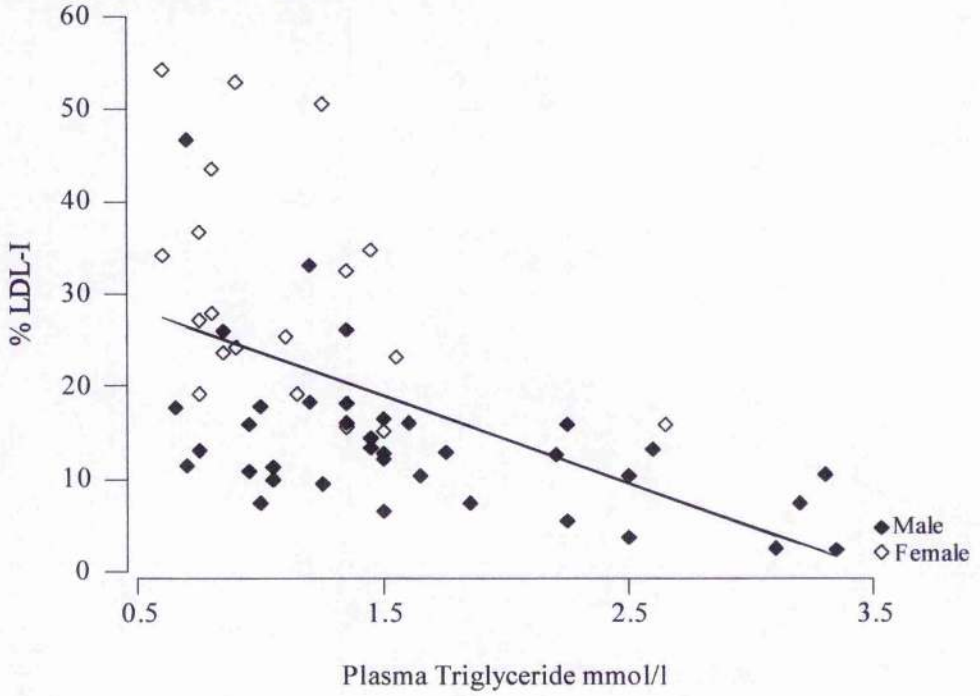


Figure 3.10
Correlation between the percentage of LDL-II within total LDL and plasma triglyceride level

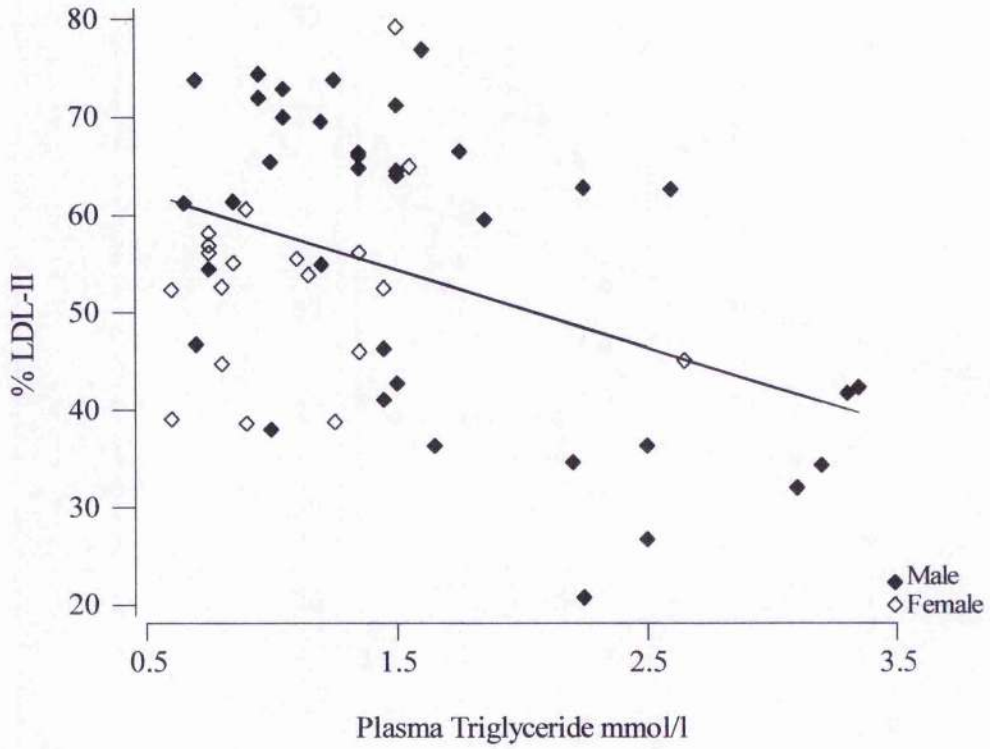
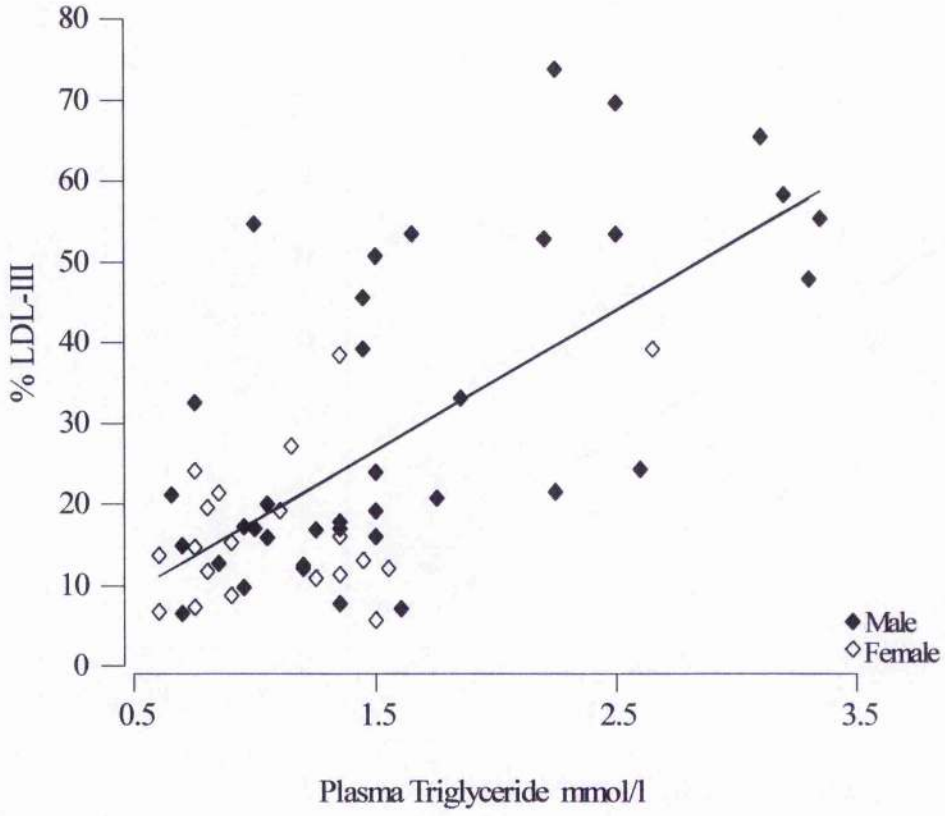


Figure 3.11

Correlation between plasma triglyceride level and the percentage of small dense LDL-III within total LDL



when the subjects were divided on the basis of gender, Plasma cholesterol , LDL cholesterol, VLDL cholesterol were similar between the sexes (Table 3.7). Male patients had higher plasma triglyceride, plasma cholesterol/HDL ratio and small dense LDLIII concentration within total LDL and a lower total HDL cholesterol and LDLI concentration within total LDL (Table 3.7). A typical LDL subfraction profile for men and women is shown in Figure 3.12.

Table 3.7

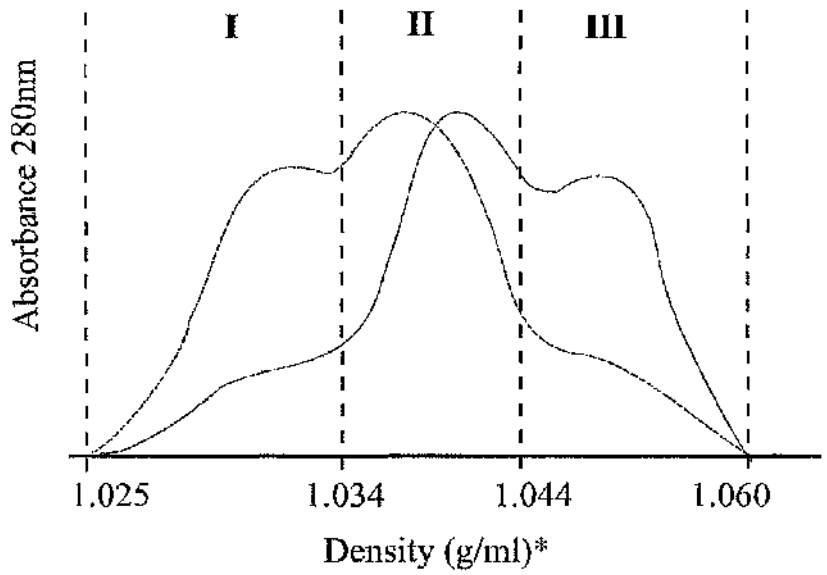
Lipid and lipoprotein profile in male and female subjects

Lipid and LDL subfraction profile		*Mean* *Male n = 39*	*Female n = 20*
Total cholesterol	mmol/l	5.7 ± 1.2	5.4 ± 1.3
LDL cholesterol	mmol/l	3.8 ± 1.1	3.5 ± 1.3
VLDL cholesterol	mmol/l	0.7 ± 0.4	0.4 ± 0.2**
HDL cholesterol	mmol/l*	1.1 ± 0.3	1.5 ± 0.4**
Plasma triglyceride	mmol/l*	1.8 ± 0.8	1.1 ± 0.5**
% LDLI		14 ± 8.3	30 ± 12.5*
%LDLII		54 ± 16.8	54 ± 10.0
%LDLIII		33 ± 21.1	17 ± 9.5*
APG-LDL complex	AU	0.4 ± 0.2	0.3 ± 0.1***

** P <0.0001, **p<0.001, *** P = 0.007, AU; absorbance units at 600 nm*

Figure 3.12

Mean LDL subfraction profile for male and female patients



Male (——) and female (·····) LDL subfraction profile.

3.7 Lipid profile and APG-LDL complex formation

The extent of APG- LDL complex formation was found to be inversely related to HDL cholesterol concentration (Fig 3.13) , positively related to triglyceride (Fig 3.14) but showed no relationship with plasma total (Fig 3.15) or LDL cholesterol (Fig 3.16) (Table 3.6).

Figure 3.13

Relationship between the extent of APG-LDL interaction and plasma HDL cholesterol level.

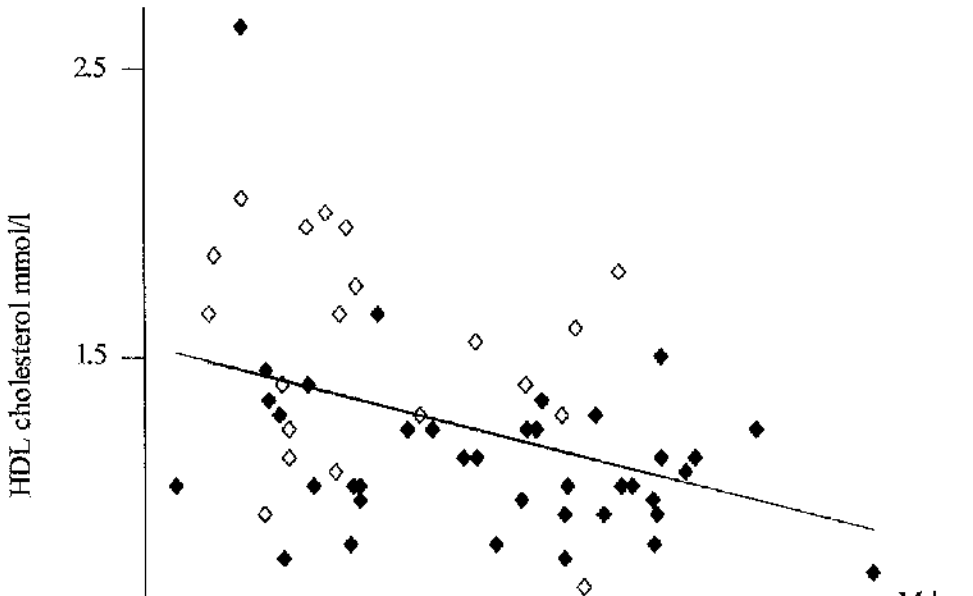


Figure 3.14

Relationship between the extent of APG-LDL interaction and plasma triglyceride level

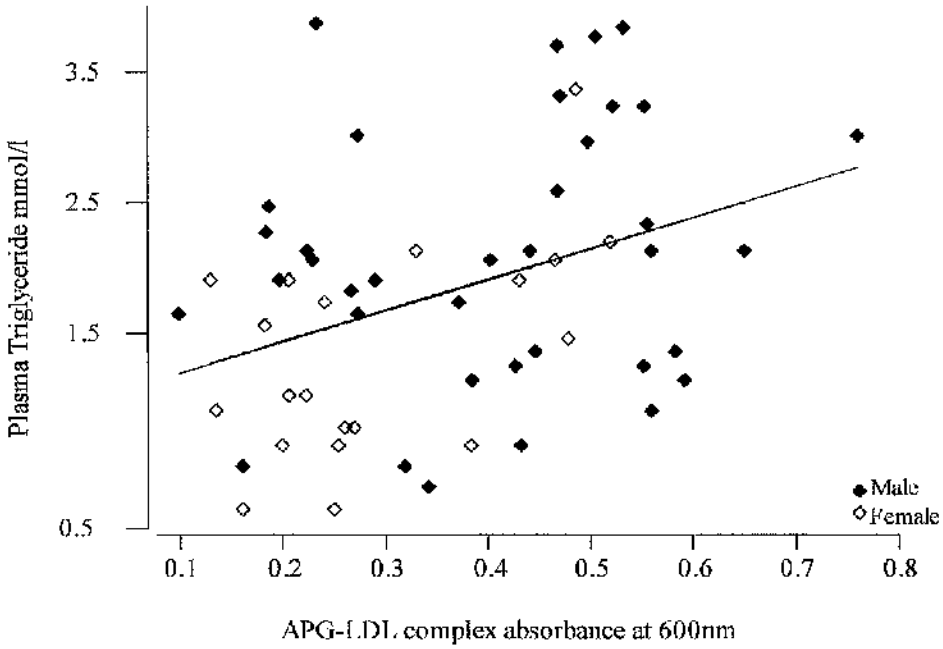


Figure 3.15

Relationship between the extent of APG-LDL interaction and plasma total cholesterol

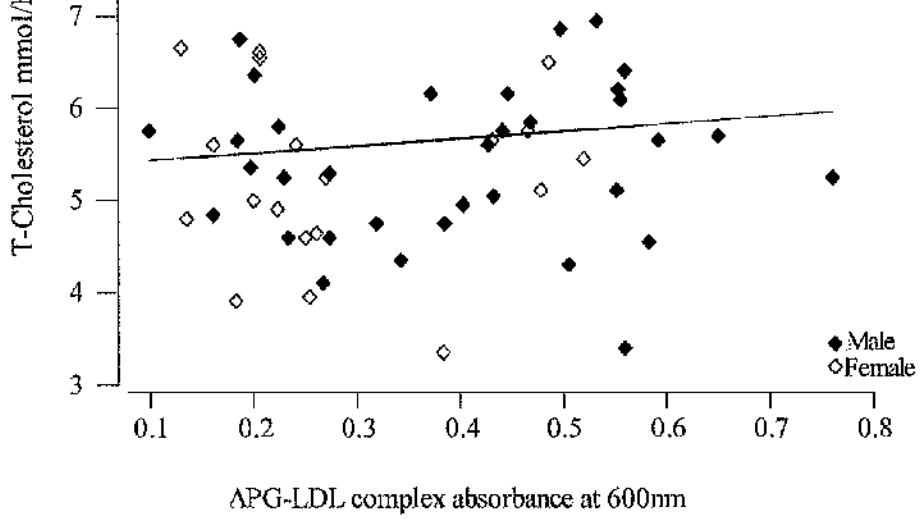


Figure 3.14

Relationship between the extent of APG-LDL interaction and plasma triglyceride level

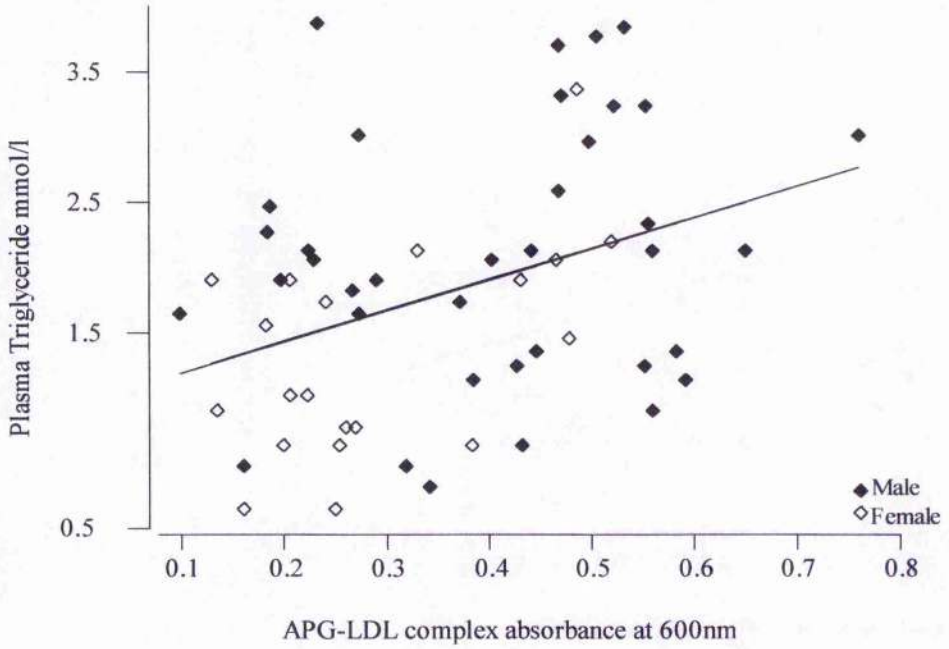


Figure 3.15

Relationship between the extent of APG-LDL interaction and plasma total cholesterol

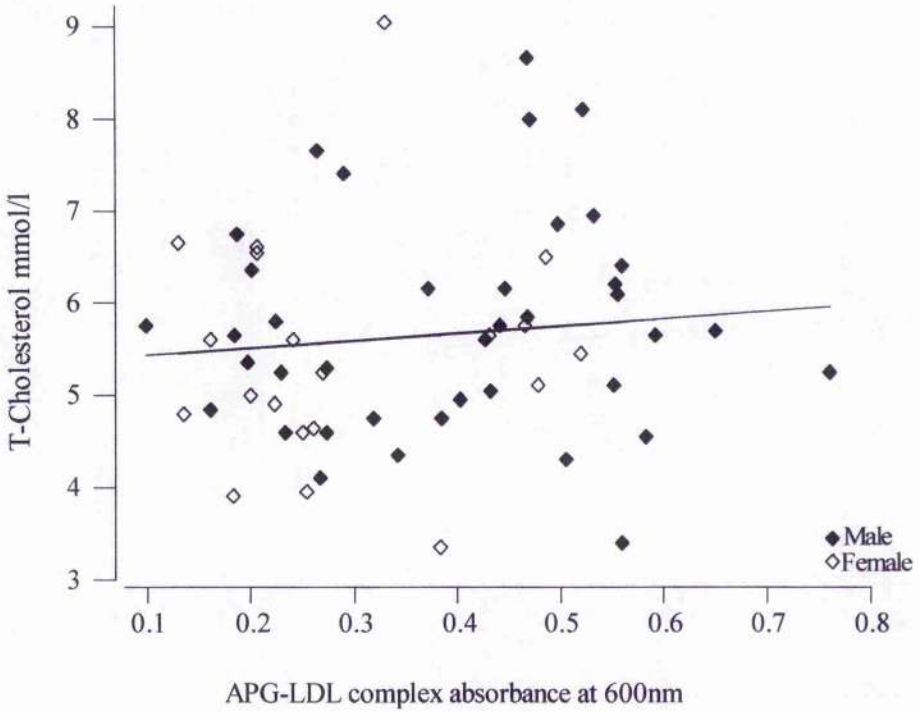
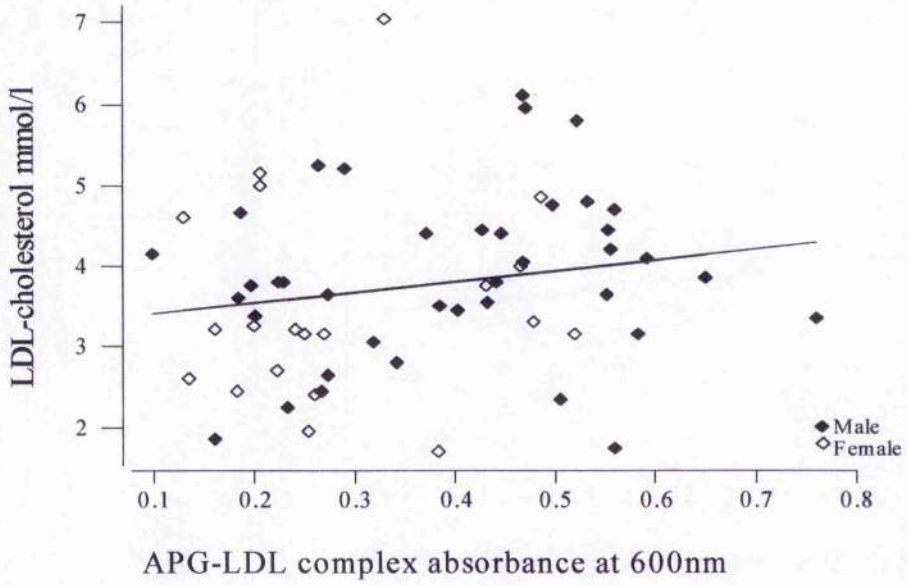


Figure 3.16

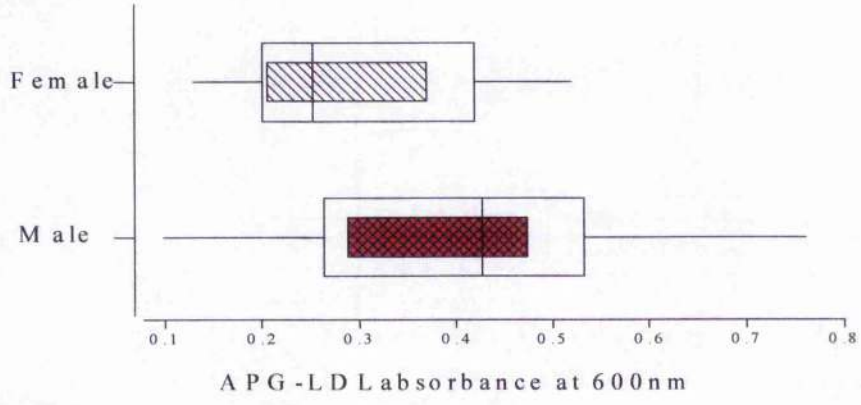
Relationship between the extent of APG-LDL interaction and plasma LDL cholesterol



In general, LDL from males with higher plasma triglyceride level and small dense LDL-III showed a greater reactivity for APG than that from females (0.40 ± 0.16 vs 0.29 ± 0.13, $p = 0.007$) (Fig 3.17).

Figure 3. 17

The extent of APG-LDL complex formation. Comparison between male and female

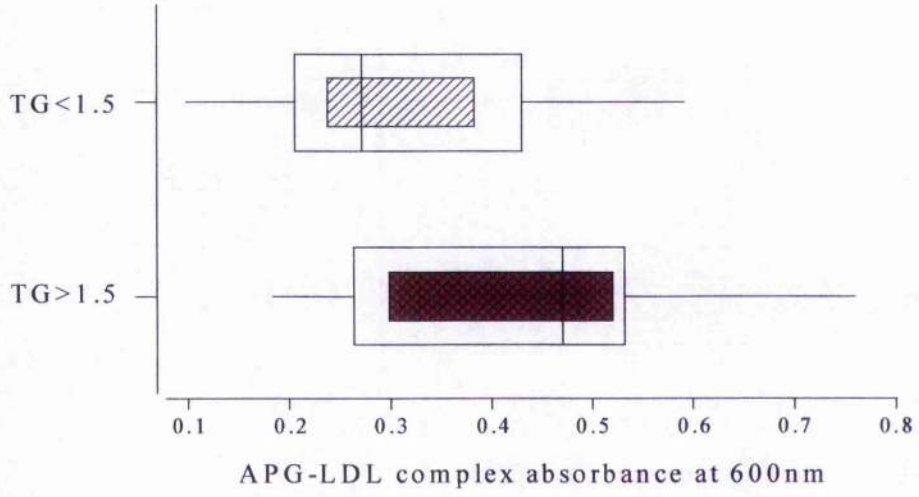


The inner box represents the 25-75 percentile, the median value is indicated by a vertical line, the outer box represents the 5th and the 95th percentile while the range of values are indicated by the horizontal lines. p= 0.007

When the subjects were divided on the basis of their plasma triglyceride being higher or lower than 1.5 mmol/l, APG-LDL complex formation was found to be higher in the subjects with the higher triglyceride level (Fig 3.18)

Figure 3.18

APG-LDL interaction in subjects with plasma triglyceride above and below 1.5mmol/l



The inner box represents the 25-75 percentile, the median value is indicated by a vertical line, the outer box represents the 5th and the 95th percentile while the range of values are indicated by the horizontal lines. p= 0.007.

3.8 Arterial proteoglycan - LDL interaction and LDL subfraction distribution

The extent to which total LDL (d = 1.019-1.063g/ml) interacted with APG was positively correlated with the percentage of small dense LDL-III ($p<0.0001$) (Fig 3.19) and inversely with the percentage of large buoyant LDL-I ($p<0.0001$) within total LDL (Fig 3.20). There was no correlation between the extent of LDL-APG complex formation and the percentage of the major, intermediate density LDL species, LDL-II (Table 3.6). Males in general had a greater proportion of LDL-III ($P<0.001$) and a lower proportion of LDL-I ($P<0.001$) percentage within total LDL than female subjects (Table 3.7) in agreement with previous studies (Griffin *et al* 1994). This finding explained the different reactivity between men and women (Figure 3.17).

Figure 3.19

Correlation between the extent of APG-LDL complex formation and the percentage of LDL-I within total LDL.

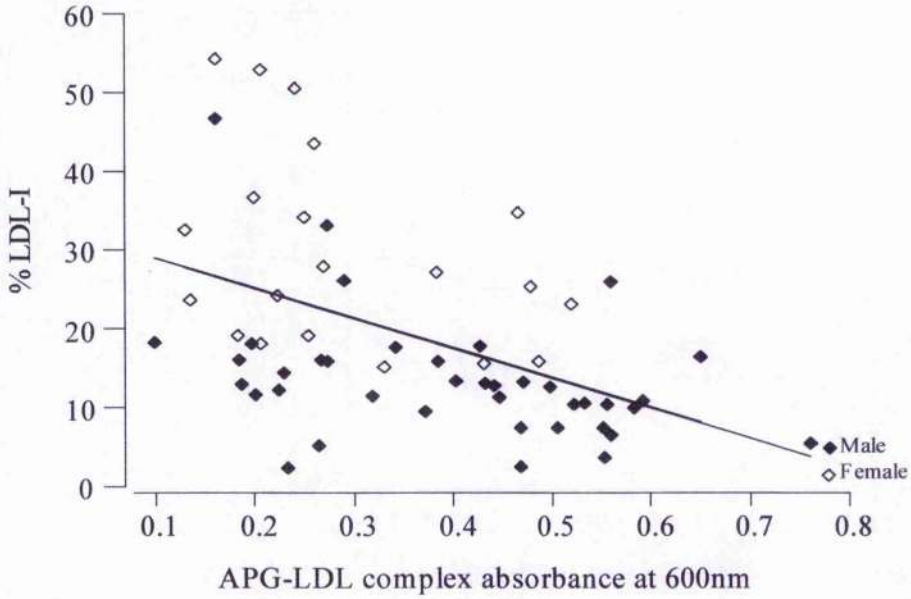
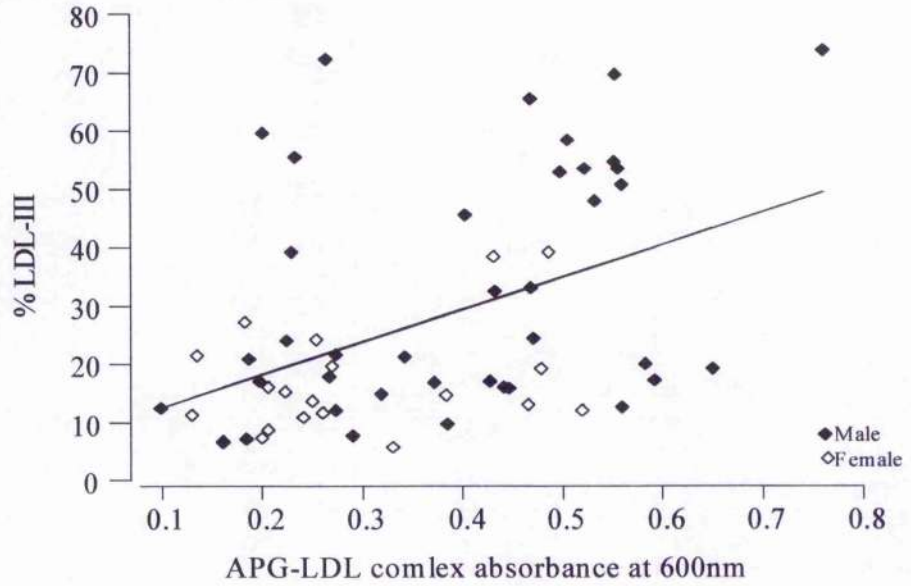


Figure 3.20

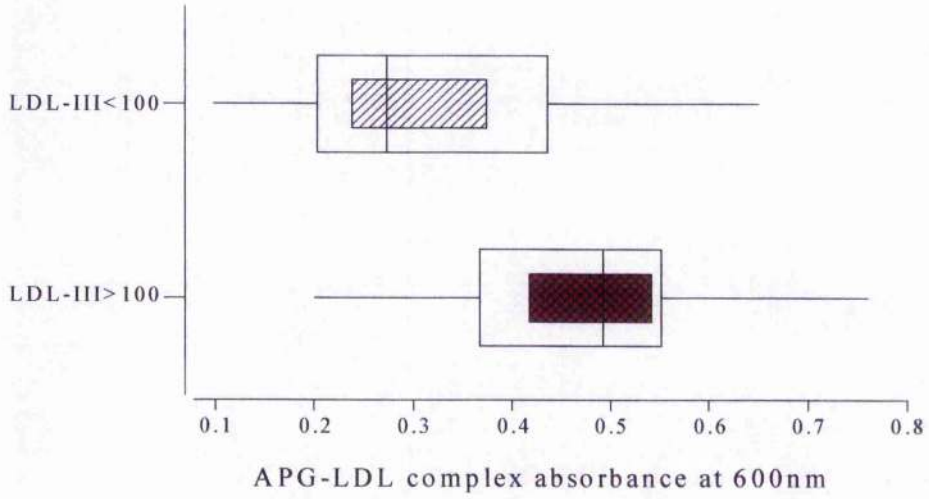
Correlation between the extent of APG-LDL complex formation and the percentage of LDL-III within total LDL.



When the subjects were subdivided on the basis of their LDL-III concentration into those with LDL-III above and below 100mg/dl as an indication of the presence of ALP (Griffin *et al* 1994), it was found that those in the high LDL-III group had an elevated plasma triglyceride ($p<0.0001$) level, a low HDL cholesterol concentration ($p<0.0001$) but the same plasma cholesterol (Table 3.8). The extent of APG-LDL complex formation seen in those with ALP was significantly different from those without ($p<0.002$) (Figure 3.21) (Table 3.8).

Figure 3.21

APG-LDL complex formation in patients with LDL-III above or below 100mg/dl.



The inner box represents the 25-75 percentile, the median value is indicated by a vertical line, the outer box represents the 5th and the 95th percentile while the range of values are indicated by the horizontal lines. *p= 0.002.*

Table 3.8

Lipid, Lipoprotein profile and APG-LDL interaction in subjects with LDL-III mass above and below 100 mg/dl within total LDL

Variable		*LDL-III>100 n=18*		*LDL-III<100 n=41*	
Age		54	± 8.8	52	± 14.3
Total cholesterol	mmol/l	6.0	± 1.4	5.5	± 1.1
VLDL cholesterol	mmol/l	1.0	± 0.4	0.4	± 0.2*
LDL cholesterol	mmol/l	4.0	± 1.2	3.7	± 1.1
HDL cholesterol	mmol/l	0.9	± 0.2	1.4	± 0.4*
Plasma Triglyceride	mmol/l	2.4	± 1.0	1.2	± 0.4*
LDL-I mass	mg/dl	31.9	± 21.3	70.5	± 37.0*
LDL-II mass	mg/dl	130.8	± 61.2	193.0	± 74.3**
LDL-III mass	mg/dl	186.3	± 80.4	47.5	± 18.8*
Apolipoprotein B	mg/ml	1.2	± 0.5	1.1	± 0.4
HDL/Apo B ratio		1.6	± 1.0	0.9	± 0.4**
APG-LDL	AU†	0.46	± 0.1	0.32	± 0.1**

** P<0.0001, **p<0.001, values represent mean ± standard deviation.*
† AU, absorbance unit determined by turbidity measurement by absorbance at 600nm.

3.9 DISCUSSION

The nature of the APG-LDL interaction is not yet understood although a number of studies have focused on elucidating factors that influence the binding reaction (Srinivasan *et al* 1982; 1989, Berenson *et al* 1986, Camejo *et al* 1980b; 1985a; 1989, Steele & Wagner 1987). Clearly variation in the structure of APG as well as lipoprotein could contribute to the variation observed. The composition of proteoglycans dissociatively extracted from arterial wall by 4.0M Gdn-HCl has been studied by a number of investigators (Ogema *et al*

1979, Salisbury & Wagner1981, Wagner *et al* 1986, Kapoor *et al* 1981). CS has been shown to be the predominant GAG of the aorta and a major component of the 4.0M Gdn-HCl extract (Wagner *et al* 1986). It binds most avidly to LDL (Srinivasan *et al* 1989, Kempen *et al* 1989, Olsson *et al* 1993) and our findings were consistent with this observation. Pool D contained ~15-times more CS-PG than pool A and showed a higher LDL binding reactivity (Table 3.2). The next most abundant GAG species was keratan sulphate as described previously (Ogema *et al* 1979, Kapoor *et al* 1981) and a small amount of hyaluronic acid (11%) (most hyaluronic acid remains in the residual tissue (Kapoor *et al* 1981)). DS-PG was present in the lower density fractions (pool A&B) (Table 3.3) but not in pool D used in the assay. Since dermatan sulphate has been shown to be on the same GAG chain as C-4-SO_4 (Berenson *et al* 1986), this finding suggest that the CS present in pool D is mainly of C-6-SO_4. In addition amino acid analysis showed similar results as the proteochondroitin-6-sulphate isolated by Kapoor *el al* (Kapoor *et al* 1981). Heparan sulphate is resistant to Gdn-HCl extraction (Berenson *et al* 1986, Salisbury & Wagner 1981, Kapoor *et al* 1981) therefore was only present in traces in the APG preparation. The CS-PG which binds most avidly to LDL has been described to be a C-6-SO4 isomer with a large protein core (Breneson et al 1986) and a high molecular weight (Wagner *et al* 1986, Alves & Mourao 1988) and oversulphation rather than chain length has been shown to be important in the binding process (Sambandam *et al* 1991). In the present study further characterisation of the CSPG with regard to chain length or the degree of sulphation was not performed, rather the limited amount of material from a single aorta was used in all binding assays to reduce the potential APG variation and reveal lipid and lipoprotein factors that affected binding. In-vitro, optimal binding of LDL to APG occurs near physiological pH and ionic strength (Steele & Wagner 1987) and the interaction is known to be a function of charge (Camejo *et al* 1985b). It has been suggested that LDL with a high isoelectric point and a low sialic acid content is particularly reactive towards APG (Camejo *et al* 1985, Hurt-Camejo *et al* 1990). Olsson *et al* (Olsson *et al* 1993) have recently identified a peptide segment of apoB, rich in arginine and lysine residues, which appears to be the principal site mediating the APG-LDL

interaction. They have also shown that patients with acute or chronic CHD show increased reactivity of their LDL for APG (Camejo *et al* 1989) and suggest that APG can distinguish from within total LDL a subclass with a higher isoelectric point, a lower ratio of surface to core lipids and a higher affinity for CS-APG that is present variably in different individuals (Hurt-Camejo *et al* 1990). The present study builds on these earlier observations by providing evidence that the APG-LDL interaction is indeed highly variable and related to the plasma lipid profile and distribution of LDL subfractions.

Total LDL was chosen as the substrate for the reaction with APG because it is the complete lipoprotein class that is presented to the arterial wall of an individual. Plasma or serum was not used to eliminate the effect of other factors present in plasma which can affect the interaction such as plasma fibronectin which inhibit LDL-APG complex formation (Kampen et al 1989, Labat-Robert *et al* 1990) or Lp(a) level which has a higher affinity to APG (Bihari-Varga *et al* 1988). LDL is known to be heterogeneous and composed of at least three subpopulations (Griffin *et al* 1990), the distribution of which in a subject is strongly influenced by plasma triglyceride and HDL levels (Griffin *et al* 1994), the presence of insulin resistance syndrome (Reaven *et al* 1993) and by gender. We found that variation in HDL (consistent with previous findings by Camejo (Camejo *et al* 1980b)) levels and plasma triglyceride was associated with altered reactivity of total LDL towards APG (Figure 3.13, 3.14) and hypothesised that these influences derived from metabolic disturbances which altered LDL structure (Griffin *et al* 1994). When the LDL subfraction pattern itself was related to APG reactivity, it was observed that there was a negative association with the relative abundance of the least dense LDL-I (which is found in high concentration in the plasma of young females and is thought not to be atherogenic (Griffin *et al* 1994)) and a positive association with the relative abundance of small dense LDL-III. This subfraction is present in high concentrations in the plasma of subjects with CHD and is believed to be the most atherogenic LDL species, in part, because it is readily oxidised (deGraaf *et al* 1991), a pre-requisite step in the uptake of lipoproteins by macrophages. The variation in the LDL-APG binding for the same value of LDL-I or LDL -III in different individuals might be due to different medications taken by the

patients such as beta-blockers which has been described to lower LDL-APG interaction (Linden *et al* 1990). An ALP is the most common dyslipidaemia seen in MI survivors (Griffin *et al* 1994, Austin *et al* 1990) and it was observed that when patients were divided into those who had an LDL-III above or below 100mg/dl, the group with the higher LDL-III concentration show significantly greater reactivity for APG. The difference in the LDL subfraction profile (LDL-III is, in general, more prevalent in males) was also the likely cause of the sex difference. If the reactivity between APG and LDL is due to specific epitopes present on apoB (Kinoshita *et al* 1990) as opposed to differences in lipid and carbohydrate content (Camejo *et al* 1985) then our findings suggest that this binding site may be differentially expressed in individuals and revealed to a greater extent in small dense LDL. This proposal accords with the knowledge that the conformation of apoB is influenced by LDL particle size (Galeano *et al* 1994). These findings help to explain, in part, the association of ALP with increased risk of CHD.

Chapter 4

Interaction of very low density, intermediate density and low density lipoproteins with arterial wall proteoglycans...

4.1 Introduction

Most investigators now agree that one or more apoB containing lipoproteins either in their native form or modified by oxidation or complex formation is responsible for cholesterol deposition in the artery. The atherogenic role of low density lipoprotein, especially small dense LDL, is well established by epidemiological studies, clinical findings and controlled trials (Gordon *et al* 1977, Frick *et al* 1987, LRCP 1984a, 1984b). One possible mechanism by which LDL can cause atherosclerosis is its interaction with arterial wall proteoglycans (APG). APG, especially chondroitin sulphate proteoglycans (CS-PG), have long been known to specifically interact with apoB containing lipoproteins mainly LDL, LP(a) and to a lesser extent VLDL, but not HDL (Vijayagopal *et al* 1981, Bihari-Varga *et al* 1988, Camejo *et al* 1980a; 1980b; 1985, Srinivasan *et al* 1989, Olsson *et al* 1993,). This interaction leads to their entrapment, modification and uptake by macrophages in the intimal extracellular space leading to foam cell formation.

The isolation of LDL from atherosclerotic lesions, the higher affinity of LDL from survivors of myocardial infarction towards APG by in-vitro binding assays (Srinivasan *et al* 1975, Camejo *et al* 1985a, Linden *et al* 1989) and our findings of the positive association between atherogenic lipoprotein phenotype (ALP) and APG-LDL complex formation (Chapter 3, Anber *et al* 1996) provides further evidence for the atherogenicity of LDL. There is now evidence that VLDL and IDL have atherogenic potential too. They have also been isolated from atherosclerotic plaque and have been shown to enter the arterial wall and share with low density lipoprotein (LDL) the potential for causing lipid accumulation (Shaikh *et al* 1991, Nordestgaard *et al* 1992, Nordestgaard& Nielson 1994). Their low efflux rate due to their particle size (Nordestgaard *et al* 1992) augments the rate of cholesterol delivery to the arterial wall as they contain 5 or more times cholesterol and cholesteryl ester per particle than LDL (Mahley *et al* 1979).

We have shown that individuals with an increase percentage of LDL III (small dense LDL) possess LDL with a greater binding affinity towards APG (Chapter 3, Anber *et al* 1996) an extension of the interesting finding by Hurt-Camejo *et al* (Hurt-Camejo *et al* 1990) that when APG was used to fractionate LDL it had a preference for small dense LDL. However the absolute reactivity of each subfraction within an individual is not known nor the relative reactivity of lipoprotein species across the S_f 0-400 spectrum. Lipid lowering treatment with fibrates affects both plasma triglyceride and cholesterol and is associated with a change in the LDL subfraction profile (Caslake *et al* 1995). Recently it has been shown that a number of lipid lowering therapies reduce total LDL binding with APG (Wiklund *et al* 1996). To explore further the mechanism of this binding process and to support the concept that triglyceride rich lipoprotein particles (TGRL) are atherogenic we undertook a series of studies, the objectives of which were: 1) to compare the reactivity of different apoB containing lipoprotein subfractions with APG, 2) to test the effects of lipid lowering treatment with ciprofibrate, which changes an individuals LDL subfraction pattern, on different apoB containing lipoprotein subfractions and their interaction with APG.

4.2 Subjects

A total of twenty eight subjects were recruited for both studies. All donated 50ml of blood after an overnight (12h) fast. Fasting blood was collected by venepuncture using K_2EDTA (final concentration 1mg/ml) as an anticoagulant. Plasma was separated at 4°C by low speed centrifugation (3000rpm) and aliquots for lipid, lipoprotein measurements and LDL subfractionation used immediately.

4.2.1 Study I: The reactivity of different apoB containing lipoproteins with APG

Eighteen subjects aged 19-60 years were divided into three groups (six subjects in each group) on the basis of their LDL subfraction profile and the presence or absence of ALP (Table 4.1). Group I were young healthy normolipidemic, aged 19-22 years, (5 female and 1 male) whose LDL profile predominated by LDL-I, group II were healthy normolipidemic (2 female and 4 male) aged 34-46 years, had mainly LDL-II profile, and group III were hyperlipidemic (1 female and 5 male) aged 39-60 years with a profile in which LDL-III was the major species present.

4.2.2 Study II: The effect of lipid lowering treatment with Ciprofibrate on APG-Lipoprotein interaction

Twelve male patients aged 40-60 yr. were selected on the basis of their cardiac catheterisation results (all with angiographically positive coronary artery disease) from the cardiac catheterisation unit at the Glasgow Royal Infirmary. They had a total cholesterol >5.2mmol/l and a plasma triglyceride <3.5mmol/l. Baseline assessment of lipid and lipoprotein levels, LDL subfraction profile and APG reactivity were performed. Patients were then commenced on ciprofibrate 100mg/day for 8 weeks. After the eighth week a second assessment of lipids and lipoprotein levels, LDL subfraction profile and APG reactivity were performed. The characteristics of each patient are summarised in Table 4.1.

All the patients studied were receiving prescribed medications for other clinical conditions, and these were continued unchanged throughout the course of the study.

Ciprofibrate was well tolerated by all subjects participating in the study. Two of the patients withdrew from the study, one had a myocardial infarction and the other could not continue because of difficulty in commuting..

The following Exclusion Criteria were applied:

1. any lipid lowering medications at the time or up to 6 weeks before recruitment.
2. known or suspected hypersensitivity to the fibrate group of drugs.
3. a recent MI (i.e. in the previous 3 to 6 months).
4. signs of renal or hepatic impairment or thyroid disease.
5. diabetics or had an impaired glucose tolerance test.
6. consumption of more than 22 units of alcohol per week.

Both studies were approved by the Research Ethics Committee of Glasgow Royal Infirmary and each volunteer gave written informed consent.

4.3 Statistical analysis

Statistical analysis and manipulation were performed using MINITAB release 10 for windows (Minitab Inc., PA). Pearsons correlation , simple regression, 2 sample t-test and one way analysis of variance (ANOVA) were used to asses the relationship between variables.

Table 4.1

Summery of patients characteristics

Subject	*sex*	*age*	*drug therapy*	*clinical diagnosis*
cip 01	M	50	Diltiazem, Aspirin, GTN	Angina, MI (January 1995)
cip 02	M	41	Imidur, Aspirin, GTN	2 vessel disease
cip 03	M	56	Atenolol, Aspirin, GTN	Ischaemic pain, positive angiogram, family history of CHD, hypertension
cip 04	M	63	Amlodipine, Tildiem retard, Isosorbide mononitrate, Aspirin	Angina, +ve angiogram
cip 05	M	51	Atenolol, Ikorel, Aspirin	Coronary angioplasty
cip 06	M	54	Prosac, Ditiazem, Enalapril, Aspirin	Angina, +ve angiogram, strong family history
cip 07	M	38	GTN spray, Isosorbide dinitrate, Aspirin, Losec	Angina, hypertension
cip 08	M	47	Captopril, Diltiazem, Aspirin	Angina, +ve angiogram
cip 09	M	63	Atenolol, Isosorbide dinitrate	Angina, MI (1992), coronary bypass surgery (1993)
cip 010	M	62	GTN	Exertional angina, +ve angiogram, nontoxic goitre

GTN; Glycerine trinitrate, MI; Myocardial infarction.Cip-o1-010 are codes for patients on ciprofibrate therapy.

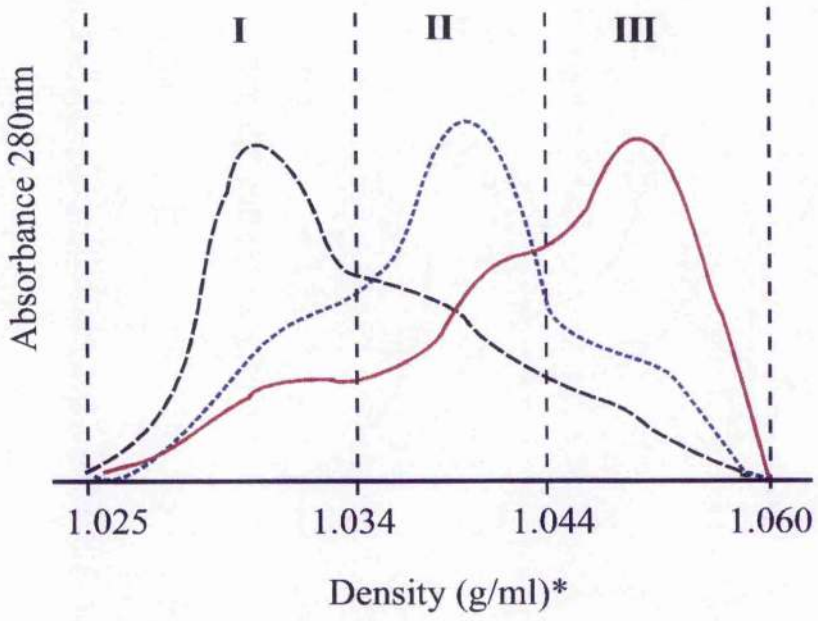
4.4 Results

4.4.1 Study I: Lipid and LDL subfraction profile

Mean plasma lipid level and LDL subfraction profile for the three groups of subjects are shown in Table 4.2. A one way analysis of variance showed that plasma total cholesterol, triglyceride level and LDL subfraction profile in Group III subjects were significantly different from groups I & II subjects ($p<0.0001$). Group I & II had similar lipid levels but differed in their LDL subfraction profile $p<0.0001$ (Table 4.2) (Fig 4.1).

Figure 4. 1

LDL subfraction profile among the three groups of subjects.



Group I (·····), Group II (—) and group III (——) LDL subfraction profile.

Table 4.2

Lipid variables and LDL subfraction distribution within total LDL for the three groups of subjects

Lipid and LDL subfraction profile		*Group I** *n = 6*	*Group II** *n = 6*	*Group III** *n = 6*	*p*
Total cholesterol	mmol/l	3.7 ± 0.5	3.8 ± 0.5	7.1 ± 0.7	<0.0001
Triglyceride	mmol/l	0.7 ± 0.2	1.1 ± 0.3	2.1 ± 0.5	=0.007
LDL cholesterol	mmol/l	1.9 ± 0.4	2.1 ± 0.4	4.6 ± 0.8	<0.001
HDL cholesterol	mmol/l	1.5 ± 0.4	1.1 ± 0.2	1.0 ± 0.1	0.1
LDLI%†		52 ± 9	22 ± 3	13 ± 1	<0.0001
LDLII%†		39 ± 7	62 ± 3	34 ± 16	<0.05
LDLIII%†		9 ± 5	16 ± 2	53 ± 16	=0.008

**Group I; group II and group III represent young healthy subjects with predominantly LDLI , middle aged normolipidemic with mainly LDLII and hyperlipidemic subjects with predominantly small dense LDL subfraction profile respectively, data represent mean ± SD (standard deviation) for each variable*

† LDLI% ; LDLII%; LDLIII%, the percentage of low density lipoprotein I, II, and III subfractions within total LDL.

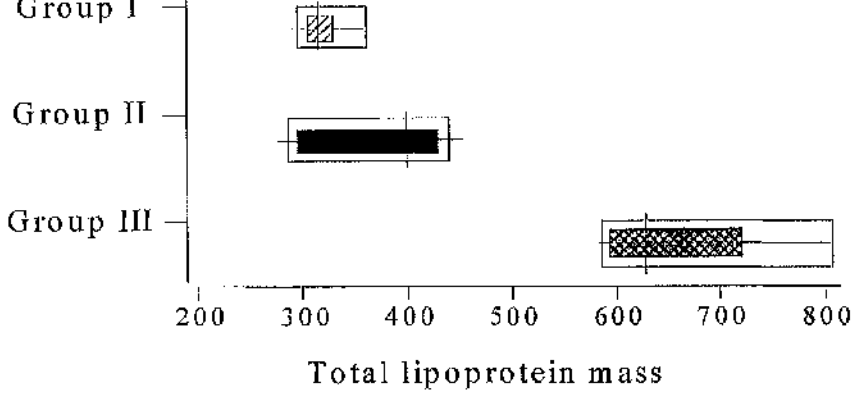
ApoB containing plasma lipoproteins (VLDL, IDL, and LDL) were subfractionated from fresh plasma by a modification of a previously published cumulative density gradient ultracentrifugation procedure (Lindgren *et al* 1972), as described in chapter 2. Six subfractions, two from each lipoprotein class were obtained from each subject, i.e. VLDL1 S_f (Svedberg flotation rate) (60-400), VLDL2 S_f (20-60), IDL1 S_f (16-20), IDL2 S_f (12-16), LDL A S_f (8-12) and LDL B S_f (0-8) (Table 4.3).

VLDL1, VLDL2 and LDLB fractions were significantly different between the three groups of subjects. They were higher in group III compared to group I & II subjects. Table 4.3 shows that there were no significant differences between the IDL1, IDL2 and LDLA mass among the three groups of subjects.

Total plasma apoB containing lipoprotein mass was significantly lower in groups I & II (327mg/dl & 334mg/dl respectively) compared to group III subjects (605mg/dl) $p<0.001$ (Fig 4.2).

Figure 4.2

Total apolipoprotein B containing lipoprotein mass among the three groups of subjects



Group I, young healthy normolipidemic with predominantly LDL-I subfraction profile, Group II, normolipidemic with predominantly LDL-II profile, Group III, hyperlipidemic with predominance of small dense LDL. The inner box represents 25-75 percentile, the median value is indicated by a vertical line, the outer box represents the 5th and the 95th percentile, while the range of values are indicated by the horizontal line. The total lipoprotein mass was lower in group I & II compared to group II ($p<0.0001$).

Table 4. 3

ApoB containing lipoprotein mass among the three groups of subjects

ApoB containing lipoproteins		*Group I** *n = 6*	*Group II** *n = 6*	*Group III** *n = 6*	*p*
VLDL1‡	mg/dl	23 ± 12	60 ± 27	126 ± 65	=0.04
VLDL2‡	mg/dl	66 ± 4	41 ± 12	76 ± 8	=0.002
IDL1‡	mg/dl	55 ± 41	18 ± 5	27 ± 8	0.13
IDL2‡	mg/dl	41 ± 52	40 ± 13	50 ± 21	0.88
LDLA‡	mg/dl	86 ± 31	96 ± 33	128 ± 64	0.48
LDLB‡	mg/dl	55 ± 11	77 ± 38	181 ± 61	<0.01

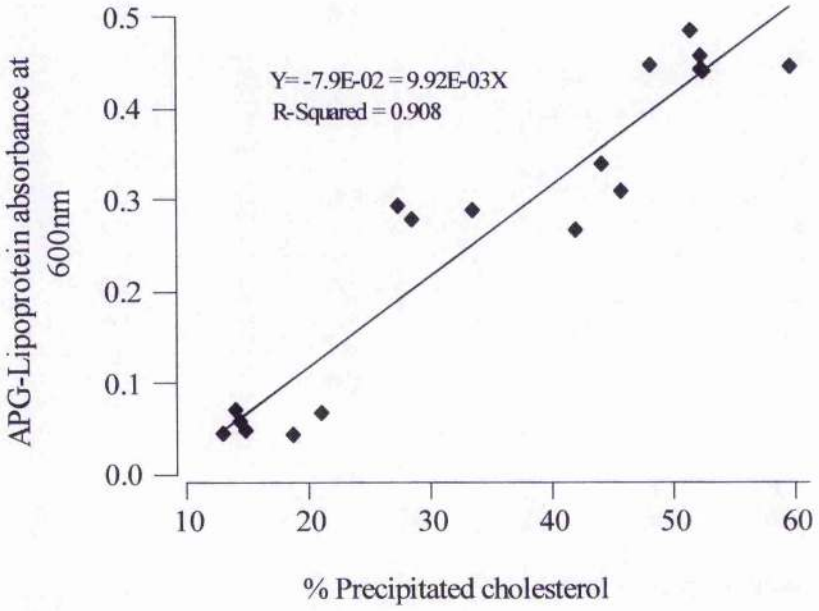
**Group I; group II and group III represent young healthy subjects with predominantly LDLI , middle aged normolipidemic with mainly LDLII and hyperlipidemic subjects with predominantly small dense LDL subfraction profile respectively, data represent mean ± SD (standard deviation) for each variable. ‡ Lipoprotein mass.*

4.4.2 ApoB containing lipoprotein subfractions and APG complex formation

The amount of cholesterol precipitated by interaction with APG measured directly in the precipitate or calculated by subtraction of the supernatant from the blank divided by the total cholesterol correlated highly significantly with the turbidity measurement by absorbance at 600nm (Fig 4.3). Therefore we used the latter in our analysis as the more convenient index for the amount of complex formed.

Figure 4.3

Correlation between turbidity as measured by absorbance unit (AU) at 600nm and the amount of cholesterol precipitated measured after the dissociation of the pellet in 2.0 mol/l NaCl.

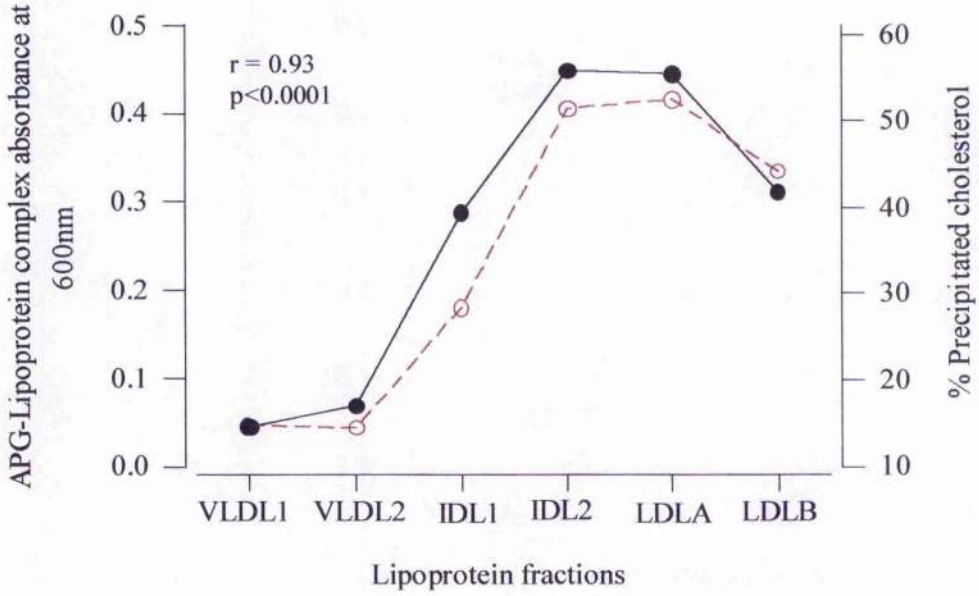


% precipitated cholesterol, the amount of lipoprotein cholesterol precipitated by APG divided by the total cholesterol applied multiplied by 100.

A similar pattern of lipoprotein-APG complex formation was observed in all individuals (Fig 4.4).

Figure 4.4

The pattern of APG-Lipoprotein complex formation in all the subjects and the amount of precipitated cholesterol



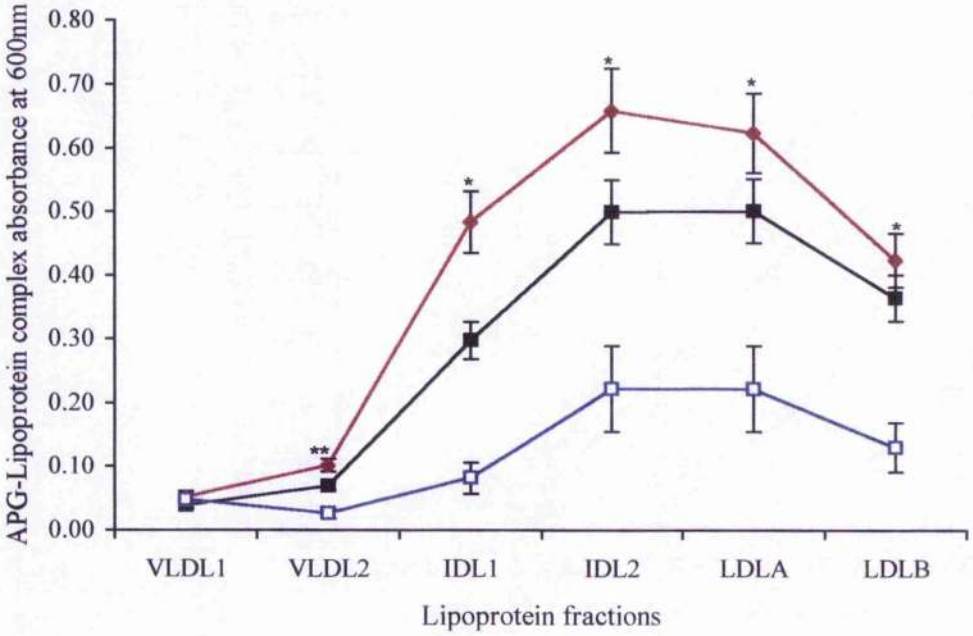
The relative reactivity of the different apoB containing lipoproteins with APG were compared by adding 0.1 mg apoB to fixed concentration of 2.5μg /ml APG. The graph represents the median value for the APG reactivity of each lipoprotein as measured by absorbance at 600nm and the % cholesterol precipitated by APG.

The mean reactivity for the lipoprotein subfractions in the three groups was highest in IDL2 and LDLA followed by LDLB and IDL1 and the least reactivity was observed in the VLDL subfractions (Fig 4.5). The highest APG reactivity for all fractions was found in group III i.e. those with raised lipid levels and a predominance of small dense LDL, intermediate in group II normolipidemic subjects with mainly LDLII subfraction and lowest in group I young healthy subjects with a high LDLI level (Fig 4.5). This pattern supports our previous findings (chapter 3, Fig 3.19; 3.20) of the positive correlation between the percentage of LDLIII, and the negative correlation between the percentage of LDLI within total LDL, and the extent of APG -LDL complex formation (Anber *et al* 1996).

the percentage of LDLI within total LDL, and the extent of APG -LDL complex formation (Anber *et al* 1996).

Figure 4.5

The pattern of lipoprotein-APG interaction of the different lipoprotein subfractions with arterial wall proteoglycans.



** p<0.0001, ** p= 0.007*

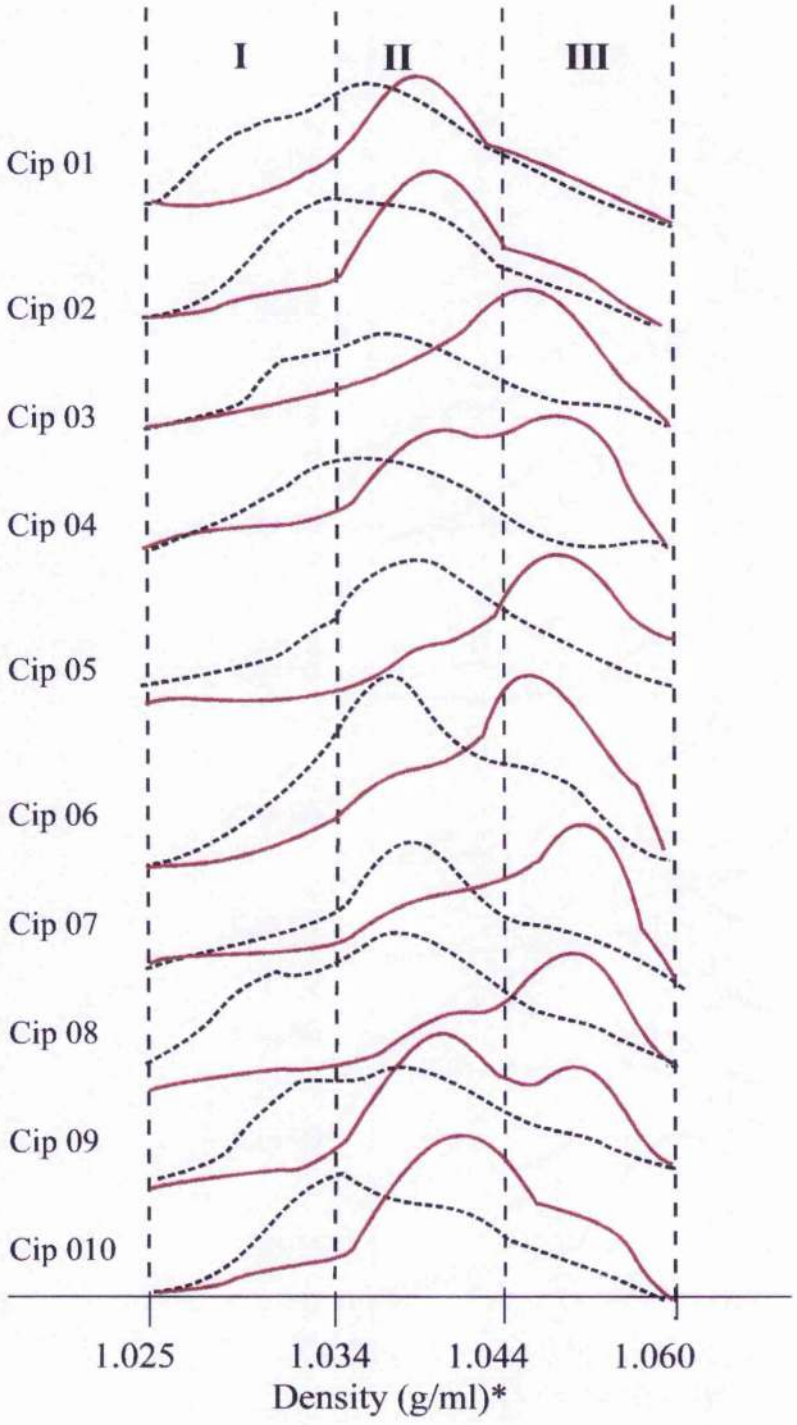
The relative reactivity between the three different groups of subjects were compared by adding 0.1 mg apoB to a fixed concentration of APG. The graph represents the median value for each lipoprotein subfractions with standard error bars in all the subjects, the line is median connect. □; group I, ■; group II, ◆; group III.

4.4.3 Study II: Effect of ciprofibrate treatment on APG-Lipoprotein complex formation

Lipid lowering treatment with ciprofibrate resulted in a significant decrease in total cholesterol (18%, p=0.039), plasma triglyceride (44%, p<0.05), a 10% reduction in

Figure 4. 6

LDL subfraction profile of the patients before and after treatment with ciprofibrate (100mg/day, for 8 weeks).



LDL subfraction profile at base line (——) and after (·····)ciprofibrate treatment.

A significant reduction in the total plasma apoB containing lipoprotein mass was found after lipid lowering treatment (703 mg/dl ± 185 vs 524 mg/dl ± 140, $p<0.04$). VLDL1 mass was significantly decreased by 57% ($p<0.05$). There was a 47% and 26% decrease in VLDL2 and IDL1 mass respectively and an 18% increase in IDL2 mass (Table 4.5). The increase in LDLA (33%) and the decrease in LDLB (25%) (Table 4.5) was due to the shift of the LDL subfraction profile from LDL-III to LDL-I with ciprofibrate treatment.

Table 4.5

ApoB containing lipoprotein mass before and after ciprofibrate

Lipoprotein subfractions		***Ciprofibrate treatment****		
		Before	***After***	***p***
VLDL1‡	mg/dl	207 ± 128	90 ± 72	=0.03
VLDL2‡	mg/dl	112 ± 83	60 ± 32	0.13
IDL1‡	mg/dl	59 ± 47	44 ± 36	0.48
IDL2‡	mg/dl	50 ± 12	59 ± 10	0.16
LDLA‡	mg/dl	112 ± 57	150 ± 32	0.14
LDLB‡	mg/dl	161 ± 65	121 ± 40	0.17

**ciprofibrate treatment 100mg/day for 8 weeks; ‡ lipoprotein mass, Very low density lipoprotein =VLDL1 (S_f 60-400) and VLDL2 (S_f 20-60), intermediate density lipoprotein = IDL1 (S_f 16-20) and IDL2 (S_f 12-16), low density lipoprotein = LDLA (S_f 8-12) and LDLB (S_f 0-8).*

The lipid lowering effect of ciprofibrate on the compositional analysis of the lipoprotein subfractions was significant mainly in reducing the percentage triglyceride ($p<0.05$) and phospholipid ($p<0.05$) in the VLDL1 fraction (Table 4.6). It also decreased the ability of each lipoprotein subfraction to form complexes with APG. IDL1, IDL2 and LDLA reactivity were significantly reduced by 54% ($p=0.03$), 49% ($p=0.006$) and 40% ($p=0.03$) respectively and that of small dense LDL by 42% but this was not statistically significant (Table 4.7) (Fig 4.7). There was little or no change in the reactivity of the VLDL subfractions with APG (Fig 4.7).

Table 4.6

Compositional analysis of the apoB containing lipoprotein before and after treatment with ciprofibrate (100mg/day for 8 weeks)

Lipoprotein subfractions	*%Total cholesterol*		*%Free cholesterol*		*%Triglyceride*		*%Phospholipid*	
	pre	*post*	*pre*	*post*	*pre*	*post*	*pre*	*post*
VLDL1	1.0 ±0.9	0.6 ±0.5	0.3 ±0.4	0.1 ±0.1	4.2 ±3.2	1.2 ±1.0*	65.0 ±43.0	22.2 ±22.4*
VLDL2	1.7 ±0.9	1.3 ±0.7	0.4 ±0.3	0.3 ±0.3	2.8 ±3.1	1.0 ±0.8	62.1 ±29.8	49.1 ±40.4
IDL1	1.0 ±0.5	1.2 ±0.8	0.1 ±0.1	0.2 ±0.2	0.8 ±1.5	0.5 ±0.7	22.5 ±10.6	18.8 ±10.1
IDL2	1.5 ±0.4	1.7 ±0.4	0.2 ±0.1	0.3 ±0.1	1.4 ±3.1	0.2 ±0.1	37.7 ±9.3	38.3 ±12.5
LDLA	3.5 ±1.8	4.6 ±1.2	1.0 ±0.8	1.3 ±0.8	1.4 ±3.1	0.4 ±0.1	87.1 ±45.9	107.9 ±22.0
LDLB	4.6 ±1.5	3.6 ±1.4	1.2 ±0.7	0.7 ±0.7	2.3 ±3.9	0.3 ±0.2	98.1 ±39.7	78.0 ±31.3

** $p<0.05$, Pre; post, pre and post ciprofibrate treatment.*

Table 4.7

APG-Lipoprotein complex formation before and after ciprofibrate treatment (100mg/day for 8 weeks).

Lipoprotein subfractions[§] *n=10*	*APG-Lipoprotein complex*[†] *Before*	*After*	*p*
VLDL1	0.05 ± 0.02	0.04 ± 0.20	= 0.34
VLDL2	0.10 ± 0.05	0.05 ± 0.03	= 0.07
IDL1	0.24 ± 0.11	0.11 ± 0.10	= 0.037
IDL2	0.45 ± 0.10	0.23 ± 0.12	= 0.006
LDL1	0.39 ± 0.10	0.23 ± 0.11	= 0.03
LDL2	0.17 ± 0.06	0.11 ± 0.03	= 0.06

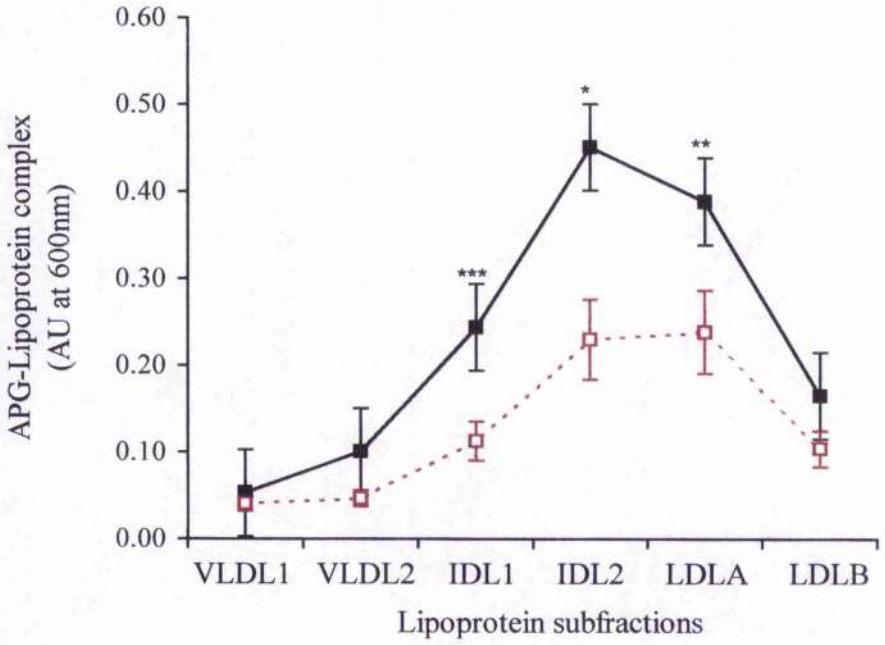
[§] *Very low density lipoprotein =VLDL1 (S_f 60-400) and VLDL2 (S_f 20-60), low density lipoprotein = IDL1 (S_f 16-20) and IDL2 (S_f 12-16), low density lipoprotein = LDL1 (S_f 8-12) and LDL2 (S_f 0-8).*

[†] *Turbidity measurement (AU) absorbance unit at 600nm*

n= number of subjects.

Figure 4.7

Lipoprotein-APG relative reactivity of the different lipoprotein subfractions before and after treatment with Ciprofibrate (100mg/day for 8 weeks) compared by adding 0.1 mg apoB to a fixed concentration of APG.

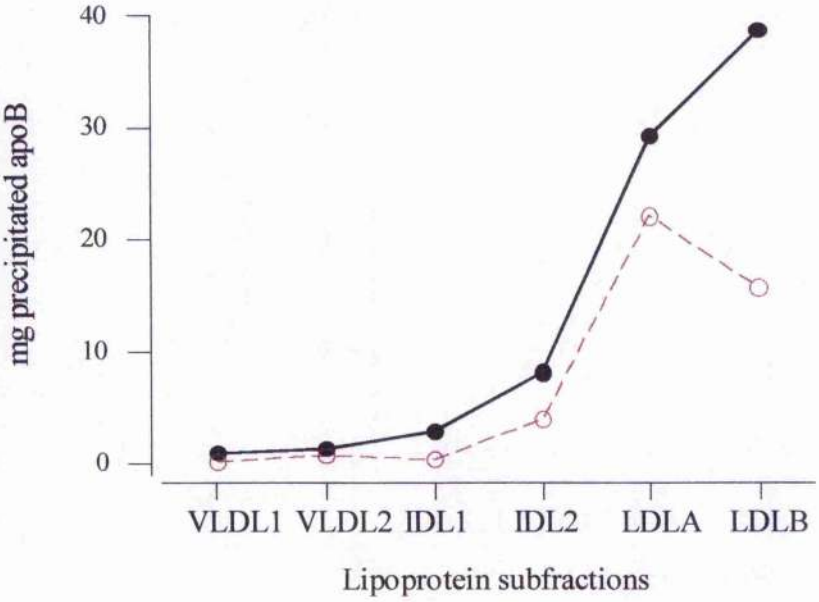


The graph represents the median value for each lipoprotein subfractions with standard error bars in all the subjects, the line is median connect. ■; *before ciprofibrate,* □; *after treatment with ciprofibrate.* * *p=0.006,* ** *p=0.03,* ****p=0.037.*

The amount of precipitated apoB in each lipoprotein subfraction was calculated by multiplying the percentage of precipitable apoB (derived from the percentage of precipitated cholesterol multiplied by the apoB/cholesterol ratio) by apoB concentration of each subfraction. From this data it was seen that the biggest changes were seen in the LDL range rather than the IDL (Fig 4.8) i.e. ciprofibrate was effective in reducing the number of APG reactive LDL particles more than any of the other lipoprotein species.

Figure 4.8

The total amount of apoB precipitated in different lipoproteins by APG, before and after lipid lowering treatment with Ciprofibrate 100mg/day for 8 weeks.



The amount of precipitated apoB in (mg) was calculated by multiplying the percentage of precipitable apoB (derived from the percentage of precipitated cholesterol multiplied by the apoB/cholesterol ratio) by apoB concentration in each lipoprotein subfraction, before (●) ; and after (○) treatment with Ciprofibrate.

4.5 Discussion

Structural diversity exists throughout the apoB containing lipoprotein spectrum. High resolution centrifugation or electrophoretic techniques have been employed to demonstrate the presence of discrete subfractions within VLDL (Lindgren *et al* 1972, Packard *et al* 1984), IDL (Musliner *et al* 1986) and LDL (Griffin *et al* 1990, Krauss 1994). These all have varying functional and metabolic properties and are likely to differ in their atherogenic potential. LDL in particular has been shown to consist of discrete subpopulations of particles, of which the small dense species have been most closely linked to increased risk of CHD (Krauss 1994). The observation that the

strength of the interaction between LDL and APG was related to the subfraction profile (Anber *et al* 1996) and the knowledge that small dense LDL was but one component of the dyslipidaemia termed the atherogenic lipoprotein phenotype (Austin *et al* 1990) prompted us to seek further abnormalities within the S_f 0-400 lipoprotein spectrum. The principal finding of the present study was that a small dense IDL fraction and large LDL were the most reactive species towards APG. This contradicted our initial supposition that there would be a monotonous increase in APG reactivity from VLDL1 through to LDLB. If APG trapping of lipoprotein is, as we believe, a key early step in atherogenesis, then the data provide strong support for the suggestion that IDL is a particularly atherogenic lipoprotein (Rapp *et al* 1994, Tatami *et al* 1981, Steiner *et al* 1987, Krauss *et al* 1987).

The pattern of APG-lipoprotein interaction was remarkably similar in all subjects studied regardless of their plasma lipid levels (Fig 4.5). However between individuals the relative reactivity of each lipoprotein subclass increased as the plasma triglyceride level and the proportion of LDLIII rose. Furthermore in subjects treated with ciprofibrate, the reactivity of all species fell in concert, with again the same general pattern being maintained. The basis of lipoprotein-APG interaction throughout the S_f 0-400 spectrum is unknown, although for LDL the work of Olsson *et al* (Olsson *et al* 1993) pinpointed certain sequences of apoB as important. What is suggested by the data in Figures 4.5 and 4.7 is that the relative reactivity of VLDL2, IDL1, IDL2, LDLA and LDLB are linked, probably by a common denominator. The findings also provide further insight into possible association between plasma triglyceride concentration and CHD risk.

On the basis of current knowledge, two mechanisms can be postulated to explain the link between the plasma triglyceride concentration and lipoprotein-APG reactivity. First, high levels of plasma triglyceride may cause remodelling of IDL and LDL to more atherogenic forms. As plasma triglyceride rises so does the extent of cholesterol ester transfer protein mediated triglyceride exchange into denser lipoproteins such as LDL and HDL. Lipolysis of these triglyceride-enriched particles results in the generation of smaller and denser lipoproteins (Patsch *et al* 1984, Tan *et al* 1995). It is tempting to speculate that IDL is affected in the same way and that small, dense IDL are active in binding APG. Second, it can be argued that APG reactive species

within the IDL and LDL density intervals are the products of the lipolysis of large VLDL1. Metabolic studies have shown that VLDL1 is converted to VLDL2 remnants, IDL and LDL particles that have a prolonged residence time in circulation compared to lipoproteins whose initial precursor is in the VLDL2 density range (Packard *et al* 1984). The properties of IDL and LDL particles which circulate for a long time in plasma may be modified, for example by altered surface glycosylation or oxidation, to enhance their APG binding. When VLDL1 levels fall on ciprofibrate (the major change in the Sf 0-400) possibly due to decreased hepatic secretion (Gaw & Shepherd 1991) so does the relative reactivity of IDL and LDL.

We conclude that an ALP leads to abnormalities throughout the apoB containing lipoprotein spectrum and that the enrichment of VLDL2, IDL and LDL in lipoprotein species which bind avidly to APG is one of the ways in which elevated plasma triglyceride levels contribute to the atherogenic process. These abnormalities are corrected by appropriate lipid lowering therapy.

Chapter 5

Sialic acid and Neutral carbohydrate concentration and APG binding...

5.1 Introduction

Low density lipoprotein (LDL) is known to be composed of a population of particles different in size and density, that can be identified either by polyacrylamide gel technique into two subclass pattern A or B (Austin *et al* 1988) or by density gradient ultracentrifugation to three LDL subfractions, LDLI, LDLII and LDLIII. These subfractions are present in each individual in differing proportions (Griffin *et al* 1990). Pattern A or LDLI are characterised by large particles with a higher buoyant density that are present mainly in young healthy males and premenopausal female subjects. Pattern B or LDLIII are smaller and denser and are present predominantly in patients with CHD (Austin *et al* 1988, Griffin *et al* 1990). Small dense LDLIII is strongly associated with the plasma triglyceride level, both of which are key components of the atherogenic lipoprotein phenotype (ALP). ALP is a recognised feature of the insulin resistance syndrome and is associated with a 3-7 fold increase in risk of MI (Austin *et al* 1990).

Although triglyceride rich particles may be atherogenic *per se*, much of the attention has been directed to their modulatory effect on the substructure of

LDL and HDL and in particular on the link between raised triglyceride and small dense LDLIII. The later has been shown to be enriched with PUFA mainly linoleic acid which increases its oxidative susceptibility leading to cholesteryl ester accumulation and foam cell formation (de Graaf *et al* 1991, Reaven *et al* 1993) compared to LDLI. In addition we have shown a positive correlation between the percentage of LDLIII within total LDL, i.e. the presence of ALP, and APG-LDL complex formation Anber *et al* 1996, chapter 3).

The question arises as to whether there are other structural differences between the LDL subfractions that may relate to the genetic basis of the LDL subfraction profile and that might contribute to differences in their atherogenicity and the coronary disease risk associated with differing patterns.

LDL subfractions have been shown to differ in their carbohydrate and sialic acid content (LaBelle & Krauss 1990, Tertov *et al* 1993). Labelle and Kraus observed that large LDL from pattern A contain higher neutral carbohydrate and sialic acid compared to LDL from pattern B (LaBeklle & Krauss 1990). This has been supported by the findings of Tertov in which sialic acid poor (SAP) LDL fraction, isolated by Ricin-agglutinin, from CHD patients contained less carbohydrate and sialic acid than sialic acid rich (SAR) LDL from the same individuals and normal subjects (Tertov *et al* 1993). Sialic acid content of LDL has been related to its capacity to stimulate intracellular lipid accumulation (Orekhov *et al* 1992, Sobenin *et al* 1991) and Camejo has suggested that LDL with low sialic acid content binds to a greater extent to APG (Camejo et al 1985b). Taken together, these findings suggest that desialation and low carbohydrate content may increase the atherogenicity of LDL.

Carbohydrate accounts for 4-9% of the mass of the single large glycoprotein (≅550KD), apoB, present in LDL (Vauhkonen *et al* 1985, Swaminathan & Aladjem 1976), 37% of which is high mannose. ApoB high mannose residue contributes about 10% of the total high mannose chains present in plasma (Vauhkonen *et al* 1985). This gives LDL the unique property of a high binding affinity to the sugar binding lectin Concanavalin-A (Con-A), a phytohemagglutinin isolated from Jack bean (Fig 2.5, Chapter 2), which has been used to characterise soluble and membrane bound glycoproteins (McConathy & Alaupovic 1974, Tavella *et al* 1991).

LDL has been isolated from human plasma by con-A affinity chromatography, since it binds avidly to the lectin in contrast to VLDL and HDL (Tavella *et al* 1991). In fact, the later does not bind at all (Mitamura 1981). The unbound fraction in LDL accounts for 4-5%, the strongly bound fraction accounts for 37% which is the high mannose structure (Vauhkonen *et al* 1985) and the rest are weakly bound including sialic acid (Harmony & Cordes 1975).

The aim of this study was: i) to determine the association between LDL total carbohydrate and LDL subfraction distribution and plasma lipid level, ii) to separate LDL subfractions on the basis of carbohydrate content by affinity chromatography on Concanavalin-A and examine the ability of the eluted fractions to interact with APG, iii) to determine the influence of sialic acid concentration of apoB containing lipoproteins and their interaction with APG, and finally iv) to examine the effect of lipid lowering treatment with ciprofibrate on the sialic acid content of apoB containing lipoproteins and their interaction with APG.

5.2 Subjects

A total of seventy five subjects were recruited for all the studies. All donated 50ml of blood after an overnight (12h) fast. Fasting blood was collected by venepuncture using K_2EDTA (final concentration 1mg/ml) as anticoagulant. Plasma was separated at 4°C by low speed centrifugation (3000rpm) and aliquots for lipid, lipoprotein measurements and LDL subfractionation used immediately. Subjects with recent MI (i.e. in the previous 6 months), renal disease, thyroid disease, diabetes or those taking lipid-lowering medications were excluded, as were those with a plasma cholesterol concentration of >9.0mmol/l and a plasma triglyceride level of >5.0mmol/l. All eligible subjects undergoing angiography were recruited to the study over a 6 month period. The study was approved by the Research Ethics Committee of Glasgow Royal Infirmary and each volunteer gave written informed consent.

5.2.1 Study Ia: LDL carbohydrate measurement, its relation to the LDL subfraction profile and APG-LDL complex formation

Thirty five subjects were recruited for this study (15 normal volunteers and 20 patients undergoing diagnostic coronary angiography in the Department of Medical Cardiology, Glasgow Royal Infirmary). Carbohydrate measurement was performed by the phenol sulphuric acid method (Dubois *et al* 1956, Beely 1985) on total and LDL subfractions prepared by sequential gradient and density gradient centrifugation respectively as described in the method section (Chapter 2).

5.2.2 Study Ib: Separation of LDL subfraction on the basis of their carbohydrate content by affinity chromatography on Con-A.

Total LDL from a subgroup of 18 subjects (9 normal and 9 CHD) was used for this. Total LDL was applied to column containing 8mls Con-A Sepharose as described in chapter 2.

5.2.3 Study II: Sialic acid concentration of apoB containing lipoproteins and its influence on APG-lipoprotein reactivity

Twenty eight subjects were examined in this study. Eighteen of which, aged 19-60 years, were divided into three groups (six subjects in each group) on the basis of their LDL subfraction profile and the presence or absence of ALP (Table 4.1, Chapter 4). Group 1 were young healthy normolipidemic, aged 19-22 years, (5 female and 1 male) whose LDL profile predominated by LDL-I, group 2 were healthy normolipidemic (2 female and 4 male) aged 34-46 years, had mainly LDL-II profile, and group 3 were hyperlipidemic (1 female and 5 male) aged 39-60 years with a profile in which LDL-III was the major species present (chapter 4). The data from the ten male subjects recruited for the ciprofibrate study prior to treatment, whose characteristics are described below, was included.

5.2.4 Study III: The effect of lipid lowering treatment with Ciprofibrate on sialic acid content of apoB containing lipoproteins and APG-Lipoprotein interaction

Twelve male patients aged 40-60 yr. were selected on the basis of their cardiac catheterisation results (all with angiographically positive coronary artery disease) from the cardiac catheterisation unit at the Glasgow Royal Infirmary. They had a total cholesterol >5.2mmol/l and a plasma triglyceride <3.5mmol/l. Baseline assessment of lipid and lipoprotein levels, LDL subfraction profile, sialic acid and APG reactivity were performed. Patients were then commenced on ciprofibrate 100mg/day for 8 weeks. After the eighth week a second assessment of lipids, lipoproteins, LDL subfraction profile and APG reactivity were performed. The characteristics of each patient are summarised in Table 4.1 (Chapter 4). All the patients studied were receiving prescribed medications for other clinical conditions, and these were continued unchanged throughout the course of the study. The exclusion criteria which applied are summarised in section 4.2.2 (Chapter 4).

Ciprofibrate was well tolerated by all subjects participating in the study. Two of the patients withdrew from the study, one had a myocardial infarction and the other could not continue because of difficulty in commuting.

5.3 Statistical analysis

Statistical analysis and manipulations were performed using the PC version of MINITAB Release 10 for Windows (Minitab Inc., PA). All variables were assessed by drawing normality plots and the ones which were not normally distributed were normalised by appropriate transformations. Plasma triglyceride was normalised by log transformation, and LDLI, LDLII, LDLIII concentrations and HDL cholesterol were normalised by taking their square root. associations between variables were tested by calculating the pearson correlation coeffecient. The significance of association between variables was determined by simple regression, 2 sample t-test and one way analysis of variance (ANOVA) were used to asses the relationship between variables.

5.4 Study I

5.4.1 lipid and LDL subfraction profile

In general (as we have shown in chapter 3, Table 3.6), plasma triglyceride level was positively correlated with total cholesterol (r= 0.45, p<0.0001) and LDL cholesterol (r=0.43, p< 0.001) within the plasma and negatively correlated with HDL cholesterol (r=-0.57, p<0.0001). Plasma triglyceride was also the strongest and most constant correlate of LDL subfraction profile showing a negative correlation with the percentage and concentration of LDL-I (r= -0.44, p<0.001) and LDL-II (r= -0.40, p<0.001) and a positive association with small dense LDL-III (r= 0.64, p<0.0001) within total LDL.

Mean plasma lipid levels and LDL subfraction profile in both groups of subjects (normal volunteers and coronary heart disease patients) are shown in Table 5.1.

Table 5. 1

Mean Plasma lipid and LDL subfraction profile in the normal volunteers and the CHD patients.

Variable		*normal volunteers n=15*	*CHD patients n=20*
Age		33.0 ± 6.6	56.4 ± 8.8*
Total cholesterol	mmol/l	4.9 ± 0.8	5.8 ± 1.1**
LDL cholesterol	mmol/l	3.0 ± 0.8	3.9 ± 0.9**
HDL cholesterol	mmol/l	1.6 ± 0.3	1.1 ± 0.3**
plasma triglyceride	mmol/l	0.9 ± 0.3	1.7 ± 0.9*
APG-LDL complex	AU	0.32 ± 0.1	0.41 ± 0.2***
%LDL-I		25 ± 12.4	15 ± 10.1***
%LDL-II		61 ± 11.7	52 ± 14.5
%LDL-III		15 ± 4.9	32 ± 19.5*

**p<0.001, **p<0.01, ***p<0.05; CHD, coronary heart disease; APG-LDL complex determined by turbidity measurement by AU, absorbance unit at 600nm visible wavelength, Values represent mean ± standard deviation.*

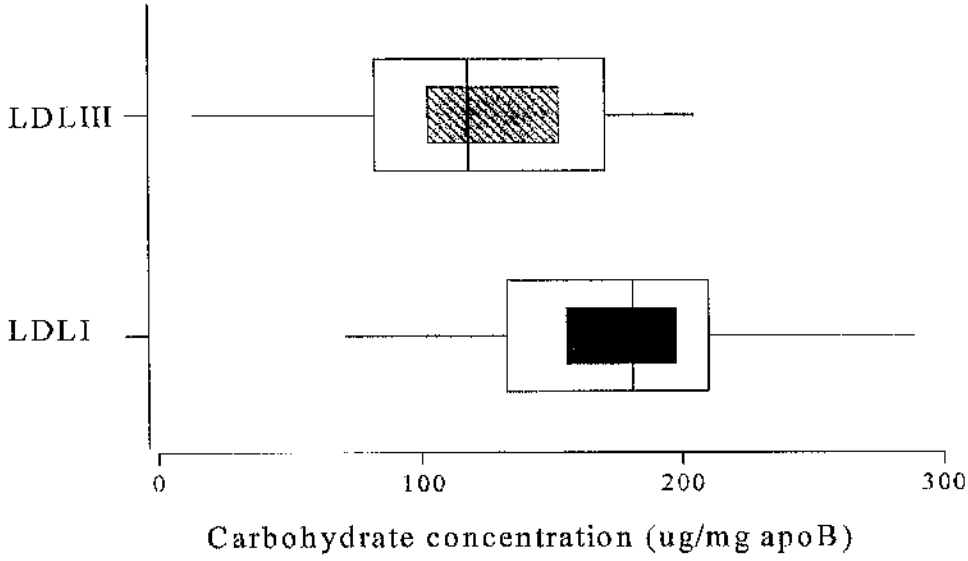
The patients with coronary heart disease were older ($p<0.0001$), had a higher plasma total cholesterol ($p<0.01$), plasma triglyceride ($p<0.001$), LDL cholesterol ($p<0.01$) and a lower HDL cholesterol concentration in plasma ($p=0.002$), compared to the normal volunteers (Table 5.1). They also had a different LDL subfraction profile where the former had lower LDLI ($p<0.05$) and a higher small dense LDLIII ($p<0.001$) within total LDL (Table 5.1).

5.4.2 Neutral carbohydrate, LDL subfraction profile and APG-LDL binding reactivity

The neutral carbohydrate concentration in the LDLI subfraction (175.4 µg/mg apoB ± 51.0) was significantly higher compared to its concentration in small dense LDLIII (123.6 µg/mg apoB ± 52.2) ($P<0.001$) in all subjects (Fig 5.1).

Figure 5.1

Carbohydrate concentration in LDL-I subfraction compared to LDL-III subfraction.



The inner box represents the 25-75 percentile, the median value is indicated by a vertical line, the outer box represents the 5th and the 95th percentile while the range of values are indicated by the horizontal lines. $p< 0.001$

Table 5.2 shows the mean neutral carbohydrate concentration of total and LDL subfractions in both normal volunteers and CHD patients. The former had higher carbohydrate in total and LDL subfractions compared to the later. This was only statistically significant for the total LDL (Table 5.2).

Table 5.2

Neutral carbohydrate concentration (μg/mg apo B) of total and LDL subfractions. Comparison between the patients and normal volunteers.

Low density lipoprotein	*Carbohydrate content*		
	normal	*CHD*	*p*
LDL-I	192.0 ± 49.4	166.7 ± 51.0	=0.21
LDL-II	183.8 ± 47.8	163.8 ± 50.8	=0.31
LDL-III	137.9 ± 66.6	111.7 ± 35.2	=0.28
T-LDL	165.4 ± 19.1	143.9 ± 33.6	<0.04

T- LDL, total low density lipoprotein (d=1.019-1.063 g/ml); LDL-I, large buoyant low density lipoprotein (d=1.025-1.033 g/ml); LDL-II, intermediate low density lipoprotein (d=1.033-1.043 g/ml); LDL-III, small dense low density lipoprotein (d=1.044-1.060 g/ml); CHD, coronary heart disease patients

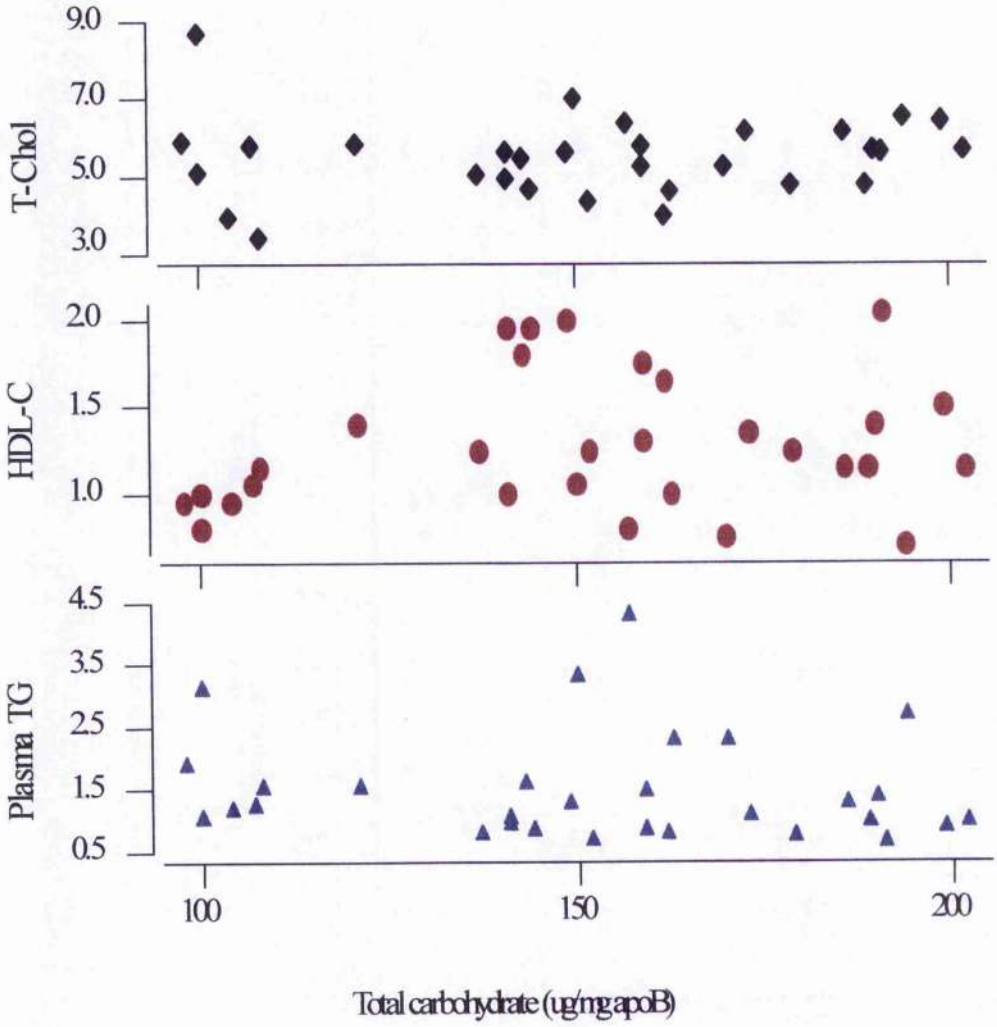
Pearsons correlation was used to examine the relationship between total LDL carbohydrate, lipid variables and the APG-LDL reactivity. Total LDL carbohydrate was not significantly correlated with plasma total cholesterol, LDL cholesterol, HDL cholesterol or plasma triglyceride (Fig 5.2) (Table 5.3). LDL-I subfraction contained more carbohydrate than LDLIII (Table 5.2), however the carbohydrate concentration of total LDL did not correlate with the LDL subfraction profile (Table 5.4) (Fig 5.3).

As we have shown before (chapter 4), the extent of APG-LDL complex formation was positively correlated with plasma triglyceride (p=0.04) and the concentration of small dense LDL (p=0.007) within total LDL and negatively correlated with HDL cholesterol (P=0.02) and LDLI concentration within total LDL (P<0.01) (Table 5.3, 5.4). However, the APG binding reactivity showed

no significant correlation with the amount of carbohydrate in total LDL (Table 5.3).

Figure 5.2

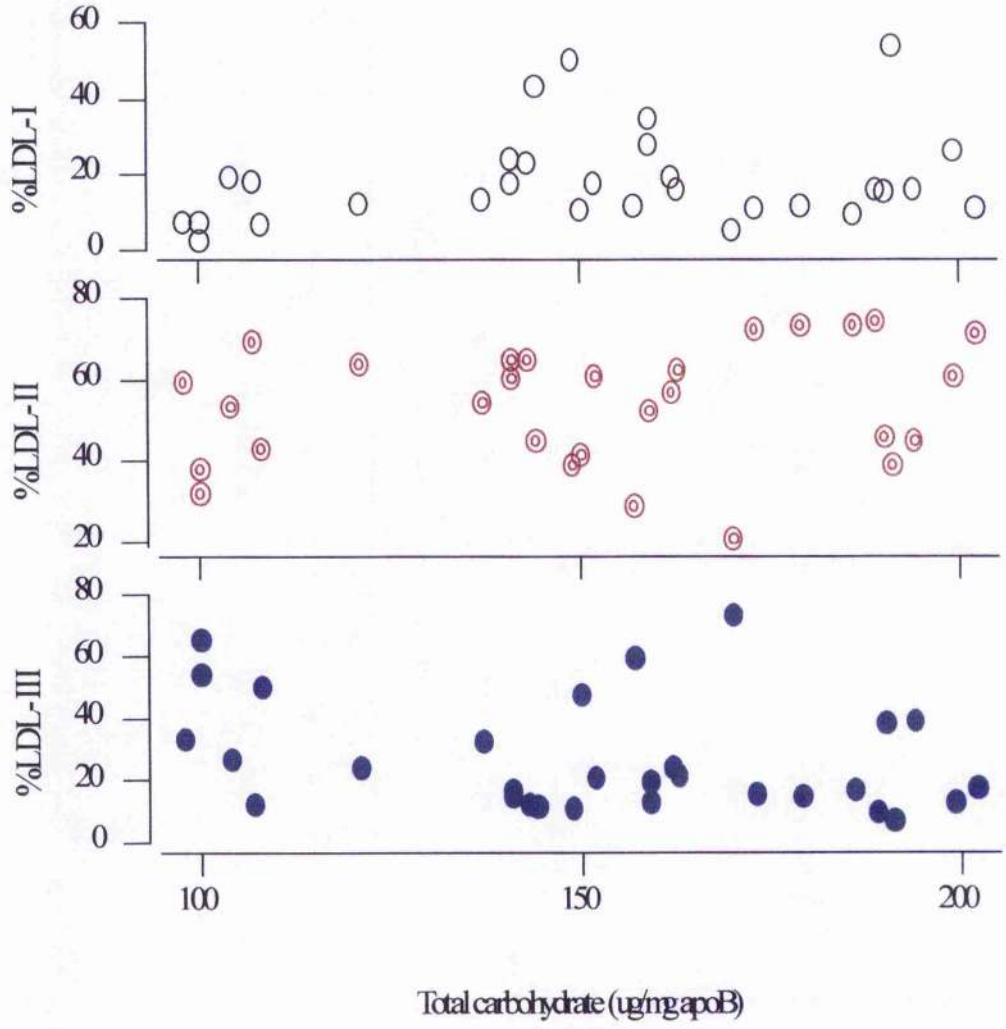
correlation between total LDL carbohydrate content and plasma lipids.



T-Cholesterol, plasma total cholesterol (mmol/l); HDL-C, high density lipoprotein cholesterol (mmol/l); Triglyceride; plasma triglyceride (mmol/l).

Figure 5.3

Correlation between total LDL carbohydrate and the LDL subfractions.



%LDL-I, %LDL-II, %LDL-III, percentage of large LDLI , intermediate LDLII and small dense LDLIII within total LDL.

Table 5. 3

Correlation between the mount of carbohydrate in total LDL, subjects lipid variables and the extent of APG-LDL interaction.

Variable		*CHO*	*APG-LDL complex*
Age		- 0.09	0.15
Total cholesterol	mmol/l	0.06	0.10
LDL cholesterol	mmol/l	0.13	0.22
HDL cholesterol	mmol/l	0.06	- 0.38*
plasma triglyceride	mmol/l	- 0.20	0.36*
T-CHO		----	0.23
APG-LDL complex	AU	0.23	---

**p<0.05; CHO, neutral carbohydrate; CHO, total LDL neutral carbohydrate concentration; APG-LDL complex determined by turbidity measurement by AU, absorbance unit at 600nm visible wavelength, values represent pearsons correlation coefficient.*

Table 5. 4
relationship between LDL subfractions, LDL total carbohydrate and APG-LDL interaction.

Variable	*CHO*	*APG-LDL complex*
%LDL-I	0.05	- 0.43*
%LDL-II	0.05	- 0.2
%LDL-III	- 0.1	0.45*

**p<0.01, CHO, neutral carbohydrate; APG-LDL complex determined by turbidity measurement by AU, absorbance unit at 600nm visible wavelength, values represent pearsons correlation coefficient.*

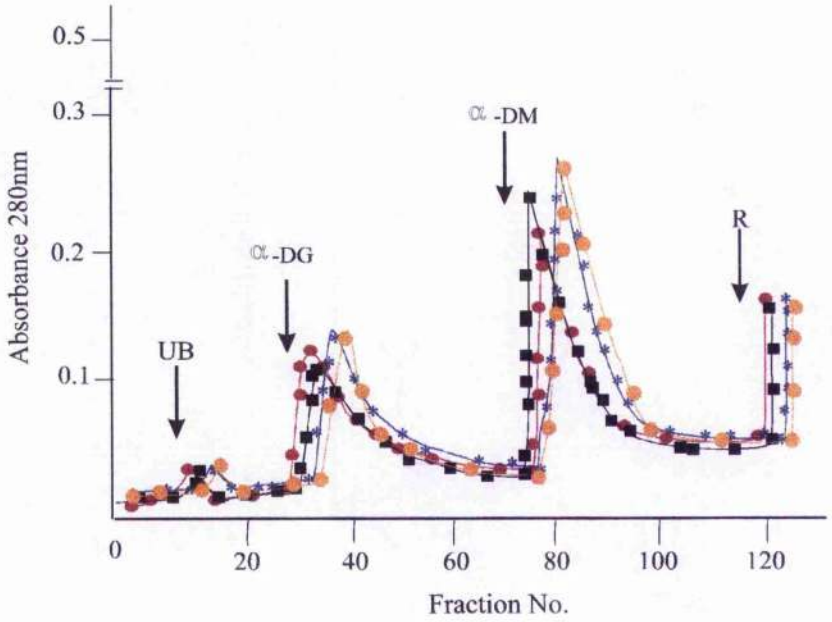
5.4.3 LDL fractions eluted from affinity chromatography of total LDL on Con-A

Affinity chromatography of total LDL (d= 1.019-1.063 g/ml), isolated by sequential gradient centrifugation, on Con-A was performed by applying 1-3 mg

of LDL protein to 10ml column of Con-A (as described in chapter 2, section 2.3.1). The unbound fraction was eluted by the equilibrating buffer pH 7.2 which accounted for 5-9% of the total protein applied to the column. The weakly bound fraction, eluted by 0.2mol/l α D-glucopyranoside, and the strongly bound fraction, eluted by 0.3mol/l α D-mannopyranoside accounted for 40-50% and 43-60% of the total LDL protein applied to the column respectively. The reproducibility of the Con-A affinity chromatography of the samples was verified with four determinations of the same sample of LDL and the calculated CV was 4.5% (Fig 5.4).

Figure 5.4

The reproducibility of the elution pattern of total LDL on affinity chromatography on Con-A Sepharose.



UB, unbound fraction eluted by equilibrating buffer, α-DG, weakly bound fraction eluted by 0.2 mol/l α-D glucopyranoside, α-DM, strongly bound fraction eluted by 0.3mol/l α-D mannopyranoside; R, regeneration buffer. Four determinations of the same LDL sample. The eluted fraction protein was determined by absorbance at 280nm.

When total LDL, from individuals with different LDL subfraction profile, was applied to the Con-A column, the percentage of weakly bound fraction was

lower in subjects with LDL-I (n=3) compared to subjects with predominantly LDL-III profile (n=3) ($p<0.05$) (Table5.5). The weakly bound fraction was significantly different from the strongly bound fraction in the same individual in subjects with LDL-I ($p=0.04$), however this significance was lost in the LDL-III predominant subjects ($p=0.22$) (Table 5.5). LDL-II elution pattern was not significantly different from that of LDL-I (Table 5.5).

Table 5.5

The unbound, weakly bound and the strongly bound fractions from total LDL affinity chromatography on Con-A Sepharose. Comparison between group of subjects with different LDL subfraction profile.

Subjects	*Unbound*	*Weakly bound*	*Strongly bound*
Group- 1 (3)	3 %	42 %	56 %*
Group- 2 (3)	2 %	45 %	53 %*
Group-3 (3)	2 %	48 %⇑	49 %⇑

** $p< 0.05$, between fractions; ⇑ $p<0.05$, between different group of subjects; unbound, percentage of unbound fraction eluted by equilibrating buffer; weakly bound, percentage of weakly bound fraction eluted by 0.2mol/l α D-glucopyranoside; strongly bound, percentage of strongly bound fraction eluted by 0.3mol/l α D-mannopyranoside; Group-1, Group-2 and Group-3, subjects with predominantly LDL-I, LDL-II and LDL-III subfraction profile respectively, number between brackets is the number of subjects in each group*

Separation of ^{125}I-labelled total LDL with Con-A Sepharose in a tube assay showed similar results to that observed with the column affinity chromatography except, that there was a small percentage (approximately 5%) of radioactivity still remained bound to the Con-A even after elution with 1mol/l α D-mannopyranoside. This could be either non-specific or a small fraction of very strongly bound LDL remaining on the lectin. Unfortunately we were not able to determine the nature of this fraction. The percentage of eluted fractions in the tube assay as determined by radioactivity measurement in counts per minute (cpm) are shown in (Table 5.6).

Table 5.6

The percentage of unbound, glucose releasable and mannose releasable fractions of ^{125}I-labelled LDL from Con-A Sepharose as measured by cpm in non-diseased and CHD subjects.

Subjects	*Total*	*Unbound*	*weakly bound*	*Strongly bound*
nl 01	100	5	40	46
nl 02	100	7	37	48
nl 03	100	7	41	53
nl 04	100	6	41	40
nl 05	100	5	41	46
nl 06	100	6	44	50
nl 07	100	5	46	46
nl 08	100	5	45	47
mean ± SD	100	6 ± 1	42 ± 3* †	47 ± 4*
CHD 01	100	4	48	44
CHD 02	100	3	49	43
CHD 03	100	2	54	37
CHD 04	100	4	50	42
CHD 05	100	3	47	43
CHD 06	100	8	42	42
CHD 07	100	9	41	48
mean ± SD	100	5 ± 3	47 ± 5	43 ± 3

**p<0.05, between normal volunteer and CHD; † p<0.01 between weakly bound and strongly bound fractions in the same group of subjects total and eluted fractions measured in cpm; the number in the brackets represent the percentage of eluted fractions; nl, represent normal subjects; CHD, represents coronary heart disease patients.*

Individual LDL subfractions, isolated by density gradient ultracentrifugation (chapter 2), showed variable elution pattern by affinity chromatography on Con-A. LDL-I subfraction bound with greater affinity to the column compared to LDL-III subfraction. Table 5.7 shows that the unbound fraction was similar in all the three LDL subfractions, while the percentage of the weakly bound fraction was highly significantly lower than the percentage of the strongly bound

fraction in LDL-I subfraction and the reverse of that was observed in LDL-III subfraction (Table 5.7) (Fig 5.5). This pattern suggests that LDL-I subfraction contain more high mannose residues compared to LDL-III.

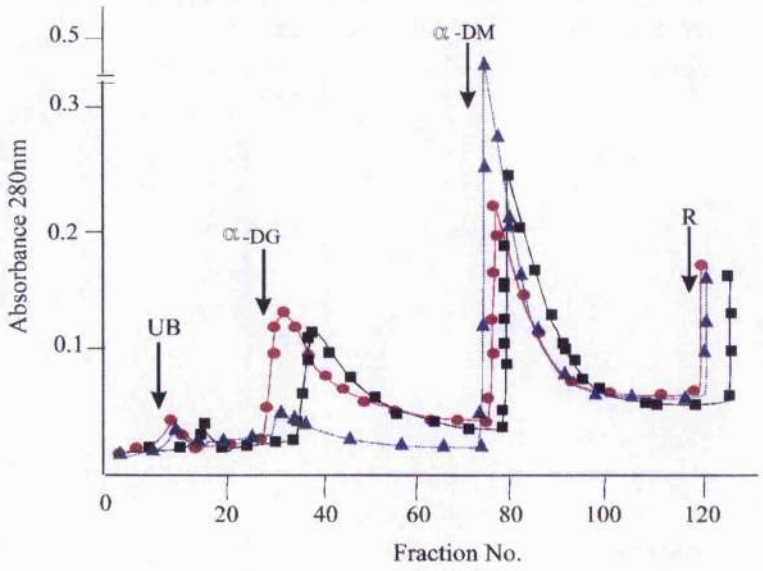
Table 5.7

The percentage of the three eluted fractions from Con-A affinity chromatography of individual LDL subfractions isolated by DGUC.

Subjects	*Unbound*	*Weakly bound*	*Strongly bound*
LDL-I	9	34* †	57*
LDL-II	3	39	56
LDL-III	9	51*†	41*

**p<0.01;between LDL-I & LDL-III, †p<0.01; between weakly bound and strongly bound fractions, unbound, percentage of unbound fraction eluted by equilibrating buffer; weakly bound, percentage of the fraction eluted by 0.2mol/l α D-glucopyranoside; strongly bound, percentage of the fraction eluted by 0.3mol/l α D-mannopyranoside; LDL-I, LDL-II and LDL-III, the percentage of LDL-I, II and III subfraction within total LDL respectively.*

Figure 5.5
The distribution of The elution pattern among different LDL subfractions



UB, unbound fraction eluted by equilibrating buffer, α-DG, weakly bound fraction eluted by 0.2 mol/l α-D glucopyranoside, α-DM, strongly bound fraction eluted by 0.3mol/l α-D mannopyranoside; R, regeneration buffer. LDL-I, (▲.....▲) LDL-II, (■——■) LDL-III subfraction (●——●)

5.4.4 APG reactivity of eluted LDL fractions from Con-A

To determine whether the two main eluted fractions from Con-A Sepharose affinity chromatography of total LDL from subjects with different LDL subfraction profile were different in their reactivity towards APG. Weakly bound (glucose releasable) and strongly bound (mannose releasable) fractions eluted from total LDL of three group of subjects with predominantly LDL-I (n=3), predominantly LDL-II (n=3) and predominantly LDL-III (n=3) subfraction profile were mixed with 2.5μg CS rich fraction of APG from human aorta and turbidity was measured, by absorbance at 600nm visible wavelength, after 30min incubation at 25°C. There was no significant differences between the extent of interaction of the weakly bound fraction compared to the strongly bound fraction towards APG. Yet the extent of complex formation in both fractions was highest in the subjects with predominantly LDL-III compared to the other two as previously noted (chapters 3 & 4) (Table 5.8).

Table 5.8

The extant of APG reactivity of the weakly bound and the strongly bound fractions eluted from affinity chromatography of total LDL on Con-A from three groups subjects with different LDL subfraction profile.

Subjects	***APG-LDL complex***	
	Weakly bound	***Strongly bound***
Group-1	0.28	0.35
Group-2	0.38	0.35
Group-3	0.44*	0.43*

**p<0.01; values represent mean duplicate measurements for APG-LDL complex formation as measured by absorbance at 600nm. Group-1, 2 and 3 were volunteers with predominantly LDL-I, LDL-II and LDL-III subfraction profile respectively.*

5.5 Study II

5.5.1 lipids, LDL subfraction profile and sialic acid content

Mean plasma lipid level and LDL subfraction profile for the three groups of subjects are described in detail in section 4.4 (Chapter 4). Plasma total cholesterol, triglyceride level was significantly higher in group III, with predominantly small dense LDLIII, compared to group I and II subjects (Table 4.2, Chapter 4).

The sialic acid content of IDL2 was positively correlated with plasma LDL cholesterol ($p<0.05$)and negatively correlated with VLDL ($p<0.05$) and HDL cholesterol ($P=0.04$). LDLA sialic acid was negatively correlated with LDL cholesterol ($p<0.01$) and that of VLDL2 was positively correlated with total and plasma cholesterol ($p<0.05$ & $p<0.01$ respectively). There was no significant correlation between the amount of sialic acid the other in apoB containing lipoproteins and the rest of plasma lipid variables (Table 5.9).

Table 5.9
Pearson correlation between patients lipid variables and sialic acid content of apoB containing lipoproteins

Variable			*Sialic acid*†			
	VLDL1	*VLDL2*	*IDL1*	*IDL2*	*LDLA*	*LDLB*
T-Chol	0.27	0.42***	0.20	0.11	-0.16	-0.11
VLDL-C	0.09	-0.18	-0.10	-0.60***	0.33	0.17
LDL-C	0.09	0.76*	0.32	0.58***	-0.68*	-0.12
HDL-C	-0.12	-0.15	-0.23	-0.46**	0.07	0.15
TG	0.14	0.13	0.05	0.09	-0.10	-0.15

** p<0.01; **p=0.04; ***p<0.05; †mol/mol lipoprotein*
T-Chol, total cholesterol (mmol/l); VLDL-C, very low density lipoprotein cholesterol (mmol/l); LDL-C, low density lipoprotein cholesterol (mmol/l); HDL-C, high density lipoprotein cholesterol (mmol/l); TG, plasma triglyceride (mmol/l)

The amount of sialic acid measured varied between the different lipoprotein subfractions isolated by cumulative density gradient ultracentrifugation (Table 5.10). It was highest in the VLDL1 and lowest in the LDL-B subfractions $p<0.0001$ in all subjects (Table 5.10) (Fig 5.7). The sialic acid concentration in VLDL1 was significantly higher compared to VLDL2 ($p<0.01$) and IDL and LDL subfractions ($p<0.0001$). The later two were not significantly different in their sialic acid content (Table 5.10)

Table 5.10

Correlation between the sialic acid content of apoB containing lipoprotein subfractions and their reactivity towards APG, as determined by turbidity measurement by absorbance at 600nm.

*Variable**	*sialic acid*	*APG-Lipoprotein complex*	*correlation r*
VLDL1	119.30 ± 127.2	0.05 ± 0.02	0.02
VLDL2	42.45 ± 16.98	0.08 ± 0.06	0.31
IDL1	20.50 ± 9.91	0.25 ± 0.16	0.26
IDL2	18.91 ± 6.97	0.45 ± 0.19	0.24
LDLA	18.39 ± 4.52	0.42 ± 0.16	0.34
LDLB	18.82 ± 4.81	0.25 ± 0.15	0.14

**VLDL1, very low density lipoprotein Sf 60-400; VLDL2, very low density lipoprotein Sf 20-60; IDL1, intermediate density lipoprotein Sf 16-20; IDL2, intermediate density lipoprotein Sf 12-16; LDLA, low density lipoprotein Sf 8-12; LDLB low density lipoprotein Sf 0-8, data represent mean ± SD (standard deviation) for each variable.*

In each individual, LDL-A and LDL-B subfractions had similar sialic acid contents (mol/mol lipoprotein). However the sialic acid content on the LDL fractions was highest in subjects with predominantly LDLI compared to subjects with predominantly small dense LDLIII, this was not statistically significant and was not related to the extent of their interaction with APG (Table 5.11) When the sialic acid content in the other lipoprotein subfractions was examined no significant differences was found among the three different groups (Table 5.11).

Table 5. 11

The sialic acid content and APG binding reactivity of apoB containing lipoprotein subfractions. Comparison between subjects with predominantly LDLI, LDLII and LDLIII subfraction profile.

Variable	*LDLI*†	*LDLII*†	*LDLIII*†
VLDL1			
SA/ApoB (mol/mol)	62.92 ± 67.41	89.19 ± 46.38	157.28 ± 168.4
APG-reactivity (AU	0.03 ± 0.01	0.05 ± 0.02	0.05 ± 0.03
VLDL2			
SA/ApoB (mol/mol)	34.51 ± 30.92	36.57 ± 4.11	48.91 ± 17.0
APG-reactivity (AU)**	0.04 ± 0.01	0.08 ± 0.04	0.11 ± 0.07
IDL1			
SA/ApoB (mol/mol)	16.21 ± 15.10	16.70 ± 2.32	24.42 ± 10.9
APG-reactivity (AU)*	0.10 ± 0.08	0.26 ± 0.09	0.33 ± 0.18
IDL2			
SA/ApoB (mol/mol)	12.51 ± 11.40	18.94 ± 2.13	20.73 ± 7.4
APG-reactivity (AU)*	0.24 ± 0.08	0.48 ± 0.06	0.57 ± 0.20
LDLA			
SA/ApoB (mol/mol)	19.83 ± 2.74	17.56 ± 2.41	18.64 ± 6.11
APG-reactivity (AU)*	0.24 ± 0.07	0.47 ± 0.07	0.50 ± 0.16
LDLB			
SA/ApoB (mol/mol)	22.31 ± 2.73	16.92 ± 1.72	19.09 ± 5.93
APG-reactivity (AU)**	0.13 ± 0.06	0.23 ± 0.1	0.36 ± 0.16

** P<0.0001; **p0.003*

†LDLI large buoyant; LDLII, intermediate density; LDLIII, small dense low density lipoproteins from Young healthy, normolipidemic and hyperlipidemic CHD patients respectively, data represent mean ± SD (standard deviation) for each variable.

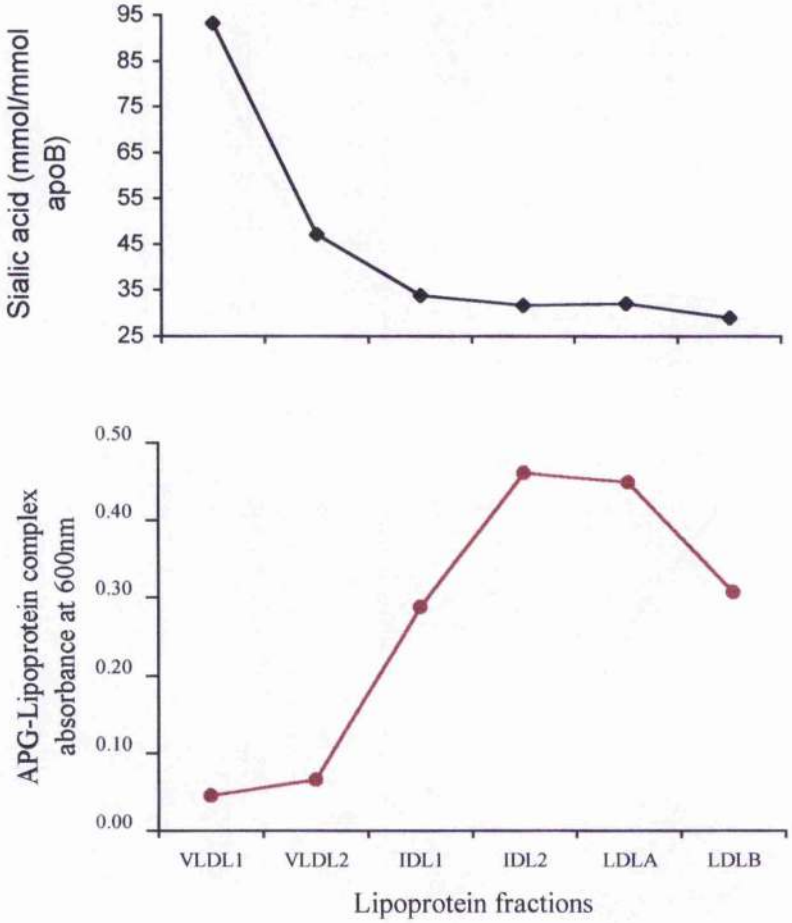
5.5.2 Sialic acid content and APG binding

The APG binding reactivity of apoB containing lipoprotein fractions from the plasma of these subjects are described in detail in section 4.2 (Chapter 4).

A similar pattern of lipoprotein-APG complex formation was observed in all individuals (also Fig 4.5, Chapter 4). The mean reactivity for the lipoprotein subfractions in the three groups was highest in IDL2 and LDLA followed by LDLB and IDL1 and the least reactivity was observed in the VLDL subfractions (Fig 4.6, Chapter 4). Although sialic acid concentration was highest in VLDL1 and lowest in LDLB, it was not correlated with the extent of APG-Lipoprotein interaction (Fig 5.6).

Figure 5.6

The amount of sialic acid content and the extent of reactivity of the different lipoprotein subfractions towards APG.



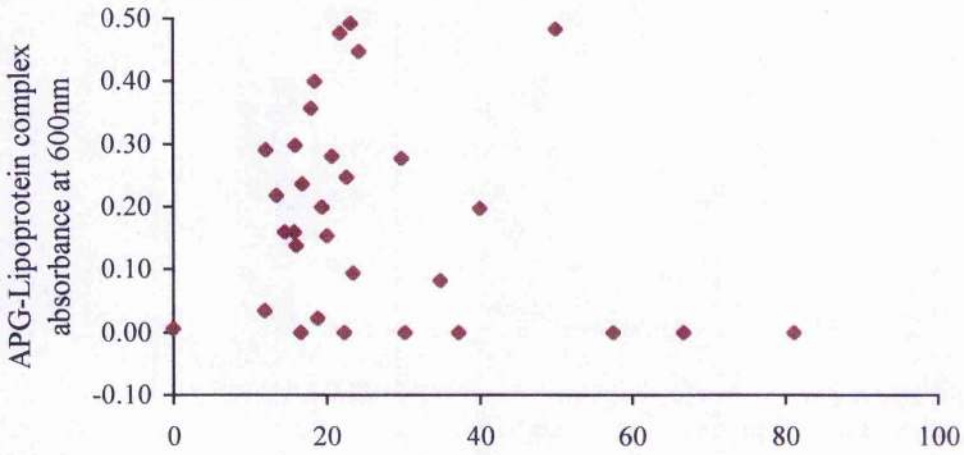
The graphs represent mean values for the sialic acid concentration and the pattern of APG-Lipoprotein complex formation in the apoB containing lipoproteins in all the subjects.

There was no significant correlation between the amount of sialic acid in the different apoB containing lipoprotein subfractions and their reactivity with APG (Table 5.11) (Figures 5.7 A & B, 5.8 A & B).

Figure 5.7

Correlation between the sialic acid concentration in IDL1and IDL2 subfractions and their reactivity towards APG.

A:IDL1



B: IDL2

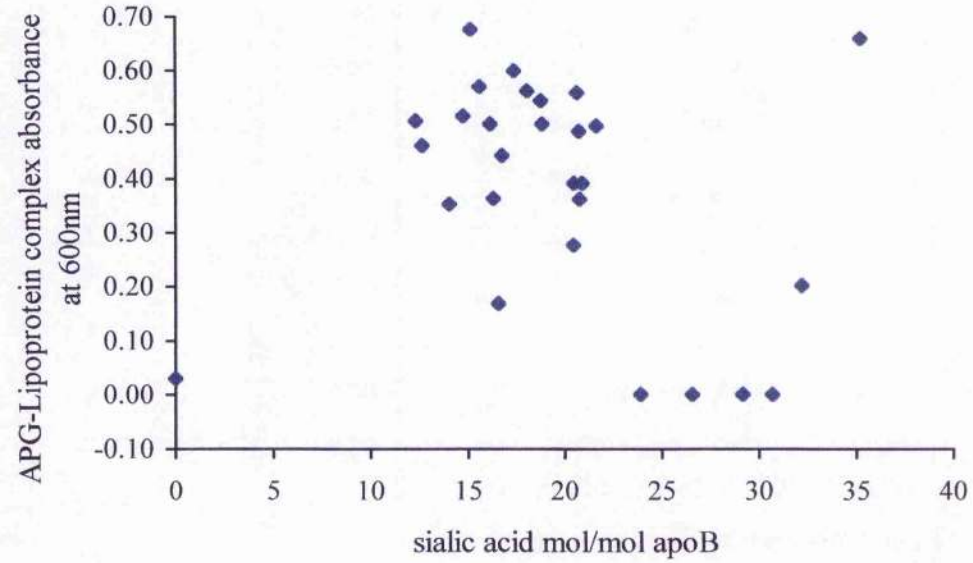
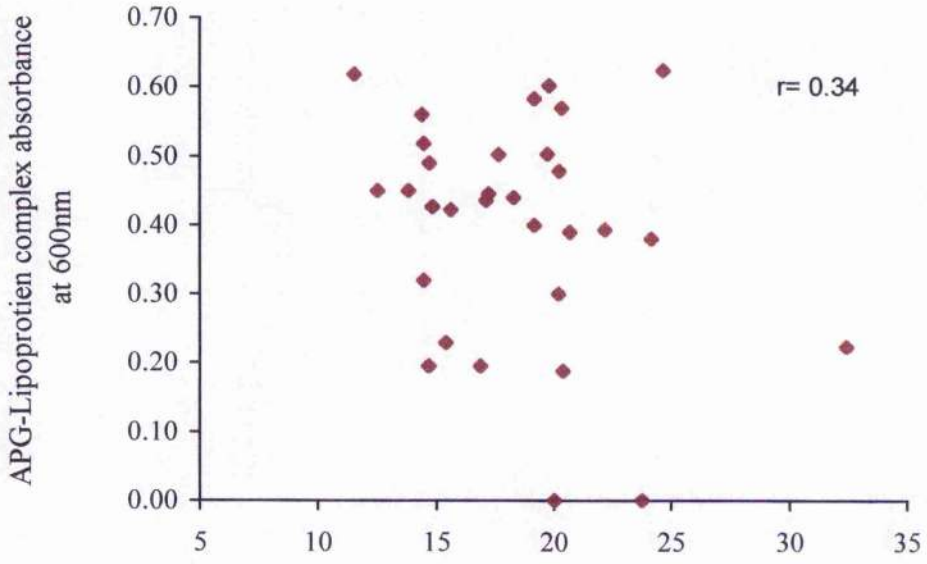


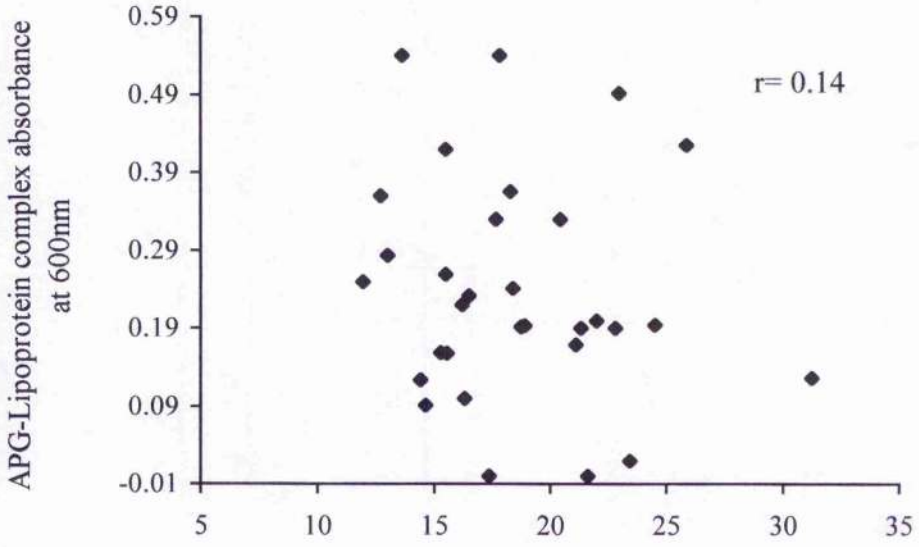
Figure 5.8

Correlation between the sialic acid concentration in LDLA and LDLB subfractions and their reactivity towards APG.

A: LDLA



B: LDLB



5.6 Study III: Effect of ciprofibrate treatment on the sialic acid content of apoB containing lipoproteins and its relation to APG binding reactivity

Treatment with ciprofibrate (100mg/day for 8 weeks), in addition to its lowering effects on plasma total cholesterol (18%, p=0.039), plasma triglyceride (44%, p<0.05) and LDL cholesterol (10%, NS) concentration and the changes in the LDL subfraction profile (Table 4.6, chapter 4), has resulted in a significant decrease in the sialic acid content of the apoB containing lipoprotein subfractions (Table 5.12). There was a 45% reduction in VLDL1 (p=0.03), a 46% in VLDL2 (p<0.001), a 46% in IDL1 (NS), a 48% in LDLA (NS) and a 24% reduction in the amount of sialic acid in LDLB subfraction (p=0.02) (Table 5.12) (Figure 5.9).

Table 5.12

Sialic acid content of apoB containing lipoprotein fractions before and after treatment with ciprofibrate

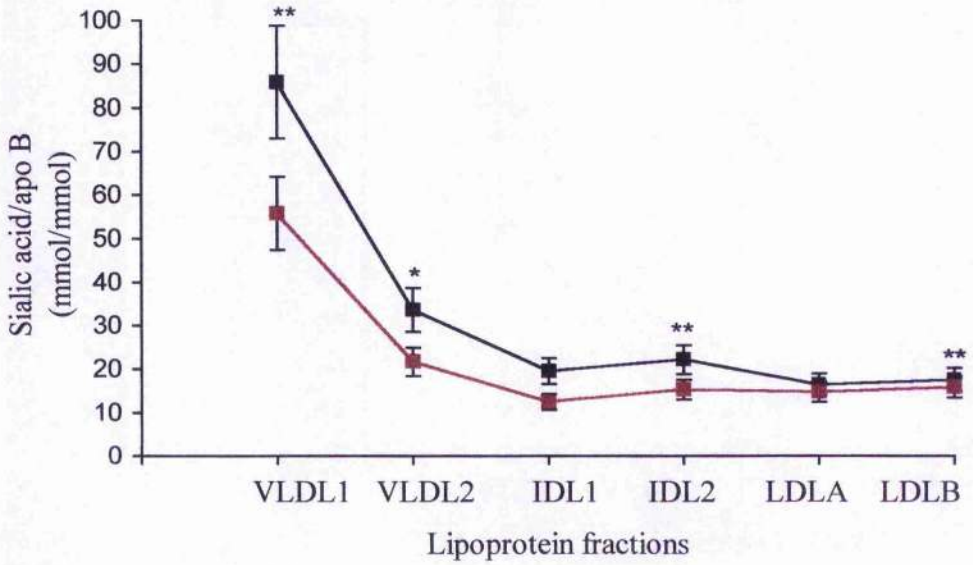
Sialic acid/apo B‡ (mol/mol)	*Ciprofibrate treatment**		
	Before	***After***	*p*
VLDL1	97.4 ± 37.3	53.3 ± 22.8	=0.03
VLDL2	37.9 ± 4.4	19.7 ± 7.6	p<0.001
IDL1	21.3 ± 14.1	11.3 ± 5.2	0.13
IDL2	23.1 ± 11.2	12.0 ± 0.9	0.04
LDLA	16.7 ± 2.3	14.1 ± 2.7	0.09
LDLB	17.9 ± 3.1	13.5 ± 2.9	0.02

**ciprofibrate treatment 100mg/day for 8 weeks; ‡sialic acid concentration mol/mol apolipoprotein B, Very low density lipoprotein =VLDL1 (Sf 60-400) and VLDL2 (Sf 20-60), intermediate density lipoprotein = IDL1 (Sf 16-20) and IDL2 (Sf 12-16), low density lipoprotein = LDLA (Sf 8-12) and LDLB (Sf 0-8). data represent mean ± SD (standard deviation) for each variable.*

ciprofibrate treatment also decreased the ability of each lipoprotein subfraction to form complexes with APG. IDL1, IDL2 and LDLA reactivity were significantly reduced by 54% (p=0.03), 49% (p=0.006) and 40% (p=0.03) respectively and that of small dense LDL by 42% but this was not statistically significant (Table 4.7, Fig 4.7 chapter 4). There was little or no change in the reactivity of the VLDL subfractions with APG. This change in sialic acid content was not correlated to the change in lipoprotein-APG interaction.

Figure 5.9

Effect of lipid lowering treatment with Ciprofibrate (100mg/day for 8 weeks) on the sialic acid content of apoB containing lipoproteins



The graph represents the median value for each lipoprotein subfractions with standard error bars in all the subjects, the line is median connect. ■ before ciprofibrate, ■ after treatment with ciprofibrate. * $p<0.001$, ** $p<0.05$.

5.7 Discussion

A wide variation in the carbohydrate content of glycoconjugates in various human LDL preparations has been shown by several investigators (Fless *et al* 1986, Marshall *et al* 1962, Fontaine & Malmendier 1975; 1978, Swaminathan &

Aladjem 1976, Filipovic et al 1979, Shireman & Fisher 1979), CHD patients have been shown to have lower LDL neutral carbohydrate and sialic acid content compared to normal subjects (LaBelle & krauss 1990, Tertov *et al* 1993). However the function of the carbohydrate moiety in LDL is not clear. In our present study we examined the carbohydrate (neutral and sialic acid) content of LDL in relation to the plasma lipid level, LDL subfraction profile and the binding reactivity towards arterial wall proteoglycans.

LDL-I subfraction contained more carbohydrate compared to LDL-III subfractions in all the subjects, in keeping with the findings by other investigators (LaBelle & Krauss 1990). However, the difference in the amount of neutral carbohydrate content of total LDL, which was slightly lower in CHD patients compared to normal subjects, was not significant. This was also observed by Labelle & Krauss when they correlated the carbohydrate content with the peak LDL particle diameter within the subclass pattern A and B phenotypes (LaBelle & Krauss 1990).

LDL subfraction profile was strongly determined by the plasma triglyceride level (Griffin et al 1994, Tan et al 1995), this has been reflected in the dyslipidemia syndrome of ALP (Austin et al 1990), in which predominance of small dense LDL-III coexists with moderate increase in plasma triglyceride and low HDL cholesterol. There was no relationship between the neutral carbohydrate content of total LDL and the presence or absence of ALP. Total LDL neutral carbohydrate content correlated neither with the plasma lipid levels nor with the LDL subfraction profile. On one hand this might suggest that the regulation and distribution of the carbohydrate residues on LDL particle might be influenced by factors other than the LDL subfraction profile and on the other hand it might be due to comparable particle size diameter, which has not been measured in this study, in both subject groups even though they differ in their plasma lipid levels. The later is supported by the findings of Labelle and Krauss that LDL particle diameter was a more significant predictor of LDL total carbohydrate content than plasma triglyceride (LaBelle & Krauss 1990). The same investigators have shown that subjects with familial hypercholesterolemia have a large LDL particle diameter and their LDL neutral

carbohydrate and sialic acid content are similar to that of normal pattern A subjects (LaBelle & Krauss 1990).

Total LDL affinity chromatography on Con-A Sepharose showed a similar elution pattern to what has been observed by other investigators (McConathy & Alaupovic 1974, Tavella *et al* 1991). The percentage of the weakly bound fraction was lower than the percentage of the strongly bound fraction, in total LDL of normal subjects and LDL-I subfraction, compared to the total LDL from CHD patients and LDL-III subfractions respectively. This suggest that the high mannose residue is greater in LDL-I compared to LDL -III subfractions regardless of the amount of carbohydrate present and because the pattern of elution did not correlate with plasma lipid level and the LDL subfraction profile, we assume that the carbohydrate content of LDL is not entirely influenced by plasma lipid levels.

When we looked at the sialic acid concentration, both LDLA and LDLB subfractions were found to have a lower sialic acid content in patients with predominantly LDL-III subfraction compared to normal subjects with predominantly LDL-I subfraction profile. This finding was consistent with earlier work that reported sialic acid poor (SAP) LDL in CHD patients and sialic acid rich (SAR) LDL in normal no-diseased subjects (Tertov *et al* 1993). Labelle and Krauss had suggested that this difference is due to the lower glycolipid content of LDL in pattern B subjects compared to normal pattern A, as there was no difference in the apoB sialic acid content in both groups (LaBelle & Krauss 1990). Therefore, we attribute the similar sialic acid concentration of LDLA and LDLB subfractions observed in our study, to the similar lipid content of the of the two subfractions.

Considering the effect of the above mentioned lipid and carbohydrate parameters on the interaction of LDL with APG, we found that APG was correlated significantly to the plasma lipid levels and LDL subfraction profile in the same way as has been reported (Anber *et al* 1996, Anber *et al* (in press)) and discussed in Chapter 4 & 5. While no significant correlations were found between the extent of APG-LDL interaction and the amount of neutral carbohydrate in total and LDL subfractions. In addition the weakly bound and

the strongly bound fractions eluted from the Con-A Sepharose affinity chromatography column showed a similar binding reactivity towards APG.

The concentration of sialic acid was highest in VLDL, which decreased progressively across the lipoprotein spectrum and was lowest in LDL. If sialic acid concentration was of major importance in the APG-Lipoprotein interaction, as was suggested by Camejo (Camejo *et al* 1985b, Camejo *et al* 1990), who observed that desialation of LDL by neuraminidase treatment increase in its reactivity with APG (Camejo *et al* 1985b), then one would speculate that the extent of lipoprotein binding reactivity towards APG should be highest in LDL compared to the other lipoprotein species. However, we found that APG-Lipoprotein complex formation was highest in IDL2 and LDLA. The former contained more sialic acid compared to the LDL subfractions. Similarly it has been shown that lp(a), with a higher content of carbohydrate and notably that of sialic acid than LDL apoB, has a greater reactivity towards APG compared to LDL (Bihari-Varga *et al* 1988). The effect of neuraminidase treatment on LDL will be discussed in detail in the next chapter (chapter 6). In addition the lipid lowering effect of ciprofibrate on apoB containing lipoproteins which decreased their sialic acid content resulted in a decrease, rather than the expected increase, in the ability of these lipoproteins to interact with APG. Our findings of the reducing effect of ciprofibrate on APG-lipoprotein interaction supports the findings by Wiklund *et al* (Wiklund *et al 1996*). All these suggest that carbohydrate and sialic acid content of lipoproteins have little, if any, effect on their interaction with APG which might be more conformation related rather than charge. It has also been shown that carbohydrate moiety had no role in the binding of apolipoprotein B to the LDL receptor (Shireman and Fisher 1979)

We conclude that the neutral carbohydrate and sialic acid content of LDL is variable and is not clear cut. The concentration of which is not related to the extent of APG-Lipoprotein complex formation which is determined to a greater extent on plasma triglyceride level and its influence on the LDL subfraction profile and other lipoproteins in plasma such as IDL. Finally the conformation of lipoprotein subfraction appears to be more important in determining their interaction towards APG rather than their carbohydrate concentration. This is discussed fully in chapter 6.

Chapter 6

Mechanisms related to APG-Lipoprotein interaction...

6.1 Introduction

In previous chapters we explored the role of plasma lipids and lipoprotein carbohydrate content in determining the interaction of apoB containing lipoproteins with arterial wall proteoglycans. High plasma triglyceride, low HDL cholesterol and predominance of small dense LDL-III, collectively described as the dyslipidemic syndrome of atherogenic lipoprotein phenotype (ALP) (Austin *et al* 1990), were shown to be associated with increase APG-LDL complex formation (Anber *et al* 1996). Reactivity was not correlated with carbohydrate concentration of the lipoprotein as was originally hypothesised (chapter 5).

The question which arises next is "how APG traps lipoproteins and what is the nature of this binding process". The mechanism by which APG interacts with lipoprotein has been of a particular focus of research over the past decade. Until now there is no comprehensive explanation as to how lipoproteins form complexes with extracellular matrix proteoglycans in the arterial intima-media. It is believed that this could be a function of the charge, sialic acid content or conformation of apoB (Fig 6.1)

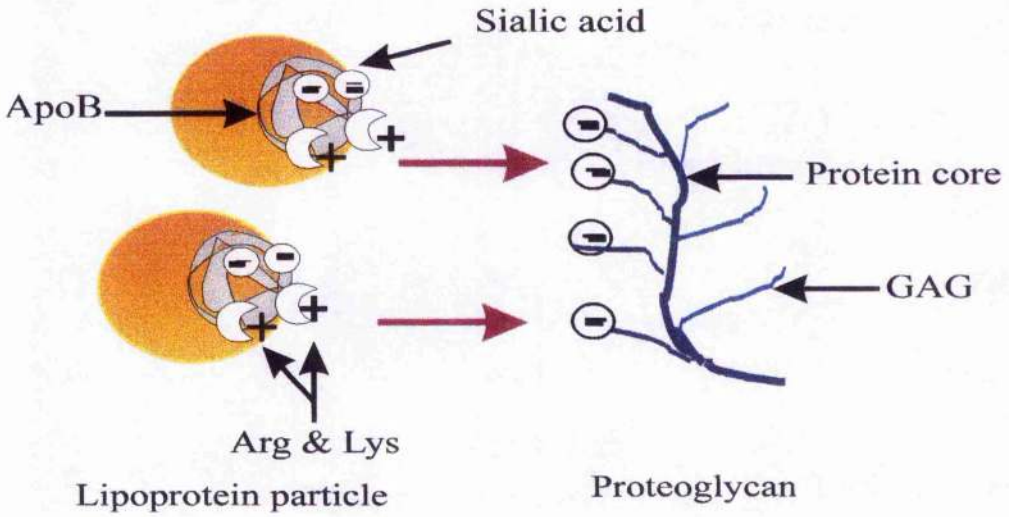


Figure 6.1

A schematic diagram illustrating the mechanisms of APG-Lipoprotein binding:

1. *Charge (positively charged arginine & lysine residues on apoB interacts with negative charges on APG)*
2. *Sialic acid (Confer negative charges on the lipoprotein thereby decreasing the net positive charge on the particle surface.*
3. *ApoB conformation*

Proteoglycans are the most negatively charged macromolecules in living tissue (Wight 1989). This gives them the property to form complexes with positively charged residues on other molecules. Therefore the reaction mechanism of LDL and APG was suggested to be an electrostatic binding involving basic amino groups on apoB and polyanionic groups of APG (Camejo *et al* 1988). Olsson *et al* have shown a positively charged amino acid sequence on apoB rich in arginine and lysine to be mainly responsible for the interaction of LDL with APG (Olsson *et al* 1993). This has supported earlier work by Vijayagopal *et al* that chemical modification involving lysine and /or arginine residues on apoB prevented LDL from forming complexes with APG (Vijayagopal *et al* 1981). In the same study it was also shown that removal of the protein core, glycosaminoglycan (GAG) chains and desulphation of proteoglycan also inhibited complex formation with LDL, further suggesting the importance of charge in the interaction process (Vijayagopal *et al* 1981).

Since apoB contains sialic acid residues (Swaminathan & Aldjamen 1976, Vauhkonen *et al* 1985) which are negatively charged, it is believed to deter

LDL from binding to APG (Camejo *et al* 1985b). In an early study Camejo *et al* examined the nature of neuraminidase treated LDL interaction with APG (Camejo *et al* 1985b). They attributed the increase in the ability of the desialated LDL to interact with APG to the increase in the net positive charge of the lipoprotein. The same group of investigators have suggested that small dense LDL, which has been shown to contain less sialic acid compared to large buoyant LDL (Labelle and Krauss 1990), has a greater affinity towards APG (Camejo *et al* 1990). This was concluded after the interesting finding by Hurt-Camejo (Hurt-Camejo *et al* 1990) that when APG was used to fractionate LDL, it had a greater preference for small dense LDL . In contrast, Bihari-varga *et al* reported that neuraminidase treatment of LDL did not interfere with GAG or chondroitin sulphate binding (Bihari-Varga *et al* 1988). In addition, when we examined the direct relative reactivity, as measured by absorbance at 600nm, of different lipoprotein subfractions towards APG, it was highest in the IDL2 and LDLA species (chapter 4, Anber *et al in press*). These subfractions contain a higher concentration of sialic acid compared to small dense LDLB (chapter 4). Similarly the reactivity of Lp(a), which also contain more sialic acid than LDL, has been shown to be greater compared to the reactivity of LDL towards APG (Bihari-Varga *et al* 1988). All the above indicate that the binding process is not entirely charge dependant.

Larger triglyceride rich particles (VLDL and IDL) have been shown to enter the arterial intima (Nordestgaard & Nielsen 1994), interact in-vitro with APG (Vijayagopal *et al* 1981, Anber et al *in press, chapter 4*) and have been isolated from atherosclerotic plaque with LDL (Srinivasan *et al* 1975, Rapp *et al* 1994) They contain apoE as well as apoB, both of which are enriched in atherosclerotic lesions, indicating that apoE might play a role in this interaction process. It has been suggested that apoE might enhance APG-LDL binding (Parks et al 1991, Giaturco & Bradley 1994). Decreasing apoE:apoB ratio of LDL by dietary polyunsaturated fat has been shown to decrease its interaction with APG (Manning *et al* 1994). In addition, it has been suggested that apoE might act as a bridging molecule between APG and the lipoprotein, possibly through heparin binding sites on both apoB and apoE (Saxena *et al* 1993, Ji *et al* 1993, Wight 1995). Therefore, an increase in the concentration of apoE is

thought to provide additional binding sites for the GAG chains in APG (Wight 1995). On the other hand, apoE rich HDL has been shown to block lipoprotein lipase-induced retention of LDL in the extracellular matrix (Saxena *et al* 1993). This is believed to be due to similar binding sites for apoE and lipoprotein lipase on APG (Mulder *et al* 1993,Wight 1995). Such competition was suggested to be one way by which apoE rich-HDL protects against atherosclerosis (Saxena *et al* 1993). Therefore, the role of apoE in influencing APG-Lipoprotein interaction remains controversial.

Data on the influence of chemical composition of the lipoprotein on APG binding is inconsistent. LDL with a high ratio of cholesteryl ester to triglyceride was shown to have an increased tendency to form insoluble complexes with APG (Avila *et al* 1978). Kostner et al have shown highly significant correlation between the content of free cholesterol and of phospholipids with the LDL:GAG ratio in the insoluble complex formed (Kostner *et al* 1985). While Wagner et al found no significant influence of the chemical composition of LDL on its binding reactivity towards APG (Wagner *et al* 1989).

Taking these findings into account, and based on our data, we hypothesise that the conformation of apoB might have a key role in this interaction process. Evidence in support of this is the suggestion by Wagner *et al* (Wagner *et al* 1989) that surface property of LDL might be responsible for increased number of LDL particles bound to APG.

To explore further the mechanism of the binding process. We undertook a series of studies the objective of which were: 1) to examine the role of sialic acid in the reactivity of LDL towards APG, 2) to determine the nature of this interaction in terms of charge and protein structure by examining the effect of modified lysine and arginine residues on the ability of LDL to interact with APG under near to physiologic conditions, 3) to determine the effect of apoE and apoC on the binding process by incubating total LDL with apoE2/3 and apoCIII, and finally 4) to determine the chemical composition of the APG bound LDL, in the pellet, and the unbound, in the supernatant, and their relation to the extent of complex formation.

6.2 Subjects

A total of thirty subjects were recruited for various studies. Total LDL from 14 normal volunteers aged 22-45 years was used for studies I, II, and III, and the rest of the subjects (n=16) recruited for the second part of study III, were CHD patients aged 45-73 years. These patients were undergoing diagnostic coronary angiography in the Department of Medical Cardiology, Glasgow Royal Infirmary. Subjects in the fasting state donated 50ml of blood which was collected by venepuncture using K_2EDTA (final concentration 1mg/ml) as anticoagulant. Plasma was harvested at 4°C by low speed centrifugation (3000rpm) and aliquots for lipid, lipoprotein measurements and LDL subfractionation used immediately. Subjects with recent MI (i.e. in the previous 6 months), renal disease, thyroid disease, diabetes or those taking lipid-lowering medications were excluded, as were those with a plasma cholesterol concentration of >9.0mmol/l and a plasma triglyceride level of >5.0mmol/l. The study was approved by the Research Ethics Committee of Glasgow Royal Infirmary and each volunteer gave written informed consent.

6.2.1 Study I: Role of sialic acid in the mechanism of APG-Lipoprotein interaction

Since we found no correlation between the sialic acid concentration and APG-Lipoprotein complex formation (chapter 5), we incubated total LDL with neuraminidase and different gangliosides to change the sialic acid content on the lipoprotein and tested the relative reactivity of this treated LDL vs control LDL. Neuraminidase treatment of LDL was performed by incubating total LDL from three normal subjects, isolated by preparative sequential ultracentrifugation at densities 1.019-1.063 g/ml, with neuraminidase type VI-A from Clostridium Perfringens for 25h at 37°C on a roller mixer as described (Camejo et al 1985b) (chapter 2).

Total LDL from another three subjects were incubated with different dilutions of crude, GM3, GM1ganglioside and asialoganglioside (mol/molLDL) (Sigma Pharmaceuticals) for 1 hr at 37°C as described in chapter 2.

6.2.2 Study II : Effect of LDL modification on APG-LDL binding

To explore further the function of charge in the mechanism of LDL-APG interaction, total LDL isolated from fresh plasma from four volunteers by preparative sequential ultracentrifugation at densities 1.019-1.063 g/ml was subjected to chemical and enzymatic modification. Cyclohexandione modification of LDL arginine residues was achieved by the addition of 1,2 cyclohexandione and carbamylation of lysine residues by mixing with potassium cyanate as described previously (Weisgraber *et al* 1978), both reactions reduce the net positive charge on LDL apoB. Reductive methylation of lysine residues was performed using sodium borohydrate and the addition of 40% formaldehyde (chapter 2).

6.2.3 Study III : Apolipoprotein E and apolipoprotein C and APG binding

Factors tested in this study were apoE, apoCIII and heparin. Total LDL isolated from the plasma of 8 subjects (normal volunteers) were incubated with different dilutions of apoE2, apoE3 and apoCIII for 1h at 37°C as described in chapter 2.

Total LDL from 16 CHD patients was examined to determine the effect of heparin on APG-LDL interaction. Each patient donated two 50 ml samples of blood, collected in to EDTA tubes, one before and the other immediately after cardiac angiogram. Heparin (1000-2000 IU, according to the patients BMI) was flushed down the femoral artery into the inferior venacava during the catheterisation procedure. Therefore plasma obtained after the angiogram was mixed with heparin. LDL isolated from the first sample (without heparin) was termed as native LDL and from the second sample, heparinised LDL.

6.3 APG-Lipoprotein complex formation

The binding assay has been described in detail in chapter 2. In all the studies a blank and a test was performed on each sample to detect non-specific LDL precipitation.

Control samples were exposed to the same buffers (without the addition of the enzymes and apoproteins listed in Table 6.2) and conditions as the modified LDL samples. The ability of these enzymes and apoproteins to form complexes with APG was tested by incubating them with 2.5 μg CS-rich APG under the same conditions of the binding assay but without the addition of LDL (chapter 2).

6.4 Statistical analysis

Statistical analysis and manipulations were performed using the PC version of MINITAB Release 10 for Windows (Minitab Inc., PA). associations between variables were tested by calculating the pearson correlation coeffecient. The significance of association between pairs of variables was determined by linear regression.

6.5 Subjects characteristics

The subjects characteristics are shown in Table 6.1. Normal volunteers had significantly higher HDL cholesterol and a lower plasma triglyceride level than CHD patients (Table 6.1). They also had a predominantly LDL-II in their subfraction profile compared to the LDL-III in the CHD patients.

Table 6. 1

Patients characteristics

Lipid variable (mmoll)	*Normal volunteers n=14*	*CHD patients n=16*
T-Cholesterol	4.6 ± 0.9	5.8 ± 1.5
VLDL-Cholesterol	0.5 ± 0.2	0.7 ± 0.4
LDL-Cholesterol	3.4 ± 0.7	3.8 ± 1.4
HDL-Cholesterol	1.2 ± 0.5	1.0 ± 0.3*
Triglyceride	1.0 ± 0.3	1.6 ± 0.8*

**p<0.01, CHD, coronary heart disease patients; n, represent number of subjects.*

6.6 Results

6.6.1 Neuraminidase treatment of LDL and APG binding

Incubation of total LDL, (d= 1.019-1.063 g/ml) with agarose bound neuraminidase for 25h released up to 37%, and incubation with the free enzyme released up to 50%, of the sialic acid content of the lipoprotein (Table 6.2). This resulted in a 54% and a 176% increase respectively in the binding reactivity of the LDL compared to the control sample (Table 6.2). No insoluble complex formation was detected between neuraminidase and APG (Table 6.2).

6.6.2 The effect of LDL incubation with ganglioside on APG binding

The reactivity of total LDL was perturbed following incubation with different gangliosides to increase the amount of carbohydrate on apoB. GM3 ganglioside from plasma resulted in only 12 % reduction in the binding reactivity (Fig 6.2a). Increasing concentrations of crude ganglioside from brain, decreased the reactivity of LDL towards APG and near abolition of the reactants at 40mol/mol LDL (Fig 6.2b). GM1 gangliosides had no effect on binding while asialo-GM1 increased the binding interaction by 10% (Fig 6.3a and b, respectively) (Table 6.2). When APG was mixed with gangliosides without LDL, no insoluble complex formation was observed (Table 6.2).

Table 6. 2

The extent of APG-LDL complex formation. Comparison between control and modified LDL.

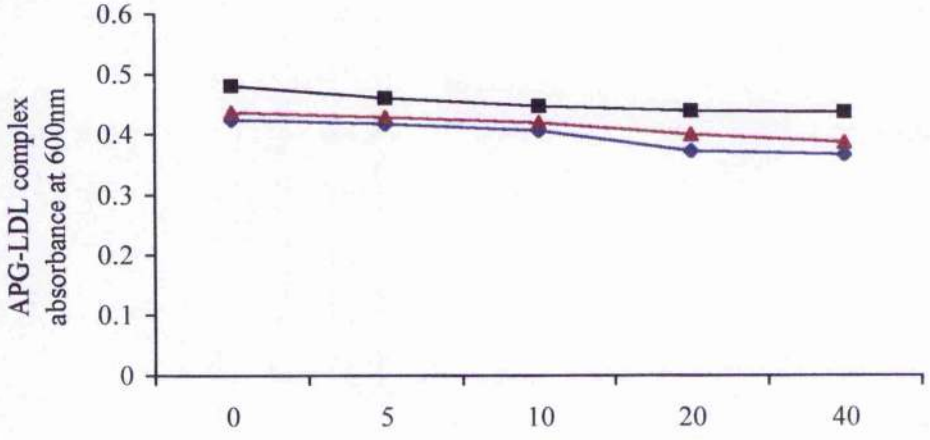
Variable	*APG-LDL complex*		*APG complex*
	*Test (% change)**	*control*	*(without LDL)†*
Neuraminidase	0.389 (+76)	0.221 (100)	0.005
GM3-ganglioside	0.397 (-12)	0.446 (100)	0.006
Crude-ganglioside	0.055 (-93)	0.602 (100)	0.003
GM1-ganglioside	0.490 (0)	0.490 (100)	0.004
Asialo-ganglioside	0.520 (+10)	0.490 (100)	0.005
ApoE2	0.049 (-92)	0.600 (100)	0.003
ApoE3	0.048 (-91)	0.470 (100)	0.004
ApoCIII	0.470 (-23)	0.606 (100)	0.003
Lp(a)	0.778 (+29)	0.602 (100)	0.666
Heparin	0.271 (-21)	0.385 (100)	-
Cyclohexandione	0.005 (-99.2)	0.580 (100)	-
Carbamylation	0.006 (-99.2)	0.580 (100)	-
Reductive methylation	0.006 (-99.2)	0.580 (100)	-

** % change vs control; † the extent of insoluble complex formation, as measured by absorbance at 600nm, was determined by incubating different amounts (discussed in the appropriate section) of the enzymes and lipoproteins listed with 2.5μg CS-rich APG (in the absence of LDL in the binding mixture) at 25℃ for 30 min. The extent of APG-LDL complex formation was determined by adding 2.5μg CS-rich APG to 0.1mg LDL protein and absorbance was measured at 600nm; the values in the brackets represent the percentage increase or decrease in the binding reactivity after modification.*

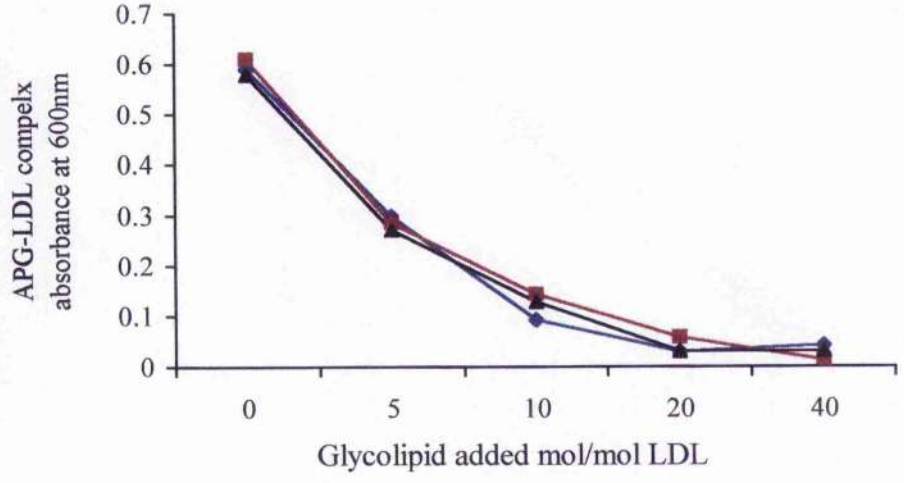
Figure 6.2

The extent of APG-LDL complex formation in the presence of different dilutions of GM3 ganglioside from plasma and crude gangliosides from brain in three different subjects

A: GM3



B: Crude ganglioside

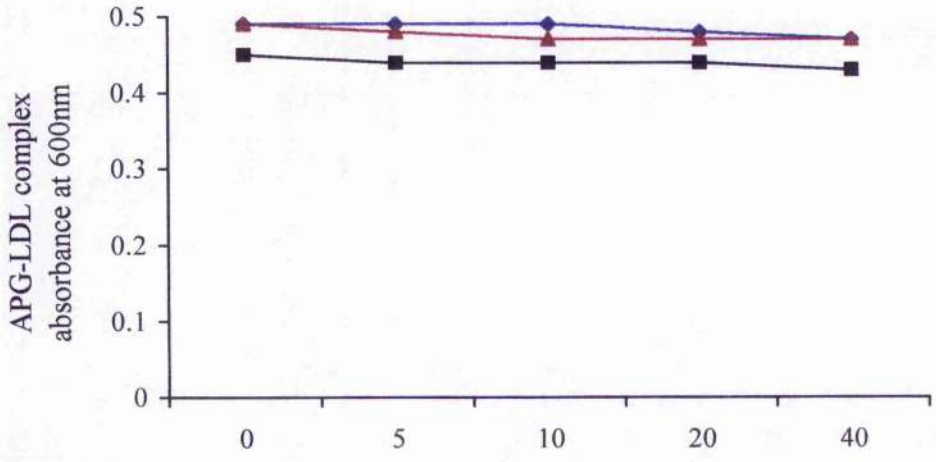


The graph represents the extent of APG-LDL complex formation in three different subjects in the presence of gangliosides. Total LDL was incubated, for 60min at 37℃, with 0-40 mol gangliosides/mol LDL (final apoB concentration 0.1mg/ml), 2.5μg APG was added and the extent of complex formation was measured by absorbance at 600nm after 30min incubation at 25℃. A) GM3 ganglioside from plasma, B) crude ganglioside from brain

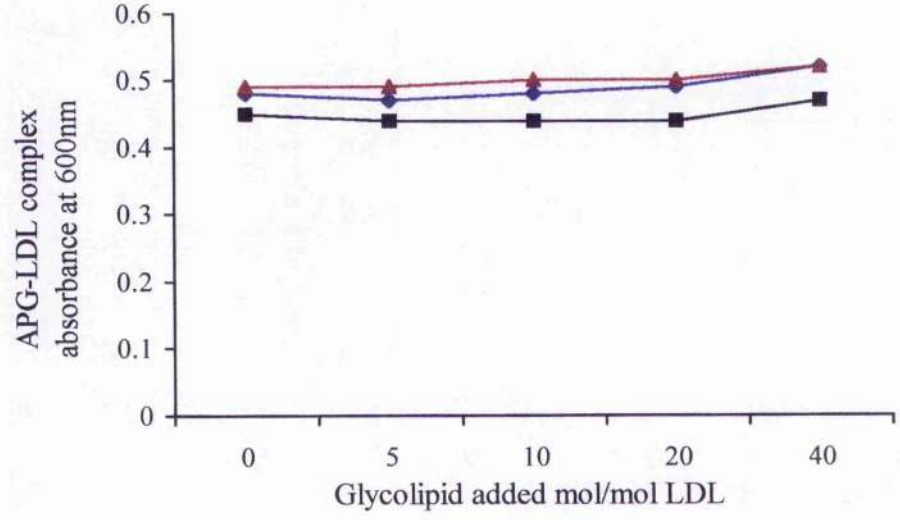
Figure 6. 3

The extent of APG-LDL complex formation in the presence of different dilutions of GM1and asialo-GM1 gangliosides in three subjects.

A: GM1



B: AsialoGM1



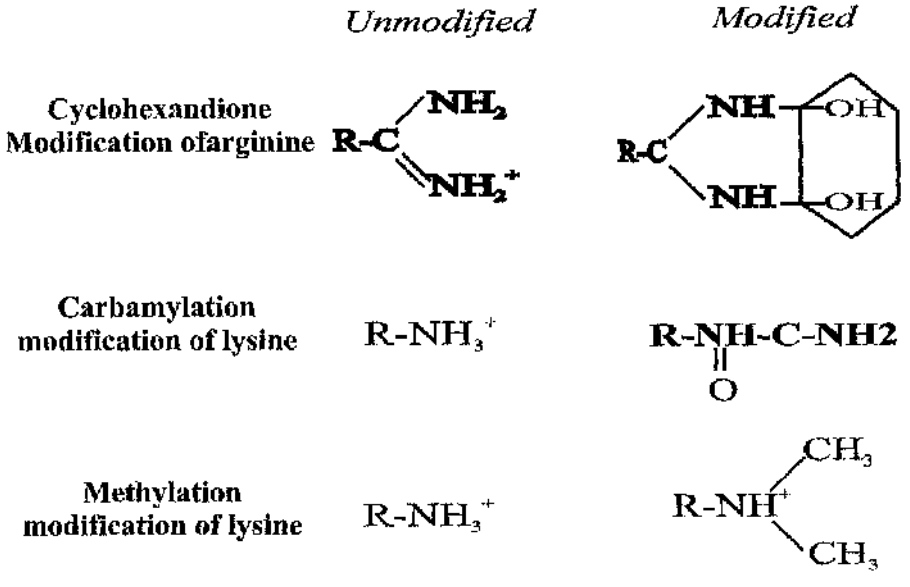
The graph represents the extent of APG-LDL complex formation in three different subjects in the presence of gangliosides. A) GM1 ganglioside, B)Asialo-GM1 ganglioside. Total LDL was incubated, for 60min at 37℃, with 0-40 mol ganglioside/mol LDL (final apoB concentration 0.1mg/ml), 2.5μg APG was added and the extent of complex formation was measured by absorbance at 600nm after 30min incubation at 25℃.

6.6.3 Chemical modification of LDL and APG binding

Total LDL isolated from the plasma of normal volunteers by sequential gradient ultracentrifugation was subjected to a number of enzymatic and chemical modifications. The extent of complex formation between the modified LDL compared to the native and neuraminidase treated LDL with APG is shown in Table 6.2. A blank and a test was performed on each sample to detect non-specific LDL precipitation. Modification of lysine and arginine residues by carbamylation and cyclohexandione treatment respectively, reduced the net positive charge of LDL on agarose electrophoresis (Fig 6.4) and abolished the ability of the LDL to form complexes with APG (Table 6.2, Fig 6.5). Reductive methylation which did not alter the charge on LDL was also effective in blocking completely the binding reactivity of LDL with APG (Table 6. 2).

Figure 6. 4

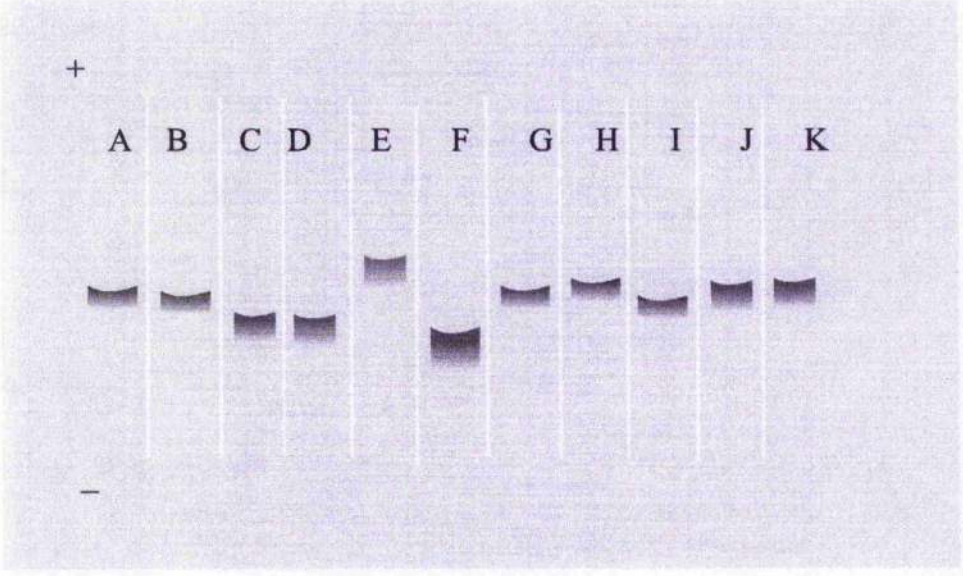
Chemical modification of LDL and effect on APG-LDL complex formation.



The extent of charge modification on the native total, chemically modified and neuraminidase treated LDL samples was tested by agarose electrophoresis (Figure 6.5).

Figure 6. 5

The diagram represents agarose electrophoresis of native and modified total LDL to determine charge modification.



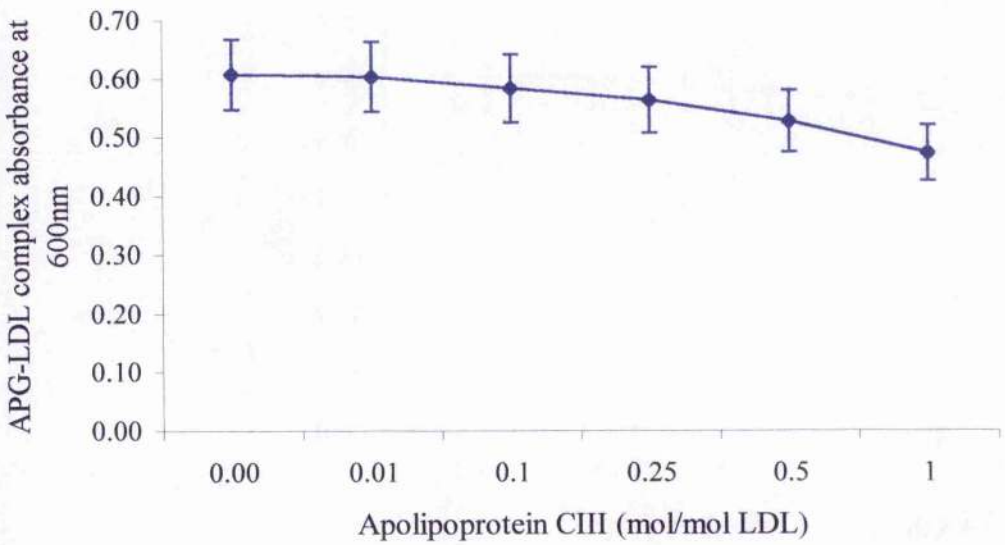
A, native total LDL; (B-K) modified total LDL as follows: B, reductive methylation; C, cyclohexandione treatment; D, carbamylation; E, neuraminidase treatment; F, incubation with crude ganglioside from the brain; G, incubation with GM1 ganglioside; H, incubation with asialo GM1 ganglioside; I, incubation with GM3 ganglioside from plasma; J, incubation with apoE2; K, incubation with apoE3.

6.6.4 Effect of apoE and apoCIII on APG-LDL interaction

Apolipoprotein E_2 and E_3 had similar effect on the binding reactivity of total LDL towards APG. Apolipoprotein CIII had little effect in blocking the ability of LDL to interact with APG (Fig 6.6). The extent of complex formation was reduced by 23% at 1mol apoCIII /mol LDL (Table 6.2). Interestingly, the extent of complex formation decreased as the concentration of apoE (E2 & E3) increased in the binding mixture and was almost completely blocked at 1mol apoE/mol LDL (Fig 6.7 A & B). There was no insoluble complex formation between APG and apoE2, apoE3 or apoCIII (Table 6.2).

Figure 6.6

Effect of apolipoprotein CIII on APG-LDL interaction.

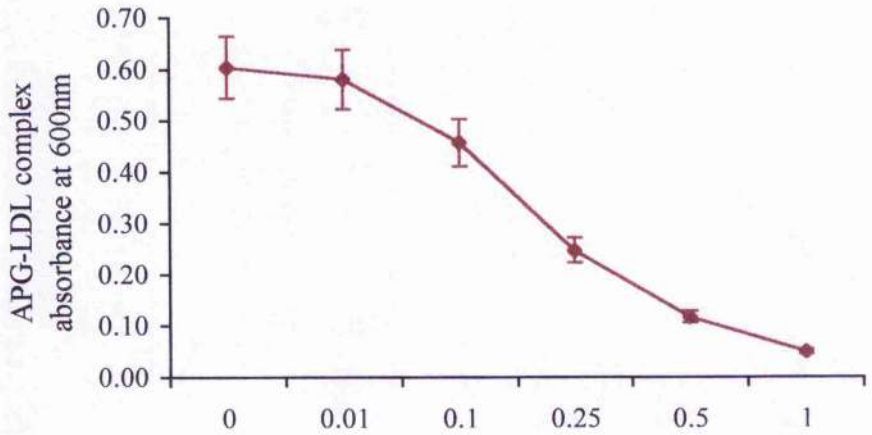


The graph represents mean values for the extent of APG-LDL complex formation, in the presence of apoCIII, in three different subjects. The bars represent standard error. 0.1mg LDL was incubated with 0-1mol apoCIII/mol LDL and mixed with 2.5μg APG. Absorbance was measured at 600nm after 30min incubation at 25℃.

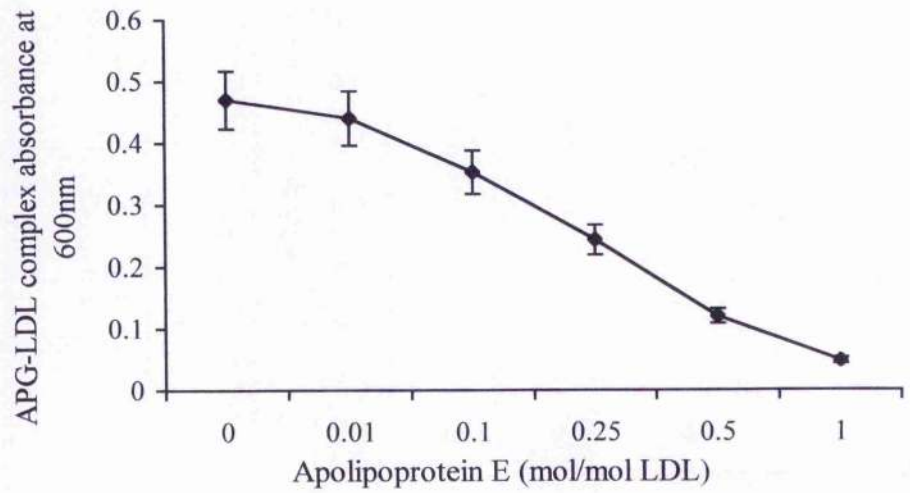
Figure 6. 7

Effect of apoE on APG-LDL interaction.

A : ApoE3



B : ApoE2

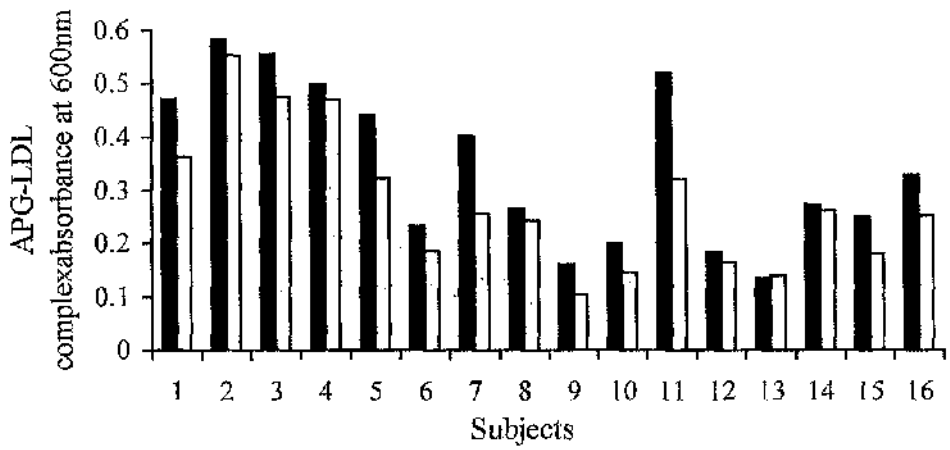


The graph represents mean values for APG-LDL complex formation in the presence of apoE3 (A) and apoE2 (B). 0.1 mg LDL incubated with apoE (0-1mol/molLDL) was mixed with 2.5μg APG and turbidity was measured by absorbance at 600nm.

Heparinisation during the procedure of cardiac catheterisation was significantly effective in reducing the reactivity of total LDL towards APG by 21 % ($p < 0.05$) (Table 6. 2). This was observed in all the patients (Fig 6.8).

Figure 6. 8

Effect of heparin on APG-LDL interaction.



The graph represents the extent of total LDL reactivity towards APG in each patient before and after catheterisation. ■ initial LDL (unheparinised), □ heparinised LDL after catheterisation.

6.6.5 Compositional analysis of apoB containing lipoproteins and binding interaction with APG.

The compositional analysis of the precipitated and the unreacted fractions from the apoB containing lipoproteins are shown in Table 6.3. Free cholesterol and protein were similar in the precipitated fraction and the supernatant in all lipoproteins (Table 6.3), while lipoprotein mass in the precipitate was lower compared to the supernatant (Table 6.3). The precipitate in the VLDL subfractions contained higher esterified cholesterol (EC) and lower triglyceride (TG) compared to the unreacted fraction while the precipitated LDL subfractions were TG-rich and EC poor (Table 6.3). This suggest that the lipoprotein particles which are more prone to be trapped by the APG appear to be TG-poor remnant particles in the TGRL spectrum and TG-rich LDL particles in the LDL species.

Table 6. 3

Compositional analysis of the precipitated and supernatant fractions of very low density, intermediate density and low density lipoprotein with APG.

Variable	*% FC*	*% EC*	*% TG*	*% PL*	*% Protein*	*mass*	*EC/TG*	*P/C*
VLDL1								
reacted	6.6 ± 7.4	12.9 ± 10.3	45.4 ± 18.7	6.3 ± 6.6	20.9 ± 12.8	2.4 ± 1.38**	0.4 ± 0.5	0.4 ± 0.8
unreacted	2.2 ± 2.6	7.4 ± 3.3	70.9 ± 3.3	15.2 ± 2.2	12.9 ± 5.5	38.0 ± 13.8	0.1 ± 0.1	2.2 ± 1.5
VLDL2								
reacted	7.3 ± 10.7	7.9 ± 9.9	53.1 ± 18.9	15.9 ± 6.3	19.8 ± 15.4	1.0 ± 0.5*	0.2 ± 0.2	0.25 ± 0.5
unreacted	4.6 ± 0.9	13.4 ± 8.6	53.7 ± 11.0	16.5 ± 4.8	15.9 ± 2.2	14.5 ± 1.85	0.3 ± 0.2	1.5 ± 0.8
IDL1								
reacted	6.4 ± 5.1	23.0 ± 0.3	26.9 ± 8.5	16.6 ± 12.2	23.4 ± 7.5	2.8 ± 0.6**	0.9 ± 0.2	1.1 ± 0.9
unreacted	5.3 ± 1.0	25.0 ± 4.6	27.8 ± 2.1	16.1 ± 3.7	21.8 ± 6.4	10.2 ± 2.9	0.9 ± 0.2	1.1 ± 0.6
IDL2								
reacted	4.6 ± 1.7	32.9 ± 2.3	17.8 ± 5.3	21.9 ± 7.3	20.5 ± 1.4	4.7 ± 0.4*	1.9 ± 0.6	0.9 ± 0.2
unreacted	7.2 ± 5.1	31.5 ± 7.7	15.6 ± 2.8	15.7 ± 6.6	19.9 ± 0.7	8.1 ± 0.7	2.1 ± 0.8	0.8 ± 0.1
LDLA								
reacted	5.5 ± 2.2	32.8 ± 2.7	16.4 ± 6.9	22.2 ± 11.3	20.5 ± 3.8	4.4 ± 0.5***	2.3 ± 1.2	0.8 ± 0.1
unreacted	3.8 ± 3.2	39.5 ± 5.7	7.8 ± 4.3	13.9 ± 6.5	21.7 ± 1.6	7.4 ± 1.5	7.6 ± 6.6	0.8 ± 0.1
LDLB								
reacted	6.4 ±0.9	29.2 ± 4.0**	15.9 ± 10.0	20.5 ± 10.4	24.7 ± 4.8	3.7 ± 0.8**	2.5 ± 1.7	1.1 ± 0.2
unreacted	3.4 ±1.2	36.5 ± 4.6	9.6 ± 4.7	12.7 ± 5.8	23.4 ± 2.0	6.6 ± 0.9	6.1 ± 6.3	1.0 ± 0.2

**p<0.001, **p<0.01, ***p<0.05 comparison between the precipitate and the unprecipitated fractions; Reacted, is the fraction of the lipoprotein precipitated by APG and dissociated in 2mol/l NaCl; Unreacted, is the fraction of the lipoprotein in the supernatant which was not precipitated by APG; FC, percentage lipoprotein-free cholesterol; EC. Percentage lipoprotein-esterified cholesterol; TG, percentage lipoprotein-triglyceride; PL, percentage lipoprotein-phospholipid; protein, percentage lipoprotein-protein within the lipoprotein; EC/TG, esterified cholesterol to triglyceride ratio; Mass, represent total mass of the lipoprotein; P/C, represents the ratio of protein to cholesterol within the lipoprotein.*

6.7 Discussion

Variability in APG-Lipoprotein interaction has been shown in several studies. This has been attributed partly to variability in APG itself (Wagner *et al* 1986, Alves & Mourao 1988, Cardoso & Mourao 1994, Sambandam 1991) and partly to variation in lipoprotein components (Linden *et al* 1989, Srinivasan *et al* 1984, Anber *et al* 1996, Bihari-Varga *et al* 1988, Vijayagopal *et al* 1981, Wagner *et al* 1989). In this study we focused on factors affecting lipoprotein, and used a single APG preparation which was fully characterised as described in chapter 3. Different mechanisms which have been implicated to play a role in this interaction process were tested. The effect of neuraminidase treatment of LDL on its reactivity with APG (Table 6.2), in keeping with the findings by Camejo (Camejo *et al* 1985b) supports the sialic acid hypothesis in which the higher reactivity of small dense LDL towards APG was attributed to its lower sialic content which increases the net positive charge of the lipoprotein particle (Camejo et al 1985b). It also supports our earlier findings (Anber et al 1996) that LDL from subjects exhibiting an ALP with a predominance of small dense LDL-III (chapter 3) is more reactive towards APG. In contrast, the higher reactivity of the IDL2 fraction, among the apoB containing lipoproteins, with a higher sialic acid content (Anber *et al (inpress)* chapter 5) towards APG goes against the above mentioned hypothesis. This is further questioned due to the variability in the APG-LDL interaction after LDL incubation with different gangliosides. Increasing concentrations of crude gangliosides was most effective in reducing the ability of LDL to interact with APG (Fig 6.2b). However this ganglioside was from brain and not plasma. GM3 gangliosides from plasma was effective in diminishing LDL reactivity towards APG by only 12% (Table 6.2a), While GM1 and asialo GM1 did not have a significant effect on the binding process (Fig 6.3 a & b). This variability in effect may be due to either lack of binding of certain gangliosides to LDL or the fact that it is not the crude overall charge on the lipoprotein that is important but critical domains that may interact in only certain circumstances with ganglioside. For example, GM3 has been shown to interact with LDL resulting in conformational changes mainly, a rearrangement of LDL surface phospholipid (Mikhilenko et al 1991). We

suggest that sialic acid on lipid is important for exposing or hiding free amino groups on the lipoprotein rather than simply affecting its net charge.

Our findings on LDL modification, in keeping with earlier reports (Vijayagopal *et al* 1981) suppose the contention that arginine and lysine residues on apoB are essential for APG-Lipoprotein interaction. This further supports the observation by Olsson et al, that positively charged aminoacid residues rich within a specific domain on apoB in LDL, are critically important in the binding process (Olsson *et al* 1993). On the other hand we found that reductive methylation of LDL, which changes the protein structure of apoB without affecting the net charge on the particle, was also effective in blocking APG-LDL interaction. It is worth noting that reductive methylation was reported by Mahley *et al* not to affect heparin (analogous to heparan sulphate) binding of LDL and HDL (Mahley *et al* 1979). However, marked differences in the ability of heparin and chondroitin sulphate proteoglycan to form soluble or insoluble complexes with LDL have been reported (Vijayagopal *et al* 1983). This is supported by the fact that HDL does not bind to CS-PG (Camejo *et al* 1980b), yet it binds to heparin (Mahley *et al* 1979). Taken all these together, suggest that although overall charge and sialic acid content of the lipoprotein may contribute to some extent to the regulation of binding, it can not be fully responsible for controlling of the lipoprotein-APG interaction.

Interestingly, modification of the lipoprotein by reductive methylation has been shown earlier to be effective in abolishing the receptor activity of both LDL and HDL (Mahley *et al* 1979), which suggests that the mechanism of APG binding might be similar to receptor binding. If that was the case, then it is reasonable to presume that the high reactivity of IDL2 towards APG (chapter 4) would be due to a high content of apoE in this lipoprotein, which also mediates receptor binding activity. An increase in apoE concentration of LDL-I in CHD patients has been shown to be related to increase receptor activity of this lipoprotein compared to small dense LDL (Campos *et al* 1996). However we found that the addition of increasing amounts of both apoE2 and apoE3, to total LDL in different subjects decreased the ability of the lipoprotein to interact with APG. The concentrations of apoE used in this study did not affect the net charge on LDL particle, as tested by gel electrophoresis. In addition, apoCIII did not have

a significant blocking effect on APG-LDL interaction, yet it has been shown to inhibit receptor mediated cellular uptake of lipoproteins (Windler *et al* 1980) Thus, conformational changes on apoB, which might be influenced by apoE, is a more likely explanation for rendering the lipoprotein particle not susceptible to APG binding. This might indicate one way by which apoE protects against atherosclerosis.

Our data on the compositional analysis, of the precipitated and unreacted fractions of different apoB containing lipoproteins is in keeping by the findings by other investigators (Avila *et al* 1978) that particles which are CE-enriched show a greater affinity to APG . In our study, the APG-reacted fractions in VLDL were relatively enriched in CE. This indicated that VLDL remnant particles were more reactive towards APG. Since it is recognised that a subpopulation of VLDL which contain no apoE exists in human plasma (Evans *et al* 1989) it is reasonable to postulate that the CE-enriched VLDL remnants might be apoE poor with impaired receptor mediated removal from plasma. CE-enriched VLDL are also thought to be unable to complete the VLDL to LDL conversion process (Oschry *et al* 1985). This may result in an increased residence time in circulation and subsequent filtration of these particles into the arterial wall leading to their entrapment by APG. On the other hand, the reacted fractions in the denser lipoprotein species were relatively TG-enriched (Table 6.3). Several investigators have reported decreased interaction of TG-enriched LDL with LDL receptor (Chait *et al* 1984, Aviram *et al* 1988, Kinoshita *et al* 1990, Galaeno *et al* 1993). This was attributed to conformational changes in apoB induced by TG with altered expression of free amino groups (Aviram *et al* 1988, Galaeno *et al* 1993). TG induced conformational change may increase expression of the domains critical in APG binding.

These observations suggest that the mechanism of interaction between APG and lipoprotein is not straight forward, and that multiple factors are responsible for controlling the process. The predominance of small dense LDL and raised plasma triglyceride are important determinants (Anber *et al* 1996). It is associated with an increased concentration of other lipoprotein subfractions important in the binding process i.e. IDL. Conformation and surface property

of the lipoprotein appears to be of major importance in the binding mechanism. If the sialic acid content of the lipoprotein has an effect on binding, it appears to be through hiding or revealing different epitopes on apoB which are important in the interaction rather than through a change in overall charge. The blocking effect of apoE on the binding process further provides evidence that the mechanism is conformation related rather than dependant on charge.

Chapter 7

Effect of EPO on plasma lipids and LDL subfractions and their susceptibility to oxidative modification in NIDDMs...

7.1 Introduction

Diabetes is a major contributor to cardiovascular morbidity and mortality and an independent factor with 2-3 fold increase in risk of atherosclerosis (Kannel & McGee 1979). Undoubtedly diabetic individuals are predisposed to premature coronary heart disease for a number of reasons. Acceleration of atherosclerosis in diabetics has been linked to the long term control of blood glucose and to lipid abnormalities (Albrink et al 1963, Santen et al 1972). The incidence of fasting hyperlipidemia in diabetes is 30-40% (New et al 1963, Hays 1972). Non insulin dependant diabetics (NIDDM) have raised plasma triglyceride and low levels of HDL cholesterol (Kennedy et al 1978, Howard et al 1978, Simpson et al 1979, Lopes-Virella et al 1977). Although non-insulin dependent diabetes mellitus (NIDDM) patients exhibit normal LDL cholesterol, there is an increased tendency to oxidise, glycosylate (Hunt et al 1990, Lyons 1991) and glycate (Tames et al 1992) the lipoprotein. The syndrome of an atherogenic lipoprotein phenotype (ALP) which has been associated with a 3-fold increase in risk of coronary heart disease (CHD), is generally considered to be the characteristic dyslipidemia associated with the insulin resistance syndrome (Reaven et al 1993a).

The mechanisms linking small, dense LDL with atherogenesis may be explained by several theories. One of which is its prolonged residence time in circulation, owing to its slow removal by cellular LDL receptors (Caslake et al 1992), thus enhancing its infiltration into the arterial wall. In addition, small, dense LDL has been shown to be more susceptible to in-vitro copper catalysed oxidative modification (DeGraff 1991), a prerequisite step for the deposition of LDL cholesterol and foam cell formation in the atherosclerotic plaque. Increased LDL copper catalysed oxidative modification in patients with ALP (Austin et al 1988) and diabetics might be partly explained by their low HDL level (Lopes-Virella et al 1977, Kennedy et al 1978, Howard et al 1978, Simpson et al 1979). This is supported by the significantly lower serum paroxonase activity in NIDDMs (MacNess et al 1991) which has been reported to be responsible for the antioxidant properties of HDL (MacNess et al 1993). In a study, monitoring peroxidative stress in LDL surface and core compartment, using oxidation labile Fluorescent probe, Tribble et al have attributed the enhanced susceptibility of small, dense LDL to oxidative stress to its surface monolayer (Tribble et al 1995). Furthermore, diabetics are prone to infection and increased superoxide production by activated monocytes which may increase lipoprotein oxidation (Hiramatsu & Arimori 1988). The increased activity of sorbitol pathway in these patients leads to increased consumption of NAPDH which is essential for the regeneration of antioxidants (Barnett et al 1986). Increasing amounts of glycated lipoprotein has been found in the serum of diabetics which is thought to render the lipoprotein more susceptible to oxidation (Lyon et al 1986). Hicks et al (Hicks et al 1988) have shown that both glucose and glycosylated collagen can catalyse the peroxidation of linoleic acid (LA) and arachidonic acid (AA) in-vitro. A similar process might occur in-vivo which might enhance lipid peroxidation in diabetes. The process of oxidative modification of LDL appears to be dependent on the peroxidative decomposition of polyusaturated fatty acids (PUFA), releasing reactive fragments such as malonyldialdehyde (MDA), some of which form covalent bonds with LDL apoB, which may in turn cause fragmentation of the apolipoprotein B (Palinski et al 1989, Yiä-Herttuala et al 1989, Steinbrecher

1987, Hoff *et al* 1989). This modified LDL is not recognised by the LDL receptor and it is taken up by alternative, scavenger receptors to be rapidly internalised by macrophages leading to cholesteryl ester accumulation and foam cell formation (Parathasarathy *et al* 1987, Sparrow *et al* 1989). Therefore the fatty acid composition of LDL is thought to be an important determinant of oxidative susceptibility.

LA (C18:2, n-6 is an essential fatty acid found in many vegetable oils) and more importantly its metabolites gamma linolenic acid (GLA) (C18:3, n-6) and dihommolinolenic acid (DGLA) (C20:3, n-6) and arachidonic acid (AA) (C20:4, n-6) may influence the pathogenesis of CHD by affecting the traditional set of coronary risk factors (Horrobin & Huang 1987) namely plasma lipoprotein levels, haemostasis and diabetes. Diets rich in LA and GLA have been shown to lower plasma total cholesterol, LDL cholesterol, apolipoprotein B (Ishikawa *et al* 1989) and plasma triglyceride (Chaintruil *et al* 1984). The TG-lowering effects of n-6 PUFA has been suggested to be due to a decrease in hepatic VLDL production (Chait *et al* 1974, Nestel & Barter 1971, Cortese *et al* 1983). Since VLDL is the precursor of much of the circulating LDL, it might explain the decrease in LDL with LA-enriched diet. These effects have been suggested to depend on the quantity and quality of PUFA supplied by the diet (Chaintruil *et al* 1984). Evening Primrose Oil (EPO), a natural vegetable oil extracted from the seeds of the evening primrose (*Oenethera* species) has been shown to have a higher lipid-lowering capacity than sunflower oil. Both of which contain linoleic acid as their principal component in common with other vegetable oils (Chaintruil *et al* 1984, Ishikawa *et al* 1989), but EPO is unique in containing about 9% gammalinolenic acid. The later is believed to be more potent in lipid lowering compared to the former. In addition GLA has been shown to induce an increase in HDL (Fragoso & Skinner 1992). Therefore, it can be speculated that the influence of dietary n-6 PUFA in EPO on the distribution of LDL subclass pattern will reflect its capacity to reduce the concentration of triglyceride rich lipoproteins which might increase the susceptibility of LDL to oxidation . However, its direct influence on discrete LDL subclasses has yet to be elucidated.

Therefore, we hypothesise that supplementation of LA in the form of EPO with GLA present might by lowering triglyceride rich lipoproteins beneficially affect the LDL subfraction profile and also be protective against atherosclerosis.

The aims and objectives of our study were to examine 1) the effects of EPO supplementation on LDL oxidisability in a subgroup of NIDDM expressing an ALP, 2) to elucidate the ability of EPO to modify components of an ALP, namely, lowering plasma TG, redistribution of LDL subclass pattern and increasing HDL cholesterol, in these patients and finally 3) to examine relationships between changes in ALP status and oxidative susceptibility.

7.2 Study design

The trial was conducted in 2 phases over 24 months. The first phase of which was double blind, parallel, placebo controlled for 12 months with the patients randomised to receive active or placebo therapy. The next 12 months was a single blind phase and all patients were on active therapy.

The dose was 12 capsules/day of either active EPO (500mg×6 bd) or placebo capsules. The capsules were identical in appearance (and taste if swallowed intact). They were packaged and individually labelled for each patient. Treatment was allocated by a computer program using block randomisation by Scotia Pharmaceuticals. All unused capsules and the completed capsule packs were returned to Scotia Pharmaceuticals

Active therapy: oral Efamol EPO (evening primrose oil) 500 mg with 10 mg vitamin- E in a soft gelatine capsule, containing as active ingredient 40mg GLA. Total dose of GLA= 500mg/day

Placebo: oral liquid paraffin 500 mg with 10 mg vitamin E in a soft gelatine capsule.

7. 3 Subjects

A total of 28 subjects were selected from those attending the diabetic clinic at the Southern General Hospital. All donated 50ml of blood after an overnight

(12h) fast. Fasting blood was collected by venepuncture using K_2EDTA (final concentration 1mg/ml) as an anticoagulant for immediate transport (on the same day) on wet ice to the Institute of Biochemistry, Glasgow Royal Infirmary. Plasma was separated at 4°C by low speed centrifugation (3000rpm) and aliquots for lipid, lipoprotein measurements and LDL subfractionation used immediately.

Patients compliance and control of diabetes were monitored by completion of diary card, measurement of plasma essential fatty acids, measurement of glycosylated haemoglobin (HbA1) and fructosamine level in plasma.

Inclusion criteria

i) Patients with idiopathic non-insulin dependent (type II) diabetes mellitus (who met the WHO criteria at the time of diagnosis).

ii) Patients of either sex aged between 18-70 years, who have given written informed consent.

iii) patients in whom control of diabetes has been stable for at least 6 months prior to recruitment, i.e. no acute metabolic decompensation, for example a hypoglycemic event requiring intervention by a second party or hyperglycemic event requiring hospitalisation, or a change in type of therapy for diabetes.

Exclusion criteria

i) Patients with acute febrile illness.

ii) active hepatic disease, renal failure (creatinine above 200μmol/l) or other intercurrent disease

iii) Patients receiving experimental drugs (other than the study capsules).

iv) Patients who are pregnant or trying to conceive.

v) Patients on lipid lowering medications.

Adverse events

No serious risks are known to accompany the ingestion of EPO. Patients may complain of mild gastrointestinal upset, e.g. flatulence, loosening of stools or

mild nausea rarely these are sufficiently troublesome to cause the patient to stop treatment.

7.4 Statistical analysis

Statistical analysis and manipulations were performed using the PC version of MINITAB Release 10 for Windows (Minitab Inc., PA). The distribution of all variables was assessed by normality plots and the ones which were not normally distributed were normalised by appropriate transformations. Plasma triglyceride was normalised by log transformation, small, dense LDLIII concentration within total LDL was normalised by square root transformation. Pearson's correlation coefficient, simple regression, 2 sample t-test and one way analysis of variance (ANOVA) were used to assess the relationship between variables.

7.5 Patients characteristics

The patients age, sex, body mass index (BMI), current illnesses, other than non-insulin dependent diabetes, and medications are listed in Table 7.1. Anthropometric indices, systolic and diastolic blood pressure of both groups (active and placebo) are shown in Table 7.2.

Age, weight, BMI, systolic and diastolic blood pressure were similar between the two groups (active and placebo) (Table 7.2). They had also similar levels of total, LDL and VLDL cholesterol, apoAI and apoB concentration in plasma (Table 7.4). Patients who were on active treatment had a lower HDL cholesterol ($p<0.02$) and cholesterol/HDL ratio ($p=0.008$) and a higher plasma triglyceride level, though the latter did not reach statistical significance.

Table 7.1

Patients characteristics at the initial visit.

Subjects	*Sex*	*Age*	*BMI*	*Clinical diagnosis**	*Medications*
EPO 01	M	66	33.1	—	D & O, Fybogel, senna
EPO 02	F	58	26.6	—	—
EPO 03	M	60	30.3	—	D & O
EPO 04	F	64	24.6	—	D
EPO 05	M	51	25.6	—	D
EPO 06	M	48	34.0	—	D & O
EPO 07	M	64	27.1	↑BP	D & O, Adalat
EPO 08	M	52	25.2	—	D
EPO 09	M	62	25.6	—	D & O
EPO 10	M	60	30.7	—	D & O
EPO 11	M	48	—	—	D & O
EPO 12	F	71	25.0	MI 9/93	D & O, Istin
EPO 13	M	55	23.5	—	D & O
EPO 14	M	66	27.6	Angina, MI, ↑BP	Insulin, Digoxin, Aspirin, Hoidaruroa
EPO 15	M	59	26.2	IHD	D & O, Isosorbide dinitrate, Diltiazam
EPO 16	M	41	22.8	↑BP	Insulin, Tenormin
EPO 17	M	60	20.3	Angina, HF, ↑BP	D, Digoxin, Tenormin, Wrfarin, Captopril
EPO 18	F	55	26.2	↑BP	D & O, Adalat
EPO 19	M	69	33.1	↑BP	D, Nifidipine
EPO 20	F	61	22.2	↑BP	Insulin, adalat retard
EPO 21	M	39	31.0	Asthma, ectopics	D & O, Adalat,ventolin inhalor, Colofac
EPO 22	M	58	31.2	Angina, ↑BP	

Clinical diagnosis other than NIDDM; BMI, body mass index; D&O, diet and oral hypoglycemics; IHD, ischemic heart disease; BP, blood pressure mmHg; MI, myocardial infarction; HF, heart failure.

Table 7.2

Anthropometric indices, plasma lipids, lipoproteins, LDL subfraction profile, blood glucose, HbA1 and Fructosamine level in both study groups

Variable	*Active n= 14*	*Placebo n= 8*
Age	57.5 ± 8.7	57.7 ± 8.4
Weight (kg)	77.5 ± 12.1	76.2 ± 15.7
BMI	27.3 ± 4.0	27.1 ± 3.9
BP systolic (mmHg)	138.7 ± 25.1	140 ± 9.3
BP diastolic (mmHg)	87.0 ± 12.8	85.6 ± 11.8

BMI, body mass index; BP, blood pressure

The lower plasma triglyceride level in the placebo group was reflected in their baseline LDL subfraction profile, with a higher LDL-I and a lower LDL-III percentage within total LDL compared to the active treatment group (Table 7.3) but this was not statistically significant.

Table 7.3

Plasma LDL subfraction profiles of the two groups of patients at the end of the first year of the trial.

Variable	*Active n=14*		*Placebo n=8*	
	Baseline	12 months	Baseline months	12
%LDL-I	16 ± 9	16 ± 9	22 ± 9	18 ± 8
%LDL-II	46 ± 17	45 ± 9	53 ± 10	55 ± 9
%LDL-III	31 ± 17	38 ± 14	26 ± 17	27 ± 10

%LDL-I, LDL-II, LDL-III, the percentage of large, intermediate and small dense LDL within total LDL; active, treatment with 500mg×12 EPO +10mg Vit.E/day; placebo, treatment with liquid paraffin capsules 500mg + 10 mg vit.E/day.

Correlation coefficient between the mean percentage of the LDL subfractions and the lipid and other parameters showed that small dense LDL-III to be positively associated with plasma triglyceride (log transformed data) ($p<0.001$), plasma apoB concentration ($p<0.05$), plasma glucose level ($p<0.05$) and

cholesterol/HDL ratio ($p<0.01$) and negatively associated with HDL cholesterol concentration ($p<0.04$) and HDL_2 subfraction mass ($p<0.05$).

Plasma triglyceride was positively related to blood glucose ($r= 0.42$, $p<0.05$), BMI ($r= 0.46$, $p= 0.03$) and apoB concentration ($r= 0.75$, $p<0.0001$). The latter was positively correlated with total cholesterol ($r= 0.52$, $p= 0.02$), weight of the patients in kg ($r= 0.42$, $p<0.05$), BMI ($r= 0.47$, $p= 0.04$) and cholesterol/HDL ratio ($r= 0.75$, $p<0.0001$).

The mean blood glucose values and glycosylated haemoglobin percentages shown in Table 7.4, indicate that the patients of both groups exhibited a satisfactory diabetic control at the start of the trial. Fructosamine level was used to monitor the long term control of diabetes.

Table 7.4

Indices of diabetic control in both groups before the start of the trial.

Variable		*Active n=14*	*Placebo n=8*
Glucose	mmol/l	8.7 ± 1.9	8.8 ± 3.8
Total HbA1	(%)	8.2 ± 1.8	8.3 ± 1.2
Fructosamine	μmol/l	328.7 ± 45.0	300.1 ± 44.5

Total HbA1, the percentage of glycosylated haemoglobin; Glucose; fasting blood glucose level mmol/l, Fructosamine; used to monitor the long term control of diabetes

7.6 Effect of EPO treatment on patients anthropometric indices, lipids and lipoproteins

No significant changes were observed in total, LDL and HDL cholesterol after treatment with 500mg EPO×12/day for 12months, and there were no differences between the active treatment and placebo group of patients (Table 7.5).

Table 7.5

Lipid and lipoprotein variables between the two groups of patients during phase 1 of the trial.

Variable		*Active*		*Placebo*	
		Baseline	*12months*	*Baseline*	*12 months*
Total cholesterol	mmol/l	6.2 ± 1.1	6.0 ± 1.2	6.1 ± 0.6	6.0 ± 0.6
LDL cholesterol	mmol/l	4.2 ± 1.1	3.8 ± 0.9	4.0 ± 0.6	4.1 ± 0.8
VLDL cholesterol	mmol/l	0.9 ± 0.4	1.1 ± 0.6	0.8 ± 0.3	0.6 ± 0.5
HDL cholesterol	mmol/l	1.0 ± 0.2	1.1 ± 0.2	1.3 ± 0.2*	1.3 ± 0.3
Triglyceride	mmol/l	2.1 ± 1.2	2.8 ± 1.7	1.7 ± 0.6	1.7 ± 0.9
Chol/HDL ratio		6.1 ± 1.5	5.9 ± 1.2	4.8 ± 0.7*	4.8 ± 1.0**
HDL2 mass	mg/dl	35.6 ± 14.2	31.7 ± 24.6	40.0 ± 21.6	28.8 ± 18.1
HDL3 mass	mg/dl	233.8 ± 62.9	181.1 ± 50.4	274.0 ± 100.9	205.1 ± 54.0
Lp(a)	mg/dl	22.9 ± 18.0	21.7 ± 18.9	53.9 ± 58.8	57.7 ± 65.8
ApoA1	mg/dl	1.2 ± 0.2	1.3 ± 0.3	1.3 ± 0.1	1.3 ± 0.1
ApoB	mg/dl	1.2 ± 0.3	1.3 ± 0.3	1.1 ± 0.1	1.1 ± 0.2

** $p<0.01$; **$p<0.05$, significance between active treatment group and placebo*

HDL_2, HDL_3, high density lipoprotein subfraction mass; Lp(a), lipoprotein (a); ApoA1, apolipoprotein A1 concentration; ApoB, apolipoprotein B-100 concentration; %LDL-I, %LDL-II, %LDL-III, percentage of large, intermediate and small dense low density lipoprotein subfractions within total LDL.

Plasma triglyceride showed a tendency to increase in the active treatment group (by 24%) compared to the placebo group (Table 7.5). Although this was not statistically significant. HDL_2 and HDL_3 mass was reduced at the end of the first year both in the active (by 12% and 23% respectively) and placebo group (by 28% and 25% respectively) (Table 7.5). There were no changes in the other lipid parameters in either group (active and placebo) at the end of the first year (Table 7.5)

The control of blood pressure in all the patients was satisfactory through out the 24 months of the trial period (Table 7.6). The mean percentages of glycosylated haemoglobin (%HbA1), used to monitor diabetic control, at the initial visit, at 12-months and 24-months sample were similar and indicated reasonable control of diabetes (Table 7.6). However blood glucose measurement was significantly higher at 12 months and 24 months compared with the initial visit ($p<0.05$) (Table 7.6).

Table 7.6

Blood pressure, blood glucose and glycosylated haemoglobin measurements during the trial period.

Variable	*baseline*	*1st year*	*2nd year*
B-glucose	8.8 ± 2.6	10.9 ± 4.2*	10.8 ± 3.9*
% HbA1	8.2 ± 1.5	8.9 ± 1.7	9.2 ± 1.9
BP systolic	139.2 ± 20.5	142.7 ± 20.8	144.3 ± 15.5
BP diastolic	86.5 ± 12.2	85.6 ± 12.6	86.7 ± 10.4

**p<0.05*
B-glucose, blood glucose mg/dl; %HbA1, the percentage of glycosylated haemoglobin; BP systolic, systolic blood pressure measured in mmHg; BP diastolic, diastolic blood pressure measured in mmHg.

During the second year of the trial, all patients were on active EPO treatment. At the end of which there were no significant changes in the plasma total cholesterol, LDL cholesterol, VLDL and HDL cholesterol (Table 7.7). There was a 24% decrease in plasma triglyceride and a 17% decrease in plasma Lp(a) but these did not reach statistical significance. Although, there were no changes in HDL-

cholesterol and apoAI, a significant increase in the HDL2 and HDL3 subfraction mass was observed ($p<0.05$ and $p<0.0001$ respectively) (Table 7.7), in keeping with findings by Fragoso & Skinner (Fragoso & Skinner 1992).

Table 7.7

Effect of EPO on lipid and lipoprotein variables in the second phase of the trial.

Variable		***12months*** n=22	***24 months*** n=22
Total cholesterol	mmol/l	6.1 ± 1.0	6.0 ± 1.3
LDL cholesterol	mmol/l	3.9 ± 0.9	3.9 ± 0.9
VLDL cholesterol	mmol/l	1.0 ± 0.6	0.9 ± 1.0
HDL cholesterol	mmol/l	1.1 ± 0.3	1.1 ± 0.3
Triglyceride	mmol/l	2.5 ± 1.5	1.9 ± 0.8
Chol/HDL ratio		5.5 ± 1.3	5.6 ± 2.1
HDL_2 mass	mg/dl	29.5 ± 21.9	51.1 ± 35.2**
HDL_3 mass	mg/dl	189.3 ± 50.8	266.3 ± 60.9*
Lp(a)	mg/dl	34.8 ± 44.5	28.9 ± 35.1
ApoAI	mg/dl	1.3 ± 0.2	1.2 ± 0.2
ApoB	mg/ml	1.3 ± 0.3	1.3 ± 0.3
% LDL-I		16.3 ± 8.7	15.3 ± 7.7
% LDL-II		47.5 ± 11.7	49.2 ± 15.5
% LDL-III		36.2 ± 16.1	31.1 ± 16.6

** P<0.0001; ** P=0.002; Lp(a), lipoprotein (a); % LDL-I, %LDL-II and %LDL-III, are percentages of low density lipoprotein-I, II and III subfractions within total LDL*

Compositional analysis of total LDL and the effect of EPO during the second phase of the trial, i.e. when all the patients were on active EPO treatment are shown in Table 7.8. There were no significant variations between the two years samples except, in the percentage of LDL-phospholipid which was reduced significantly by 29% ($p=0.002$) (Table 7.8).

Table 7.8

comparison between the compositional analysis of the LDL samples at the end of 1st and 2nd year of the trial

Variable	*12 months* n=22	*24 months* n=22
% protein	24.9 ± 5.7	27.5 ± 2.9
% F-Chol	13.1 ± 2.6	14.5 ± 6.0
% E-Chol	33.9 ± 4.8	35.9 ± 6.7
% Triglyceride	7.4 ± 2.7	7.5 ± 2.1
% Phospholipid	20.7 ± 2.6	14.8 ± 7.8**
Protein/Chol ratio	0.8 ± 0.3	0.8 ± 0.2

** *P=0.002; %FChol, the percentage of free cholesterol; %E-Chol, the percentage of esterified cholesterol; Protein/Chol, protein/ cholesterol ratio*

7.7 Effect of EPO on LDL oxidation

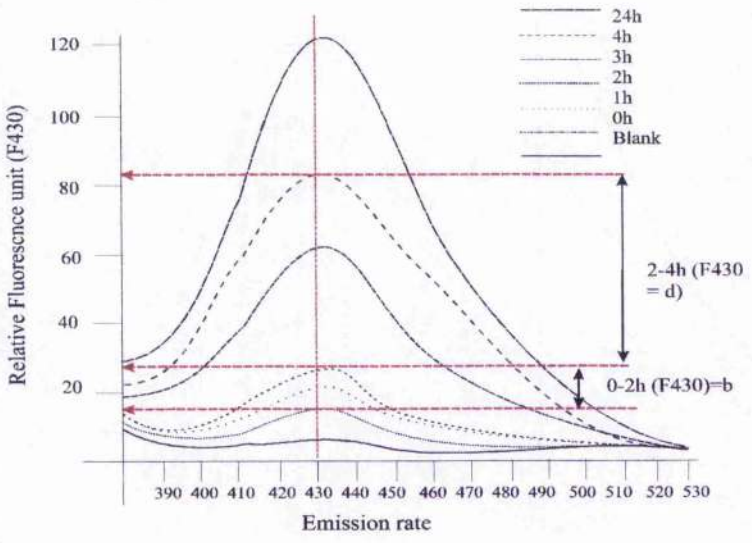
The susceptibility of LDL to oxidative modification was tested by incubation (0.2 mg apo LDL/2ml) at 37°C in the presence of copper ions following the method described by Cominacini *et al* (Cominacini *et al* 1991) (chapter 2, section 2.5). The fluorescence curve from each individual patient was determined by measuring emission rate at 430nm (excitation at 350) at time intervals (fig 7.1) using Perkin-Elmer Ls-50 spectrophotofluorimeter. The excitation and emission slits were both maintained at 5nm. During measurement the instrument drift of sensitivity was checked by measuring the fluorescence intensity of a quality control sample of quinine sulphate (Chapter 2, section 2.5) and the instrument was adjusted accordingly before reading the samples. The initial fluorescence was read at 0h and thereafter every hour for the first 4-hours, then at 24h (Fig 7.1A). The development of fluorescence was "biphasic" and thus subdivided into an inhibitory and propagatory phases. The inhibitory phase fluorescence development rate (IP-FDR), was calculated by dividing the fluorescence development rate (FDR) between 0-2h by time (t), and the propagatory phase fluorescence development

rate (PP-FDR) was calculated by dividing the FDR between 2-4h by time (Fig 7.1B).

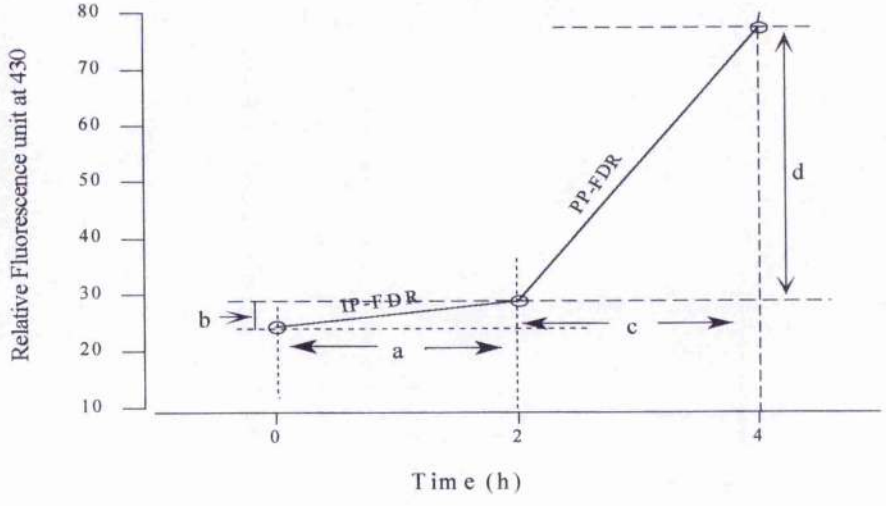
Figure 7.1

Fluorescence intensity curve and FDR.

A:



B:



A, represents fluorescence curve determined by fluorescence measurement at 430 emission, 350 excitation (F430). (b), relative fluorescence at 430 between 0-2h; (d) relative fluorescence unit at 430 between 2-4h. B, determination of the IP-FDR and PP-FDR.. IP-FDR = b (F_{430})/a(t in h), -PP-FDR = d (F_{430})/c(t in h).

The mean fluorescence rate for both patient groups (active and placebo) is shown in Table 7.9. There were no significant differences between the fluorescence intensity, as measured by relative fluorescence units, at hourly intervals and at 24h between the active EPO treatment group and the placebo group (Table 7.9, Fig 7.2) at the initial visit.

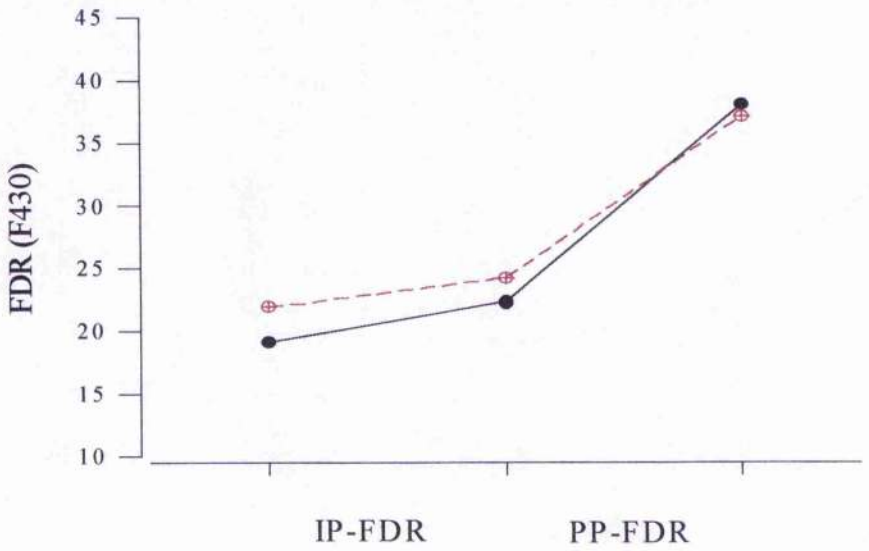
Table 7. 9

Relative fluorescence at different time intervals in the active EPO treatment and the placebo group of patients.

Patients n=22	*Fluorescence (relative Fluorescence Units)**					
	0h	*1h*	*2h*	*3h*	*4h*	*24h*
Placebo	22.6 ±4.6	22.4 ±6.8	25.0 ±6.7	30.6 ±19.5	41.0 ±18.4	70.7 ±26.8
Active	23.4 ±15.1	27.5 ±15.3	31.8 ±18.9	39.1 ±22.8	49.1 ±28.9	97.0 ±49.8

**Relative Fluorescence Units measured by measuring emission rate at 430nm and excitation at 350*

Figure 7.2
Mean fluorescence rate for the two groups of subjects at the initial visit



IP-FDR (F430/t), inhibition period fluorescence development rate, PP-FDR (F430/t), propagation period fluorescence development rate. ○····○ *, active;* ●—● *, placebo.*

Treatment with EPO enhanced the rate of oxidation significantly in the active treatment group by 40-50% ($p<0.001$) (Fig 7.3). Patients who were on placebo treatment showed a significant increase in the IP-FDR ($P<0.01$) and PP-FDR ($p<0.001$) during the second phase of the trial i.e. after treatment with EPO (Table 7.10, Fig 7.4).

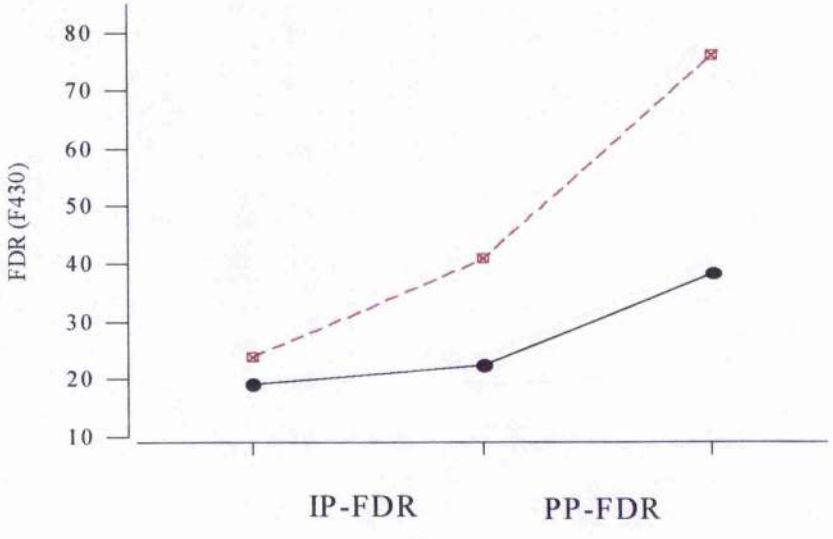
Table 7.10

Fluorescence development rate in both groups of patients after active EPO treatment.

FDR	*Patients*			
	Placebo → Treatment *n=8*		*Active → Active* *n=14*	
IP-FDR	13.7 ± 6.2	28.5 ± 15.4**	20.1 ± 16	33.2 ± 20.1*
PP-FDR	28.7 ± 13.2	58.5 ± 9.7*	27.7 ± 7.6	55.8 ± 21.4*

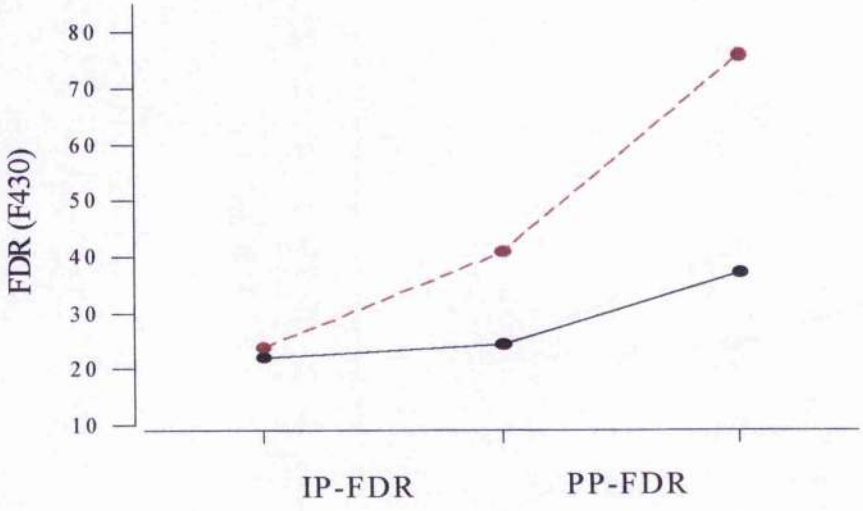
**p<0.001; **p<0.01; IP-FDR; PP-FDR,DP-FDR, Inhibitory phase; propagatory phase and degradation phase fluorescence development rate respectively measured in relative fluorescent units at 430/hr (Rel. F_{430}/hr).*

Figure 7.3
Mean fluorescence rate for the active group after treatment with EPO at the end of the first 12 months.



IP-FDR, inhibition period fluorescence development rate, PP-FDR, propagation period fluorescence development rate. ● *Before and* ⊞ *after EPO supplementation $P<0.001$ and $p<0.01$ for IP-FDR and PP-FDR respectively.*

Figure 7.4
Mean fluorescence rate for the placebo group after treatment with EPO at the end of the trial (24months).



IP-FDR, inhibition period fluorescence development rate, PP-FDR, propagation period fluorescence development rate. ● *before and* ● *after treatment with EPO.*

During the second year of the trial all the patients were on active EPO treatment. The mean rate of oxidation (± standard deviation) at hourly measurements and FDR for all the patients at the end of the first and second year of the trial are shown in Table 7.11. There was a significant increase in both the fluorescence intensity, as measured by relative fluorescence units and FDR (IP-FDR and PP-FDR), in the second year compared to the first year samples (Table 7.11, Fig 7.5).

Table 7.11

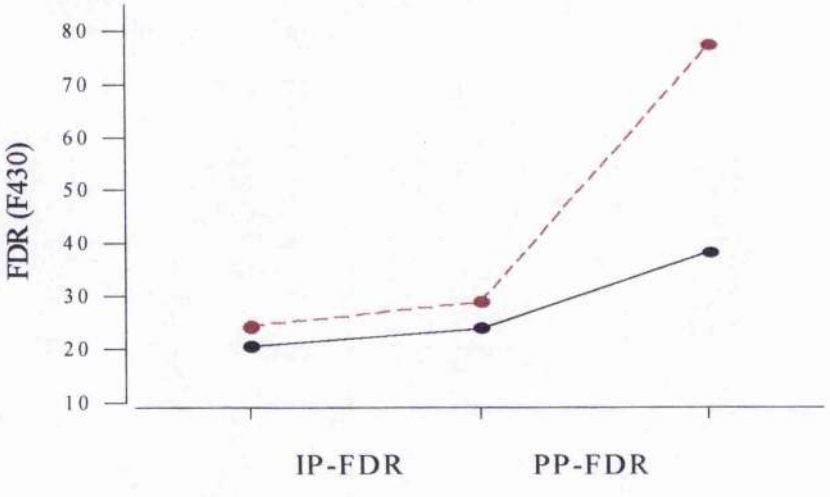
Patients mean fluorescence emission rate and fluorescence development rate during phase II of the trial.

	12 months	*24 months*
0h	23.1 ± 12.0	24.7 ± 3.2
1h	25.6 ± 12.8	33.9 ± 13.0***
2h	29.2 ± 15.5	40.4 ± 9.2**
3h	38.5 ± 21.1	57.9 ± 11.2**
4h	46.0 ± 25.2	77.0 ± 18.6*
24h	87.0 ± 43.7	118.6 ± 15.3**
FDR 0-2	17.7 ± 13.3	28.0 ± 9.7**
FDR 2-4	31.4 ± 18.2	56.8 ± 17.8*

**P<0.0001, **P<0.01, ***P<0.05*

Figure 7.5

Effect of EPO supplementation on IP-FDR and PP-FDR in all the patients at the end of the 2nd year..



IP-FDR, inhibition period fluorescence development rate, PP-FDR, propagation period fluorescence development rate. ●, *Baseline and* ●, *after EPO supplementation for 24 months in all the patients. P<0.01 and p<0.001 for IP-FDR and PP-FDR respectively.*

7. 8 Determinants of LDL oxidation

IP-FDR (0-2h), was negatively correlated with the percentage of LDL-I (NS) within total LDL and positively correlated with apolipoprotein B concentration in plasma (p= 0.03) and the percentage of glycosylated haemoglobin (%HbA1, P<0.05) (Table 7.12). PP-FDR (2-4h) was positively correlated with the percentage of small dense LDL-III within total LDL (p<0.05), plasma triglyceride level (p<0.05), apoB concentration in plasma (p= 0.02), blood glucose level and %HbA1 measurement in plasma (Table 7.12). However, the correlation between PP-FDR and blood glucose level and glycosylated haemoglobin (%HbA1) did not reach statistical significance. There were no significant correlation with HDL and LDL cholesterol levels (Table 7.12).

Table 7.12

Correlation between inhibition and propagation fluorescence development rates and lipid and lipoprotein variables

Variable		*IP-FDR*	*PP-FDR*
Total-C	mmol/l	0.16	0.33
VLDL-C	mmol/l	0.07	0.31
LDL-C	mmol/l	0.1	0.17
HDL-C	mmol/l	0.15	0.07
Triglyceride	mmol/l	0.11	0.43**
Apo B	mg/ml	0.46*	0.50*
Glucose	mg/dl	0.22	0.33
%HbA1		0.40**	0.35
% LDL-I		- 0.26	- 0.50*
% LDL-II		- 0.08	- 0.19
% LDL-III		0.19	0.40**

**p=0.02, **p<0.05; Total-C, plasma total cholesterol; VLDL-C, LDL-C and HDL-C, plasma very low density, low density and high density lipoprotein cholesterol level respectively; ApoB, plasma apolipoprotein B-100 concentration; %HbA1, the percentage of glycosylated haemoglobin in plasma; % LDL-I, %LDL-II and %LDL-III, are percentages of low density lipoprotein-I, II and III subfractions within total LDL*

The relationship between IP-FDR (0-2h) and PP-FDR (2-4h) and compositional analysis of total LDL, after treatment with EPO, is shown in table 7.13. IP-FDR showed a positive correlation with the percentage of LDL-esterified cholesterol and the PP-FDR was correlated positively with the percentage of LDL- triglyceride and LDL-phospholipid and negatively with the percentage of LDL-protein and esterified cholesterol (Table 7.13). There was no correlation between the extent of oxidation and the other compositional parameters in LDL.

Table 7.13

Correlation between the compositional analysis of total LDL and IP-FDR and PP-FDR at the end of the second year

Variable	*IP-FDR*	*PP-FDR*
% Protein	- 0.07	- 0.35
% FC	- 0.16	0.02
% EC	0.30	- 0.22
% TG	- 0.19	0.35
% PL	- 0.07	0.22
P/C ratio	- 0.11	- 0.08

% FC, percentage of LDL free cholesterol; % EC, percentage of LDL esterified cholesterol; % TG, percentage of LDL triglyceride; % PL; percentage of LDL phospholipid; P/C ratio, protein/ cholesterol ratio

7. 9 Discussion

The need to lower plasma lipid levels in management of CHD is well documented. In general, dietary recommendations have been designed to decrease total dietary fat intake and to replace dietary saturated fats with polyunsaturated fats (National cholesterol education program 1988; 1994). The identification of triglyceride as the major underlying determinant of an ALP, suggest that this lipid should be a target for dietary fatty acid modifications as a means of correcting lipoprotein abnormalities seen in an ALP. The triglyceride lowering effect of n-3 PUFA have

been given little attention because of its minimal effect on HDL and their potentially adverse effect on raising LDL cholesterol and enhancing LDL oxidation (Suzukawa et al 1995). The latter has been shown to occur also with dietary supplements enriched with linoleic acid (Reaven et al 1993b). However, the replacements of saturates with LA (n-6, PUFA) has been shown in most studies to reverse nearly all the effects of saturated fatty acids on lipids and lipoproteins. In the present study we have examined the effect of EPO on lipids, lipoproteins and LDL subfraction profile and LDL oxidative modification in NIDDM patients.

In contrast, to the findings of other investigators (Ishikawa et al 1989, Chaintruil et al 1984), our study showed that EPO had no effect on plasma total LDL and HDL cholesterol and apolipoprotein B concentration. This could be due to two factors, firstly, most of the studies performed were either on animals or non-diabetic patients. The lipid lowering effects of LA might be through its conversion to its metabolites by sequential desaturation and elongation steps. This is supported by several studies performed both in animals and human which have reported GLA and AA to be more potent, as lipid-lowering agent, compared to LA (Chaintruil et al 1984). The first step of conversion of LA to GLA, regulated by the enzyme delta-6-desaturase, is defective in diabetic patients and experimental animals (Jones et al 1986, Mercuri et al 1966). Therefore diabetic patients might be more resistant to the lipid-lowering effect of LA than normal subjects. Secondly, EPO supplementation containing 500mg GLA, the dosage which was used in this study, has been shown to have no significant change in lipid parameters compared to 2g GLA/day (Chaintruil et al 1984). In this case our finding is in keeping with the results observed by Chaintreuil et al. In addition, the patients examined in our study were not hospitalised and were not monitored for their dietary intake, as it was done by other groups (Reaven et al 1993b, Chaintruil et al 1984) during the trial period. Therefore, the increase in triglyceride observed in the first year is more likely to be due to inappropriate or lipid raising dietary habits, than the effect of EPO. However, plasma triglyceride showed a tendency to decrease after EPO supplementation for the second year. This might be due to the longer term effect of EPO rather than the dosage, as 500mg EPO supplementation has been shown to have no acute effects (Chaintruil et al 1984). This could also account for the significant increase in the HDL2 and HDL3 subfractions at the end of the second

year of the trial, i.e. when all the patients were on active EPO treatment, compared to the first year. This suggestion supported by the findings of Fragoso & Skinner though they used a higher concentration of GLA (70%) in the dietary supplementations of their experimental animals (Fragoso & Skinner 1992). With regards to the effects of EPO on LDL subfraction profile, the absence of any change in the LDL subfractions would mainly reflect a lack of influence of EPO on plasma triglyceride, which has been shown to be a major determinant of the LDL sufraction profile (Griffin et al 1994). These findings indicate that EPO supplementation at 500mg/day has no significant effect on the modification of ALP Therefore, a higher concentration of GLA in EPO preparations is needed to exert beneficial effect in terms of lipid lowering especially in diabetics.

The increased in-vitro copper catalysed LDL oxidation after EPO supplementation observed in this study is in keeping with the findings by other investigators (Reaven et al 1993b, de Graaf et al 1991). The likely explanation of this is the absence of a significant modification of the components of ALP, discussed above, by EPO supplementation and the persistence predominance of small dense LDL in these patients. In addition, inhibition of the 6-desaturation of LA, in diabetics and hyperlipidemic patients, might be responsible for its accumulation in LDL rendering it more susceptible to oxidation, as was reported by Reaven et al (Reaven et al 1993b). Unfortunately we did not measure the LDL fatty acid composition after EPO supplementation. Furthermore, these patients have low plasma HDL cholesterol level which has been reported to confer a low antioxidant activity (MacNess et al 1991).

The positive association between the extent of LDL oxidation, as measured by the rate of fluorescent development (FDR), and the percentage of small dense LDL found in our study, is in agreement with the increased susceptibility of small dense LDL to oxidative modification observed by others (DeGraaf 1991, Tribble et al 1992). Reaven et al, has suggested that increased susceptibility of small dense LDL to oxidation was linked to the preferential enrichment of this subfraction with linoleic acid (Reaven et al 1993b). This has supported earlier work by DeGraaf, which attributed the increased susceptibility of small dense LDL was partly due to higher ratios of arachidonic acid (AA), a metabolite of linoleic acid, to vitamin E (an antioxidant) levels (DeGraaf 1991). Our observation of the positive

correlation between plasma triglyceride and LDL oxidation supports the findings by Hiramatsu and Arimori that monocytes from diabetic patients with hypertriglyceridemia exhibited increased superoxide production and that this response was determined to a greater extent by hypertriglyceridemia than by the presence of diabetes (Hiramatsu & Arimori 1988). This is in keeping with the role of triglyceride as the major determinant of ALP and further provides evidence of the positive association between ALP and CHD. On the other hand, we have found a positive correlation between the PP-FDR and blood glucose level. The latter was higher at the end of the second year compared to the initial visit. This might be yet another suggestion as to why these NIDDM patients exhibit an increase in LDL oxidation.

We conclude that EPO supplementation at 500mg /day does not affect lipid and lipoprotein concentration in plasma. It does increase LDL oxidation as has been reported previously possibly due to enrichment of LDL with LA. The increase in lipid peroxidation of LDL seems to be related to plasma triglyceride which is elevated in diabetics.

Chapter 8

Conclusions...

8.1 Introduction

Modern models of atherogenesis place apoB containing lipoproteins in an etiological role. Despite the progress that has been made in elucidating the underlying mechanism of atherogenesis no single cogent hypothesis has been described that accounts for all clinical, epidemiologic and laboratory observations. However, two well documented mechanisms, APG-Lipoprotein interaction and lipoprotein oxidative modification, are believed to have a central role in the initiation and advancement of the disease process. The role of the two mechanisms in lesion development suppose to be dependant upon rate and nature of apoB containing lipoproteins generated through delipidation of secreted VLDL from liver. This is modulated through genetic and environmental factors as a result of which heterogeneous particles different in structure and function are produced within each lipoprotein class. Factors determining the make-up of these particles have important implications for their potential atherogenicity which we have addressed throughout this work.

8.2 Determinants of APG-LDL interaction

Several theories have been described to explain the atherogenic process. A schematic representation of the atherosclerotic lesion development on the basis of current knowledge and concepts is illustrated in Fig 8.1.

APG- Lipoprotein interactions under near to physiological ionic concentration and temperature were investigated to simulate the in-vivo process (Chapter 4). Attention was focused on the binding of a chondroitin sulphate rich proteoglycan fraction isolated from human aorta, since it binds most avidly to LDL.

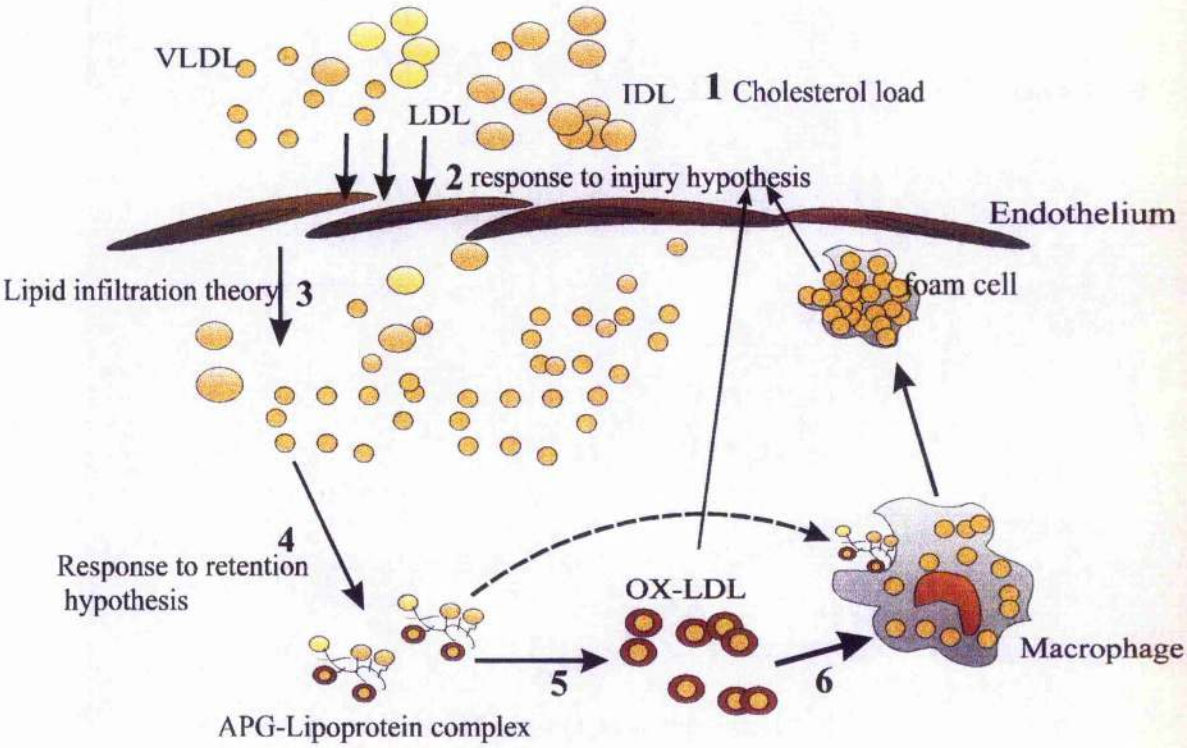


Figure 8.1

Schematic diagram illustrating mechanisms and theories related to atherosclerotic lesion development. Increased concentration of lipids and lipoproteins confer a 1) "Cholesterol Load" on the vascular endothelial surface leading to: endothelial dysfunction together with the (2)"Response to Injury" hypothesis increases endothelial permeability leading to the influx of lipoproteins into the arterial wall (3) "Lipid Infiltration theory", within the arterial wall as a (4)"Response to Retention hypothesis the lipoproteins are trapped by extracellular matrix components particularly APG, this is charge related and dependant on the conformation of the lipoprotein particle, a consequence of which is (5) lipoprotein "oxidative modification", which is enhanced in small dense LDL, subsequently these modified lipoproteins are taken up by macrophages leading to lipid accumulation and deposition characteristic of atherosclerotic lesions

An important observation in the present study was the positive association between the extent of APG-LDL reactivity and the percentage of small dense LDL-III subfraction within total LDL supporting the suggestion provided by Camejo *et al* (Camejo *et al* 1993a) that CS-PG interaction favours small, dense LDL. An even greater reactivity was associated with the concentration of small dense LDL above 100mg/dl (Fig 3.21). Furthermore, plasma TG was positively associated and HDL cholesterol was inversely related to the extent of APG- Lipoprotein interaction. When we examined the direct reactivity of different apoB containing lipoprotein subfraction including small dense LDL, a pattern of reactivity which was similar in each individual was observed with IDL2 and LDLA Sf 8-16 being the most reactive species (Fig 4.5). However, the magnitude of this pattern was highest in subjects with a predominance of small dense LDL. Since VLDL represents the major precursor of LDL, this provides evidence that a predominance of small dense LDL in circulation is associated with the presence of potentially more atherogenic apoB containing lipoproteins, such as IDL, which are produced during the delipidation cascade of VLDL to LDL. The effect of triglyceride on the modulation of LDL subfraction has been shown to be mediated through large TG-rich VLDL1 particles. These particles are suggested to be a major determinant of remodelling of LDL to smaller dense particles (Patsch *et al* 1984, Tan *et al* 1995) by mechanisms that involve the donation of TG in exchange for CE in the process of neutral lipid exchange. This results in the production of IDL and VLDL remnant particles with a prolonged residence time in plasma which promote their infiltration to the arterial wall and subsequent entrapment by APG. In addition, they undergo further delipidation by the action of hepatic lipase enzyme resulting in the production of small LDL.

The different APG-LDL reactivity between male and female could be attributed to the difference in lipid and lipoprotein levels in those two sex groups. The high reactivity of IDL from male subjects towards APG compared to female subjects is due to the high level of small dense LDL-III which is more prevalent in male patients as well as their lower HDL cholesterol level. This could partly explain the high incidence of CHD rates in men compared to women below the age of 60years.

8.2.1 Carbohydrate on LDL

The LDL-I subfraction contained more neutral carbohydrate compared to small dense LDL-III (Table 5.2). This was also observed by subfractionation of LDL by affinity chromatography on Con-A (Table 5.5) which we attributed to the high mannose structure of LDL-I compared to LDL-III. The lack of any association between the extent of APG-LDL interaction and the lipoproteins neutral carbohydrate and sialic acid concentration indicate that the "anti atherogenic" effects, if any, of these carbohydrate residues are not related to their reactivity towards APG. This is supported by the sialic acid lowering effect of ciprofibrate on the lipoprotein which also reduced its ability to interact with APG as opposed to increasing it. It is worth noting, that neither neutral carbohydrate nor sialic acid content of the lipoprotein were related to the plasma lipid levels which suggest that the carbohydrate content of the lipoprotein is a function of particle size rather than abnormalities in lipoprotein metabolism.

8.2.2 Lipid lowering treatment

The rate and nature of LDL generated from VLDL can be perturbed pharmacologically, as we have shown by lipid lowering treatment with ciprofibrate. The reduction of plasma triglyceride in the form of VLDL1 after 8 weeks treatment was possibly due to decreased hepatic secretion (Gaw & Shepherd 1991). This was reflected in the change in the LDL subfraction profile from small dense LDL to a more buoyant LDL-I profile. We implicated this in the reduced reactivity of all apoB containing lipoproteins towards apoB which fell in concert after treatment (Fig 4.7). This is another favourable effect of treatment with ciprofibrate. It is worth noting that ciprofibrate treatment was more effective in reducing the number of APG-reactive circulating apoB particles in the LDL species compared to IDL (Fig 4.8).

8.3 Mechanisms related to APG-Lipoprotein interaction

The mechanism of APG-Lipoprotein reactivity is complex. Several factors are implicated in mediating this interaction process. Like receptor mediated uptake of LDL particles the mechanism is charge related involving lysine and arginine residues on apoB which is indicated by the reduced reactivity of charge modified LDL towards APG. However, we believe that APG-Lipoprotein interaction is different from receptor mediated pathways. This can be explained by the significant effect of apoE2 and apoE3 (which act as a ligand for receptor mediated uptake) on reducing the reactivity of LDL towards APG rather than increasing it. This could be either due to the effect of apoE in inducing conformational changes on apoB since no charge modification was observed as tested by gel electrophoresis, or that the apoE is competing with the lipoprotein for proteoglycan binding sites. The latter possibility is a little remote since no insoluble complexes were formed between APG and apoE (Table 6.2). In addition apoCIII did not have any effect on the binding process.

The controversy over the effect of sialic acid on APG-Lipoprotein interaction was resolved in this work. Although the interaction is a function of charge, the net charge of the lipoprotein particle, particularly through the effect of sialic acid has little contribution in controlling the APG-lipoprotein interaction. Despite the higher reactivity of neuraminidase treated LDL towards APG, the sialic acid concentration of the apoB containing lipoproteins was not associated with the extent of APG-Lipoprotein complex formation. In addition, incubation with GM3 gangliosides had little effect in reducing the ability of the lipoprotein towards APG. Our data suggest that conformation is pivotal in determining the interaction process by exposing positively charged free amino groups on critical domains on apoB particle to mediate the interaction process. This is supported by several findings. First, modification of apoB by reductive methylation which affects the protein structure was effective in blocking the interaction process. Second, we believe that the blocking effect of apoE is more conformation related (mentioned above). Finally, we implicate conformational changes

induced by TG enrichment of the precipitated fraction in the denser lipoprotein species for their reactivity towards APG.

8.4 LDL oxidative modification

The second main aim of this work was to illustrate the effect of EPO (n-6 PUFA) on LDL subfraction profile and in relation to its oxidative modification in a group of NIDDM patients. An ALP was the characteristic feature in these patients as it has been shown before (Reaven *et al* 1993a). In keeping with the finding by other investigators (Reaven *et al* 1993b), we found an increased in-vitro copper catalysed LDL oxidation on EPO supplementation. We attribute this to a predominance of small dense LDL within the total LDL of these patients (Table 7.3) which was positively correlated with PP-FDR. In addition increased LDL oxidative modification might be due to enrichment of small dense LDL with linoleic acid as reported by others (Reaven *et al* 1993b) and low HDL level found in NIDDM with low antioxidant activity. Our observation on the positive correlation between plasma triglyceride and LDL oxidation is in keeping with the most recent report on the link between ALP and enhanced LDL oxidation (Tribble 1995).

The lack of any effect of EPO supplementation on modifying lipid abnormalities in these patients is mainly due to the lower dose which we have used in the study.

8.5 ALP and atherosclerotic lesion formation

A most contentious issue which we addressed in this work is the dyslipidemic syndrome of an ALP because of its strong association with CHD and its importance in modulating apoB containing lipoproteins particularly that of LDL subfraction profile discussed above. It is evident now that an ALP confer changes on apoB containing lipoproteins rendering them more atherogenic. Therefore it is reasonable to summarise the role of an ALP in lesion development through APG-Lipoprotein interaction and its link to oxidative modification in the schematic diagram below.

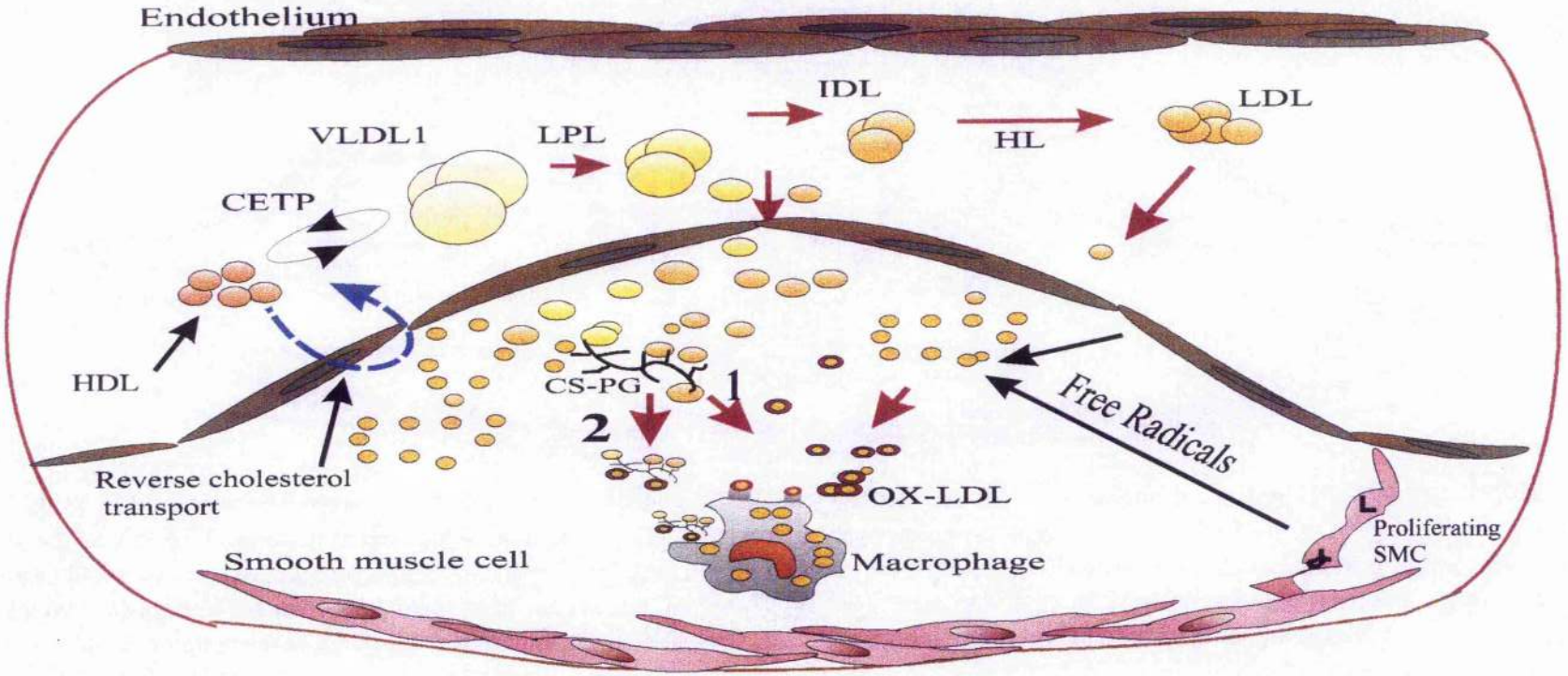


Figure 8.2 *Schematic diagram of lipoprotein abnormalities encountered an ALP in inducing lesion development. Oxidative modification of lipoprotein is thought to be a prerequisite for macrophage uptake of lipoproteins in the process of lipid deposition and plaque formation. We suggest that APL promote this process by a Direct (1) mechanism through enhance oxidation of small dense LDL by free radicals in the arterial extracellular matrix and indirectly (2) the higher reactivity of TG-rich remnant particles, lipoproteins in the Sf 8-12 range towards APG which subsequently lead to their oxidative modification and uptake by macrophages.*

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Appendix

Suppliers of Reagents, Equipment and Software.

Aldrich Chemical Company
A division of the Sigma-Aldrich Company Ltd
The Old Birickyard, New Road, Gillingham, Dorset, SP8 4XT, England
D-Mannose

Amersham International plc
Amersham Place, Little Chalfont, Amersham, Bucks. HP7 9NA, UK.
Na[^{125}I]

Beckman Instruments, Spinco Division
Beckman Instruments (UK) Ltd Analytical Sales and Service Operation
Progress Road, Sands Industrial Estate, High Wycombe, Bucks. HP12 4JL, UK.

L8-60M ultracentrifuge	L8-70 ultracentrifuge
Prep UV Scanner	AnF rotor
SW 40 rotor	Ti 60 rotor
Ultra-Clear™ centrifuge tubes	Ti 50.4 rotor
Polycarbonate centrifuge bottles and caps	Spinkote
Silicone vacuum grease	Tube slicer

Bio-Rad Laboratories Ltd
Bio-Rad House, Marylands Avenue, Hemel Hempstead, Herts. HP27TD,UK.
Bio-gel HT hydroxylapatite AG® 1-X8 200-400 mesh chloride form
Econo-columns

Denley Instruments Ltd.
Natts lane Billingshurst, West Sussex RH14 9EY, UK.
Denley orbital mixer.

Dynatech Laboratories Ltd
Daux Road, Billingshurst, West Sussex RH14 95J, UK,.
mr5000 microtitre plate reader

Jenway UK
Jenway , Dunmow, Essex, England CM6 3LD.
Jenway 3020 pH meter

Microsoft Corporation .
1 Microsoft Way, Redmond, WA, USA.
MS Word Version 4.0

M S E
Fisons Instruments, Sussex Manor Park, Gatwick Rd, Crawley, Sussex, RH10 2QQ, UK.
Mistral 4L refrigerated centrifuge.

Paar Scientific Ltd
594 Kington Road, Raynes Park, London SW20 8DN, UK.
Digital densitometer DMA 35

Perkin Elmer Ltd.
Maxwell Rd., Beaconsfield, Buckinghamshire, HP9 1QA.
Perkin Elmer LS - 50 Fluorimeter.

Pharmacia
Pharmacia Ltd, Pharmacia LKB Biotechnology Division, Midsummer Boulevard Central, Milton Keynes, Bucks. MK9 3HP, UK
Sephadex G-25(PD10 columns)

Sanofi - Winthrop
Onslow St, Guilford, Surrey, GU1 4YS, UK.
Ciprofibrate

Scotia Pharmaceuticals Ltd
Scotia House, Castle Business Park, Stirling, FK9 4TZ, Scotland
Efamol (Evening Erimrose Oil, EPO)

Sigma
Sigma Chemical Co. Ltd, Fancy Road, Poole, Dorset BH17 7TG, UK
Iodine monochloride (ICI) Dithiothreitol (DTT)
Trishydroxymethylaminomethane(TRIS) Tetramethylurea(TMU)

Spectrapor
Orme Technology, PO Box 3, Stakehill Industrial Park, Middleton, Manchester, M24 2RH,UK
Dialysis tubing

Technicon (Ireland) Ltd.
Swords Co. Dublin, Eire.
12 channel Peristaltic pump

Glossary

α-DG ..... α- D-methyl glucopyranoside
α-DM ..... α-D-methyl mannopyranoside
ALP ..... atherogenic lipoprotein phenotype
APG ..... arterial wall proteoglycan
Apo ..... apolipoprotein
BMI ..... body mass index
BP ..... blood pressure
BSA ..... bovine serum albumin
CHD ..... coronary heart disease
CHO ..... neutral carbohydrate
CM ..... chylomicron
Con-A ..... concanavalin-A
CS-PG ..... chondroitin sulphate proteoglycan
CV ..... coefficient of variation
d ..... density
Da ..... Dalton
EDTA ..... ethylene diamine tetra-acetic acetate
EPO ..... evening primrose oil
FDR ..... fluorescence development rate
FH ..... familial hypercholesterolaemia
Gdn-HCL ..... guanidine hydrochloride
GLA ..... gamma linolenic acid
HDL ..... high density lipoprotein
HL ..... hepatic lipase
HMG CoA ..... hydroxy methyl glutaryl coenzyme A
IDL ..... intermediate density lipoprotein
IP-FDR ..... inhibition period fluorescence development rate
IRS ..... insulin resistance syndrome
LA ..... linoleic acid
LDL ..... low density lipoprotein
Lp(a) ..... lipoprotein (a)
LPL ..... lipoprotein lipase
NIDDM ..... non insulin dependent diabetes
PBS ..... phosphate buffered saline
PP-FDR ..... propagation period fluorescence development rate
QS ..... quinine sulphate
SD ..... standard deviation
SDS ..... sodium dodecyl sulphate
TC ..... total cholesterol
TG ..... triglyceride
VLDL ..... very low density lipoprotein