LIPOPROTEINS, APOPROTEIN A-I CONTAINING PARTICLES AND THE DEVELOPMENT OF ATHEROSCLEROSIS

A thesis submitted to the University of London in part fulfilment for the degree of Doctor of Philosophy in the Faculty of Medicine

1993

David Thomas Vallance

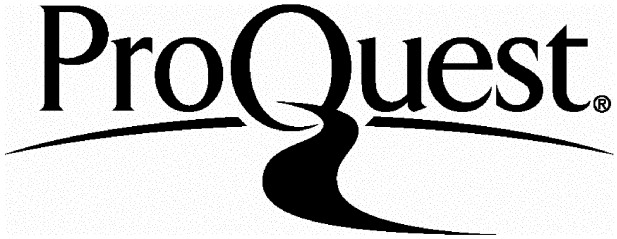
Department of Chemical Pathology
and Human Metabolism

Royal Free Hospital School of Medicine

Pond Street

London

NW3 2QG



Abstract

This study concerns the measurement and composition of high density lipoprotein (HDL) and mechanisms through which HDL might exert the proposed protective effect against coronary heart disease (CHD). The composition of HDL, particularly in respect of particles containing apoA-I but not apoA-II (LpAI) was studied after precipitation of apoB-containing lipoproteins. Differences in HDL cholesterol, apoA-I and LpAI between commonly used methods were modest and those differences were not due to selective precipitation of LpAI. All precipitation methods were equally effective at precipitating Lp(a).

Families with a history of premature CHD but without perceived risk factors showed an enhanced expression of two established risk associations - hypercholesterolaemia and/or reduced levels of HDL. LpAI concentrations were reduced in males with premature CHD and LpAI was marginally superior to apoA-I and HDL cholesterol in correctly discriminating between those with CHD and those without.

Moderate exercise, sufficient to increase HDL cholesterol, could potentially reduce CHD risk. However twelve months of moderate exercise in previously sedentary, middle-aged men did not produce any significant changes in serum lipids or apolipoproteins suggesting that the potential for modifying lipoprotein profiles in men using low intensity exercise is modest.

Exercise might produce changes in lipoprotein concentrations by modifying lipoprotein lipase (LPL) and hepatic lipase (HL) activity. In individuals with low and high HDL cholesterol, apoA-I and HDL cholesterol concentrations were correlated with post heparin plasma

LPL and inversely correlated with HL. Furthermore HL, but not LPL, activities were inversely correlated with LpAI concentrations in both groups.

The mechanisms by which HDL may protect against atherosclerosis are unclear. However the data reported here on the discriminating power of LpAI particles, the failure of exercise to influence LpAI particle concentrations and the factors which may influence LpAI concentrations in plasma, indicate that further work will be required before LpAI can truly be described as the anti-atherogenic fraction of HDL.

Contents

Acknowledgements

I wish to thank my supervisor, Professor A.F. Winder, for giving me the opportunity to undertake this work and for his help, guidance and encouragement, all of which ensured that this project was completed. I acknowledge the support and help of my many colleagues within the Department of Chemical Pathology and Human Metabolism, especially Mrs P. Wisner for secretarial help and to Mr D.J. Byrne, Dr L.H. Breimer, Dr D.M. Mikhailidis, Mr M.A. Barradas and Dr J.Y. Jeremy. I am grateful to Miss A. Staunton (St. George's Hospital, London) for recruiting families for the coronary heart disease register, Dr M. Seed (Charing Cross Hospital, London) and Professor S. Humphries (University College, London) for recruiting volunteers to undergo heparin infusions to measure post heparin lipolytic activities. Drs A. Hardman and D. Stensil (Loughborough University) recruited the men and supervised their training for the study on the effects of exercise on serum lipoproteins. I thank Dr I. McDowell (University Hospital, Wales) for helpful discussions on apoE phenotyping. Many other people, too numerous to mention, were very generous with their time and willingly and sometimes unknowingly helped me to complete this project.

Finally I thank my family for their support and forbearance and special thanks to Michaela without whom none of this would have been possible.

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Apo	Apolipoprotein
ACE	Angiotensin converting enzyme
BMI	Basal metabolic index
BP	Blood pressure
CABG	Coronary artery bypass graft
CDC	Centre for Disease Control
CETP	Cholesterol ester transfer protein
CHD	Coronary heart disease
CV	Coefficient of variation
DS	Dextran sulphate-magnesium chloride
EDTA	Ethylenediamine tetra acetic acid
FCR	Fractional catabolic rate
FED	Fish eye disease
HDL	High density lipoprotein
HL	Hepatic lipase
HM1	Heparin-manganese chloride method 1
HM2	Heparin-manganese chloride method 2
HMGCoA	Hydroxy-methyl-glutaryl co-enzyme A
IDL	Intermediate density lipoprotein
LCAT	Lecithin:cholesterol acyl transferase
LDL	Low density lipoprotein
Lp(a)	Lipoprotein (a)
LPL	Lipoprotein lipase
LTP	Lipid transfer protein
MI	Myocardial infarction
min	Minute

mm Hg	Millimetres of mercury
mRNA	Messenger ribonucleic acid
MW	Molecular weight
PAGE-IEF	Polyacrylamide gel isoelectric focusing
PEG	Polyethylene glycol
PHLA	Post heparin lipolytic activity
PKC	Protein kinase C
PTA	Phosphotungstic acid-magnesium chloride
RCF	Relative centrifugal force
s	Standard deviation
SDS	Sodium dodecyl sulphate
tPA	Tissue plasminogen activator
VLDL	Very low density lipoprotein

Chapter 1: Introduction

1.1 Objectives of the work described in the thesis

Coronary heart disease (CHD) is the commonest single cause of death and a major cause of morbidity in both men and women in the United Kingdom. Studies that have shown that high density lipoprotein (HDL) cholesterol is inversely correlated with the risk of CHD. Accurate and precise measurements of HDL cholesterol are therefore important in determining the risk of premature CHD. However, despite the apparent simplicity of the precipitation procedures used to isolate HDL, external quality assessment schemes have reported unacceptably high coefficients of variation between participating laboratories. There is therefore a case for re-examining HDL isolation procedures, incorporating recent modifications, to determine if between laboratory variability is due to incomplete precipitation of apoB-containing lipoproteins or due to inappropriate precipitation of HDL. There are at least two sub-populations of HDL which contain apolipoprotein (apo)A-I; those particles containing apoA-I in association with apoA-II, designated LpAI:AII and those containing apoA-I but not apoA-II, designated LpAI. It is important to determine if some of the reported differences between HDL cholesterol are due to selective precipitation of LpAI, a potentially anti-atherogenic lipoprotein particle. Similarly, failure to completely precipitate Lp(a), a potentially atherogenic apoB-containing lipoprotein, would lead to overestimation of HDL cholesterol and high between-laboratory imprecision. Precipitation procedures will be investigated to determine the composition of HDL isolated by various procedures and to assess the ability of each procedure to precipitate apoB-containing lipoproteins.

Much of the controversy about CHD has centred on the relative importance of each individual risk factor and the mechanisms through

which those factors operate. Some individuals may develop premature CHD without any obvious involvement of the major risk factors. A register of families with a history of premature CHD but without perceived risk factors was established. The aims were to store material for future genetic studies and to re-assess known risk factors in families with severe expression of CHD. Male family members with premature CHD were studied in more detail to determine if there were any associated changes in lipid profiles, specifically LpAI and to determine if LpAI particles could discriminate between those with CHD and those without.

As serum HDL cholesterol concentrations are inversely related to the risk of developing CHD, any intervention which would increase HDL cholesterol could potentially reduce the risk of developing CHD. A study was undertaken, using moderate exercise in previously sedentary middle aged men, to determine if regular moderate exercise could influence serum lipid and apolipoprotein concentrations, particularly HDL and HDL subfractions containing apoA-I.

One of the mechanisms by which exercise may produce favourable changes in serum lipids and apolipoproteins is by influencing the activity of lipoprotein lipase (LPL) and/or hepatic lipase (HL) and by altering the processing of triglyceride-rich lipoproteins. In addition, given that HDL2 cholesterol concentrations are linked to the turnover of triglyceride-rich lipoproteins, investigations were undertaken to determine if the levels of LpAI particles were correlated with post heparin plasma lipolytic activity. A method for measuring LPL and HL activities was developed and validated for normolipaemic adults and then used to study the relationship between plasma lipolytic activity and plasma HDL cholesterol, apoA-I and LpAI concentrations.

1.2 The lipoproteins

The lipoproteins are macromolecular complexes consisting of a hydrophobic core of cholesterol ester and triglyceride stabilised by polar components of phospholipid and cholesterol. The protein component of lipoprotein particles are specialised proteins called apolipoproteins. The major lipoprotein fractions are conventionally classified according to the floatation properties in salt solutions during ultracentrifugation (Havel et al., 1955) or by their respective electrophoretic mobility (Blix et al., 1941), the five major classes being chylomicrons (density <1.006g/ml and which remains at the origin on electrophoresis), very low density lipoprotein (VLDL, density <1.006g/ml, pre-β electrophoretic mobility), intermediate density lipoprotein (IDL, density 1.006-1.019g/ml), low density lipoprotein (LDL, density 1.019-1.063g/ml, β electrophoretic mobility) and high density lipoprotein (HDL, density 1.063-1.21g/ml, α electrophoretic mobility). Apolipoproteins were originally classified into group A which were found to be associated with lipoproteins with α-electrophoretic mobility and group B which were found to be associated with lipoproteins with β-electrophoretic mobility. Other apolipoproteins were designated C, D and E as they were discovered. This system has been rationalised into a nomenclature, referred to as the ABC nomenclature (Alaupovic, 1971), which designates apolipoproteins by capital letters, non-identical polypeptides within the groups with Roman numerals and polymorphic forms by Arabic numbers.

It is now apparent that the individual lipoprotein classes contain a complex mixture of lipoproteins of varying protein and lipid content rather than discrete particles of fixed composition.

(a) Chylomicrons

Chylomicrons are secreted by the intestine and enter the bloodstream via the thoracic duct. Chylomicrons are the largest lipoprotein particles and consist mostly of triglyceride with much smaller amounts of cholesterol, cholesterol ester, phospholipid and protein. The main protein components of nascent chylomicrons are apoB-48, apoA-I and apoA-IV. Each chylomicron particle probably contains only one molecule of apoB-48 which unlike some other apolipoproteins, does not transfer to other lipoprotein particles (Elovson et al., 1988). After secretion of the particle, other apolipoproteins, apoE and the C apolipoproteins, are acquired from HDL.

The first step in the catabolism of chylomicrons is the hydrolysis of triglycerides by LPL which is present on the surface of capillary endothelial cells. During this process, some surface components such as phospholipid, and apolipoproteins A and C are transferred to HDL, the remaining constituents of chylomicrons forming chylomicron remnant particles. The remnants are rapidly removed by the liver, possibly by a receptor based process involving a putative apoE receptor, also known as the remnant receptor (Mahley et al., 1981).

(b) Very low density lipoprotein

VLDL particles are secreted by the liver and provide a pathway to export endogenously synthesised triglyceride from the liver. The particles contain less triglyceride but proportionately more cholesterol, cholesterol ester, phospholipid and protein than do chylomicrons. The main protein components of nascent VLDL particles are apoB-100 with small amounts of E and C apolipoproteins, further amounts of apoE and apoC apolipoproteins being transferred

from HDL after secretion. The initial phases of the catabolism of VLDL are similar to that of chylomicrons with LPL hydrolysing VLDL triglyceride and the surface components of VLDL, including phospholipids, free cholesterol and C apolipoproteins transferring to HDL. As triglyceride is removed, VLDL simultaneously acquires cholesterol ester from HDL, the overall process leading to the formation of VLDL remnant particles.

The ultimate fate of the VLDL depends on both the initial size and composition of the particle. The larger VLDL particles produce larger VLDL remnants, containing several apoE molecules, which bind to the hepatic apoB,E receptor leading to rapid removal of the large remnants from the bloodstream. However, hydrolysis of the smaller VLDL particles produces smaller VLDL remnants, containing fewer apoE molecules than the larger remnants. These smaller remnants remain in the circulation longer because of reduced affinity for the hepatic receptor. The smaller VLDL remnants contain IDL particles which are converted to LDL particles (Packard et al., 1984).

(c) Intermediate density lipoprotein

As suggested by the name, IDL particles have both density and composition between that of VLDL and LDL and are produced during the catabolism of VLDL. Some IDL particles, probably those richest in apoE and which will interact more readily with the apoB,E (LDL) receptor, are removed by the liver. However, the majority of the IDL particles are converted to LDL particles, probably by the action of hepatic lipase (Goldberg et al., 1982), although the proportion of VLDL particles that go on to form LDL particles varies between individuals (Packard et al., 1984).

(d) Low density lipoprotein .

LDL is the main cholesterol carrying lipoprotein in humans and has at least four discernable subclasses within the density range of 1.019-1.063g/ml, designated LDL-I to LDL-IV (Krauss and Burke, 1982). The larger subclasses, LDL-I and LDL-II (pattern A), predominate in approximately 75% of the population with LDL-III (pattern B) predominating in the remainder. Pattern B, which appears to be inherited as a single gene trait with a dominant mode of inheritance, has been termed the atherogenic lipoprotein profile (Austin et al., 1990) as elevation in plasma cholesterol and apoB are far more common in individuals with pattern B than pattern A.

Each LDL particle is normally derived from one VLDL particle during the progressive removal of triglyceride and other components from VLDL, although there is some evidence that LDL may be directly secreted by the liver at least in certain disorders of lipoprotein metabolism (Soutar et al., 1977). Unlike VLDL, LDL contains only one apolipoprotein (apoB-100), is relatively rich in cholesterol and cholesterol esters and has a much smaller triglyceride content than VLDL. LDL can be taken up by cells either by a non-receptor mediated pathway or via a specific receptor called the apoB,E or LDL receptor which can be expressed on the surface of many types of cell. Synthesis of new LDL receptors is down regulated by high intracellular cholesterol concentrations, possibly due to the interaction of sterols with sterol regulatory elements within the promotor region of the LDL receptor gene (Goldstein and Brown, 1990). However non-receptor mediated uptake of cholesterol continues and this route is probably of increasing metabolic importance as the extracellular LDL cholesterol concentration increases.

(e) High density lipoprotein

HDL is the smallest of the lipoprotein particles. HDL particle concentrations in plasma are usually similar to those of LDL but particles numbers in tissue fluids are usually much greater than those of LDL because the small size of HDL enables the particle to cross the vascular endothelium more readily than LDL. Two major HDL subfractions can be isolated by density gradient ultra-centrifugation. The smaller of the two major particles is called HDL3 (density range 1.12-1.21g/ml) and the larger particle, called HDL2 (density range 1.063-1.12g/ml), contains more lipid but less protein than HDL3. HDL1 (density range 1.053-1.063g/ml) is a third, minor, relatively lipid-rich, HDL subfraction. The relevance of HDL1 in lipid metabolism is unclear.

HDL precursors are secreted by both liver and intestine, both making an approximately equal contribution to the circulating plasma HDL pool. HDL has been traditionally thought to be secreted as lamellar discs composed mainly of phospholipid and protein, with apoA-I and apoA-II present as the major apolipoproteins. HDL is then converted to mature spherical HDL by acquiring surface components, including phospholipids, apolipoproteins and cholesterol, released during lipolysis of triglyceride-rich lipoproteins. Recent evidence, from experimental work with rats, suggests that small spherical HDL can be secreted directly by the liver and that larger triglyceride-rich spherical HDL particles, also secreted by the liver, become disc shaped during lipolysis of triglyceride contained within the particle and subsequently become small spheres following enrichment of the particle with cholesterol (Winkler and Marsh, 1989). Similarly, the intestine may secrete small spherical HDL particles directly and it

may also secrete larger triglyceride-rich lipoprotein containing components of HDL which are then released as disc shaped HDL precursors (Forté et al., 1979) or as small spherical HDL particles (Schaefer et al., 1982) during lipolysis of triglyceride-rich lipoproteins.

Although the exact mechanism by which HDL is secreted is still unclear, once secreted HDL acquires phospholipids, apolipoproteins and free cholesterol from VLDL during the lipolysis of VLDL triglyceride (Patsch et al., 1978; Taskinen et al., 1982). Cholesterol is esterified by lecithin:cholesterol acyl transferase (LCAT) and esterified cholesterol may either be transferred to other lipoprotein particles by the action of cholesterol ester transfer protein (CETP), or be incorporated into the core of HDL particles. This process of lipolysis of triglyceride-rich lipoproteins and transfer of components between lipoproteins is associated with the production of HDL particles of increasing size and decreasing density, eventually producing HDL2. The formation of HDL2 from HDL3 depends therefore at least in part on the activity of LPL and the rates of hydrolysis of triglyceride-rich lipoproteins. This is supported by clinical observations that HDL concentrations in plasma are proportional to both adipose tissue (Nikkila et al., 1978a) and post heparin plasma (Kekki, 1980) LPL activity and to rates of VLDL triglyceride lipolysis (Huttunen et al., 1976). As HDL2 acquires more lipid it may also acquire apoE leading to the displacement of apoA-I (Blum, 1982; Rubinstein et al., 1982) and the formation of HDL1. This lipoprotein is probably only formed when larger quantities of lipid are carried as a similar lipoprotein called HDL_c accumulates in the plasma of animals fed cholesterol-rich diets (Mahley, 1982).

Removal of lipid from HDL2 particles, probably mediated by hepatic lipase (HL), is part of the process converting larger HDL2 particles to smaller HDL3 particles. *In vitro* HL hydrolyses HDL phospholipids and HDL triglyceride which *in vivo* may create suitable conditions for transfer of cholesterol esters to the liver without the need to internalise the particle (Groot et al., 1983). Furthermore, HDL cholesterol ester may be transferred to triglyceride-rich lipoproteins in exchange for triglyceride molecules generating a cholesterol ester-poor, triglyceride-rich HDL intermediate prior to the action of HL. The overall effect of this process would be the conversion of HDL2 to small, dense HDL3 and the removal of cholesterol acquired by HDL from the circulation by transferring cholesterol esters to other lipoprotein particles which will eventually be taken up by the liver or by direct transfer of cholesterol esters from HDL to liver cells. These mechanisms probably account for the low plasma concentrations of human HDL1 as cholesterol ester is transferred from HDL2 to other lipoproteins or the liver, reducing the need to form cholesterol-rich HDL1.

(f) Lipoprotein(a)

Lipoprotein(a) [Lp(a)] particles can be isolated by density gradient ultracentrifugation in the density range from 1.047-1.10g/ml and consists of LDL linked to one molecule of apo(a) by a single disulphide bond (Fless et al., 1984). The major site of synthesis of Lp(a) is the liver (Kraft et al., 1989) although the processes involved in the intracellular formation of the apoB-100-apo(a) complex and the assembly and secretion of Lp(a) particles are not yet known. Apo(a) does not circulate exclusively with LDL as apoB-100-apo(a) complexes

have also been found in triglyceride-rich particles, especially post-prandially (Pfaffinger et al., 1991). Triglyceride-rich Lp(a) particles probably arise during intravascular remodelling of lipoproteins in addition to *de novo* synthesis by the liver. The site of catabolism of Lp(a) is not clear although the LDL receptor appears to be much less important in the catabolism of Lp(a) than that of LDL, even though both lipoproteins contain apoB-100 (Scanu and Fless, 1990).

Although the mechanisms by which Lp(a) increases the risk of premature cardiovascular disease is unknown, it may be atherogenic because of the LDL content or it may influence thrombotic and fibrinolytic events through apo(a) which has certain structural similarities with plasminogen. However although Lp(a) particles have been found in atherosclerotic plaques, it is not clear if native Lp(a) is atherogenic or if complex formation with proteoglycans (Kostner and Biharri-Varga, 1990) and glycosaminoglycans (Biharri-Varga et al., 1988) or if oxidation of Lp(a) (Haberland et al., 1989) is required before Lp(a) becomes atherogenic. Also, although Lp(a) may modulate fibrinolytic events on the surface of endothelial cells *in vitro* (Scanu and Fless, 1990), there is no correlation between plasma Lp(a) concentrations and the activity of the fibrinolytic system *in vivo* (Armstrong et al., 1990).

1.3 The apolipoproteins

Apolipoproteins are important in maintaining the structural integrity of lipoprotein particles and they also have important functions as ligands for specialised receptors on the surface of cells and as co-factors for certain key enzymes involved in lipoprotein metabolism.

(a) The A apolipoproteins

(i) ApoA-I

The major protein component of HDL is apoA-I. Following intracellular cleavage of preproapoA-I, it is secreted as pro-apoA-I by the liver into plasma in association with nascent HDL particles, and into lymph by the intestine in association with chylomicrons (Karathanasis et al., 1983). ProapoA-I is then cleaved extracellularly by proteases circulating in the plasma to produce mature apoA-I (Bojanovski et al., 1985). ProapoA-I accounts for approximately 4% of total apoA-I in plasma (Sprecher et al., 1984). ApoA-I associated with chylomicrons transfers to HDL during the intravascular lipolysis of chylomicron particles. A large percentage of the structure of apoA-I is composed of α-helixes which consist of tandemly repeated 22 amino acid segments interrupted by proline residues (Baker et al., 1975). These helical regions are probably important in lipid binding, maintenance of the structural integrity of HDL and the activation of LCAT (Fielding et al., 1972). The lipid composition and/or size of the HDL particles probably determines the conformation and epitope expression of the apolipoproteins associated with the particle.

(ii) ApoA-II

ApoA-II is the second most abundant protein in HDL and is synthesised by the liver and intestine as a prepropeptide which is cleaved to produce a propeptide (Knott et al., 1984, Lackner et al., 1985). However, unlike apoA-I which is produced intravascularly from proapoA-I, proapoA-II is cleaved post-translationally within the cell to produce mature apoA-II before secretion into plasma and lymph (Lackner et al., 1985) as a dimer consisting of two identical subunits

covalently linked by a disulphide bridge (Lux et al., 1972). ApoA-II is an important structural component of HDL and can activate hepatic lipase (Jahn et al., 1983). However a mutation causing familial apoA-II deficiency appears to have little effect on lipoprotein metabolism or on the occurrence of coronary artery disease (Deeb et al., 1990).

(iii) ApoA-IV

ApoA-IV is synthesised in the intestine and is secreted in association with chylomicrons (Karathanasis et al., 1986). Some apoA-IV is probably transferred to HDL during the lipolysis of chylomicrons. A small quantity can be found in the lipoprotein-deficient fraction of plasma. Early studies which showed that the majority of apoA-IV was associated with the lipid deficient fraction of plasma were probably caused by artifactual loss of weakly bound apoA-IV from HDL during sequential ultracentrifugation (Lagrost et al., 1989). Although apoA-IV will activate LPL (Goldberg et al., 1990a) and LCAT (Steinmetz and Utermann, 1985) *in vitro*, the precise metabolic role of apoA-IV is unclear. The α-helical structure suggests it is involved in the maintenance of the structural integrity of HDL and in lipid binding (Weinberg and Spector, 1985).

(b) ApoB

ApoB has two main isoforms, designated apoB-100 and apoB-48 (Kane et al., 1980). Apo B-100, molecular weight (MW) 549,000, is found in LDL and VLDL. All other apoB fragments and sub-species are designated on a centile scale according to their molecular weight compared with apoB-100, as determined using sodium dodecylsulphate-polyacrylamide gel electrophoresis (Kane, 1983). The

apoB (MW 264,000) found in chylomicrons, is approximately 48% of the molecular weight of apoB-100 and is thus designated apoB-48. Both forms of apoB are synthesised from transcripts of the same gene located on the short arm of chromosome 2 (Law et al., 1985). A stop codon inserted post-translationally in intestinal mRNA, terminates synthesis of intestinal apoB producing apoB-48 which is totally homologous with the amino terminal portion of apoB-100 (Powell et al., 1987, Chen et al., 1987). Studies, using monoclonal antibodies, indicate that there is one copy of apoB-100 per molecule of LDL (Wiklund et al., 1985) although the number of copies of apoB-48 in chylomicrons has been more difficult to determine because of the heterogeneity of the particle size.

ApoB is more hydrophobic and contains a smaller proportion of helical structures than the other apolipoproteins (Scanu and Hirz, 1968). These hydrophobic regions interact with lipid at the core of the lipoprotein particle leaving hydrophilic regions to extent into the aqueous phase. Binding sites on apoB-100 which interact with the apoB,E receptor are not present in apoB-48 (Marcel et al., 1987). This lack of the apoB,E binding domain in apoB-48 probably ensures rapid delivery of dietary lipid in chylomicron remnants to the liver (Brown and Goldstein, 1987). Furthermore chylomicron, VLDL and VLDL remnants deficient in apoE bind poorly to the apoB,E receptor (Krul et al., 1985; Schaefer et al., 1986), indicating that conformational changes to apoB-100, brought about during intravascular conversion of VLDL to LDL, are required to expose and re-organise the receptor binding domains of apoB-100.

(c) The C apolipoproteins

The C apolipoproteins are a group of low molecular weight proteins (MW 6,600-8,800) that are mostly associated in the fasting state with HDL but which transfer from HDL to VLDL and chylomicrons when these triglyceride-rich lipoproteins enter the circulation. The C apolipoproteins then transfer back to HDL along with other surface components during the lipolysis and metabolism of VLDL and chylomicrons.

(i) ApoC-I

ApoC-I is the smallest of the C apolipoproteins (MW 6,600). ApoC-I can activate LCAT *in vitro* and may explain the normal plasma esterified cholesterol levels in individuals with apoA-I deficiencies (Soutar et al., 1975)

(ii) Apo C-II

The apoC-II gene is on chromosome 19, closely linked to the genes for apoE and apoC-I (Humphries et al., 1984) and is synthesised as preproapoC-II which is cleaved to produce proapoC-II. This undergoes further post-translational modifications before mature apoC-II is secreted (Fojo et al., 1986). ApoC-II circulates in association with HDL and is transferred to nascent chylomicrons and VLDL (Havel et al., 1973). ApoC-II is an activator for LPL and contains binding sites for LPL at the carboxyl terminal. ApoC-II concentrations in plasma greatly exceed that which is required to activate of LPL and it has been estimated that only 10% of circulating apoC-II is required for complete activation of LPL (Jackson et al., 1986).

(iii) ApoC-III

ApoC-III is the most abundant of the C apolipoproteins and circulates in plasma in three forms designated apoC-III_0, apoC-III_1 and apoC-III_2 depending on the number of sialic acid residues attached (Brewer et al., 1974). ApoC-III is sialylated intracellularly and is secreted as disialylated apoC-III_2, indicating that apoC-III_1 and apoC-III_0 are produced by desialylation in plasma (Hussain and Zannis, 1990). The disialylated form, apoC-III_2, accounts for approximately 27% of plasma apoC-III while the monosialylated apoC-III_1 and the non-sialylated apoC-III_0 account for 59% and 14% respectively (Zannis and Breslow, 1985). The physiological significance of the presence or absence of sialic acid groups is unknown. ApoC-III may regulate, possibly by inhibiting LPL, the catabolism of triglyceride-rich lipoproteins, as transgenic mice overexpressing human apoC-III become severely hypertriglyceridaemic (Ito et al., 1990). Plasma apoC-III levels are probably too low to inhibit LPL *in vivo* (Cardin et al., 1982), but more recent work has shown that LPL is inhibited by physiological concentrations of synthetic polypeptides of apoC-III (McConathy et al., 1992).

ApoC-III may also inhibit the uptake of triglyceride-rich remnants by hepatic receptors (Shelbourne et al., 1980, Windler et al., 1980) or it may act as a co-factor for LCAT although it is much less effective than apoA-I as an activator of the enzyme. (Jonas et al., 1984). The significance of any of these possible metabolic roles for apoC-III is not yet clear.

(d) ApoD

ApoD is a glycoprotein which normally circulates in association with HDL. The major site of synthesis is unknown although messenger ribonucleic acid (mRNA) has been detected in adrenal, kidney, pancreas and small intestine with lesser amounts of mRNA in liver (Drayna et al., 1986). It is probably part of the cholesterol ester transfer complex, consisting of LCAT, apoA-I, apoD and CETP, and which is thought to esterify cholesterol and distribute cholesterol ester between lipoprotein particles (Fielding and Fielding, 1980).

(e) ApoE

ApoE is synthesised by many tissues, the liver being quantitatively the most important source of plasma apoE (Blue et al., 1983). ApoE is primarily associated with chylomicrons, VLDL and HDL and acts as a ligand for the apoB,E (LDL) receptor and for the putative apoE (remnant) receptor. It is a genetically polymorphic protein with three common alleles (ϵ-4, ϵ-3 and ϵ-2) coding for three proteins designated E-4, E-3 and E-2 (Zannis et al., 1982). Single amino acid substitutions occur at two sites (amino acid residues 112 and 158) producing charge differences in the apo E isoforms. E-4 (arginine at amino acid residues 112 and 158) is the most basic (pI=6.0) and E-2 (cysteine at amino acid residues 112 and 158) is the most acidic (pI=5.7). E-3 is the most common isoform (cysteine at residue 112 and arginine at residue 158). Other much rarer variants which may cause type III hyperlipoproteinaemia include apoE-2^t (cysteine instead of arginine at residue 145) (Weisgraber et al., 1981) and apoE-3/E-3 homozygotes who have an amino acid substitution that does not affect the isoelectric point of protein (Havel et al., 1983).

Substitution of neutral amino acids for basic residues within the receptor binding domain of apoE alters binding to the apoE receptor (Innerarity et al., 1983, Weisgraber et al., 1983). The different apoE phenotypes are one of the major genetic determinants of the variation in plasma cholesterol concentrations due to kinetic differences in the metabolism of the different forms of apoE with apoE-2 being catabolized more slowly than apoE-3 which in turn is catabolized more slowly than apoE-4 (Gregg and Brewer, 1988). The ϵ-2 allele is associated with lower total and LDL cholesterol concentrations and the ϵ-4 allele with higher concentrations than the population mean (Davignon et al., 1988). The lower plasma LDL cholesterol concentrations associated with apoE-2 are due to both a decreased production rate and an increased fractional catabolic rate of LDL (Gregg et al., 1984).

Individuals who are ϵ-2 homozygotes may develop Type III hyperlipidaemia, characterised by the accumulation of abnormally cholesterol-enriched VLDL and chylomicron remnants, known as ß-VLDL, which has the hydrated density of normal VLDL but which has ß rather than pre-ß electrophoretic mobility. However another factor which predisposes to hyperlipidaemia, such as a genetic, hormonal or dietary factor must usually be present in ϵ-2 homozygotes before hyperlipidaemia and the clinical features associated with Type III hyperlipidaemia develop (Havel et al., 1980; Gregg et al., 1981).

(f) ApoJ

ApoJ is a recently discovered apolipoprotein which circulates in plasma in association with HDL, tightly associated with apoA-I (de Silva et al., 1990a; Jenne et al., 1991). The liver is thought to be the

major source of plasma apoJ although apoJ mRNA has been detected in many tissues including brain, testis, ovary kidney, heart, spleen and thymus (de Silva et al., 1990b). ApoJ has, in common with other apolipoproteins, amphipathic helices thought to be important in lipid binding but unlike other apolipoproteins, apoJ circulates as a disulphide-linked heterodimer (de Silva et al., 1990b) and shares some homology with complement proteins C7, C8 and C9 (Kirszbaum et al., 1989). The physiological function of apoJ is unknown.

(g) Apo (a)

Apo(a) is a glycoprotein, synthesised mainly if not exclusively by the liver (Kraft et al., 1989). The structure of apo(a) is quite unlike that of any of the other apolipoproteins, containing structures called kringles, similar to those found in proteins of the thrombotic, fibrinolytic and complement systems (Patthy, 1985). Protein sequencing (Eaton et al., 1987) and complementary DNA sequencing (McLean et al., 1987) indicates a high degree of homology between the structures of plasminogen and apo(a). Plasminogen has a domain with protease activity and five kringle domains each held together by three internal disulphide bonds, while apo(a) contains a protease domain and one copy of a kringle with a high degree of homology to kringle 5 of plasminogen and multiple repeats of a kringle homologous to kringle 4 of plasminogen. Apo(a) can exist as several isoforms, ranging in molecular weight from 400,000-800,000, caused by variations in the number of kringle 4 repeats (Lindahl et al., 1990). Using sodium dodecylsulphate-polyacrylamide gel electrophoresis, the isoforms can be classified according to the mobility of apo(a) compared with the mobility of apoB-100 as F (faster mobility), B

(similar mobility) and four isoforms, S1-S4, with slower mobility (Utermann et al., 1987). Recently, 23 apo(a) isoforms have been identified using high resolution SDS-agarose gel electrophoresis and immunoblotting (Kamboh et al., 1991).

The *in vivo* function of apo(a) and Lp(a) is unknown. The protease domain of apo(a) does not have plasmin-like activity against fibrinogen and the plasminogen activators streptokinase, urokinase and tissue plasminogen activator (tPA) will not activate the protease domain of apo(a) (Eaton et al., 1987). Both apo(a) and Lp(a) will bind to fibrin (Harpel et al., 1989) and also inhibit the activation of plasminogen by competing with plasminogen for binding to endothelial cell receptors (Gonzales-Gronow et al., 1989; Hajjar et al., 1989; Miles et al., 1989). It has been suggested that the binding of apo(a) to fibrin helps wound healing by delivering cholesterol to sites of recent injury (Brown and Goldstein, 1987). Whether any of these actions have important effects *in vivo* remains to be established.

1.4 Lipoprotein processing enzymes

Lipoprotein lipase, hepatic lipase and lecithin:cholesterol acyl transferase have key roles in the intravascular metabolism of lipoproteins and the distribution of lipids and apolipoproteins between lipoprotein particles.

(a) Lipoprotein lipase

LPL is synthesised by various tissues including adipose tissue, skeletal muscle, heart, lung and lactating mammary glands. The enzyme is synthesised in the endoplasmic reticulum as an inactive proenzyme and is then activated in the Golgi apparatus by

glycosylation prior to secretion although some secretion of inactive, non-glycosylated enzyme probably occurs. ApoC-II is required for maximum activity of LPL although the binding site on LPL has not yet been identified.

Following secretion, LPL is bound to heparan sulphate on the surface of endothelial cells (Klinger et al., 1985). This enables LPL to extend into the capillary lumen where triglycerides in chylomicrons and VLDL are hydrolysed, the 1 and 3 ester bonds in triglyceride and the 1 bond in monoglyceride being the preferred substrates. The lipid binding domains are very similar in LPL, HL and pancreatic lipase (Wion et al., 1987). Intravenous injection of heparin releases LPL (and HL) into the circulation.

Total LPL deficiency is rare, the incidence of homozygous LPL deficiency being about 1 per million and is characterised by fasting chylomicronaemia, eruptive xanthoma, recurrent pancreatitis and hepatosplenomegaly (Olivecrona and Bengtsson-Olivecrona, 1990). Absence of apoC-II produces a syndrome clinically indistinguishable from LPL deficiency (Breckenridge et al., 1978; Yamamura et al., 1979).

Heterozygous LPL deficiency occurs in about 1 in 500 individuals (Brunzell, 1989). Such heterozygous individuals would be expected to have approximately half the LPL activity of a healthy reference population. However some heterozygotes are hyperlipidaemic while others are normolipaemic, suggesting that some individuals with reduced LPL activity, can metabolise triglyceride-rich lipoproteins adequately (Olivecrona and Bengtsson-Olivecrona, 1990).

(b) Hepatic lipase

HL is synthesised in hepatocytes and activated by glycosylation prior to secretion (Verhoeven and Jansen, 1990). The enzyme is then transported to hepatic endothelial cells where it is bound to heparan sulphate and can be displaced from this binding site by intravenous injection of heparin. Although HL has been less extensively studied than LPL, there is some evidence that HL is involved in the later stages of the hydrolysis of triglycerides in chylomicron remnants and IDL (Demant et al., 1988) and more importantly in the hydrolysis of phospholipids and triglycerides in HDL (Clay et al., 1989). The role of HL in the metabolism of HDL particles is supported by a study which has shown a strong negative correlation between HL activity and plasma levels of HDL, particularly HDL2, indicating that HL participates in the conversion of HDL2 to HDL3 (Kuusi et al., 1989). Homozygous HL deficiency is very rare although a few cases have been reported (Breckenridge et al., 1982; Carlson et al., 1986). Lipoprotein abnormalities in this syndrome include cholesterol enrichment of VLDL, triglyceride enrichment of LDL and accumulation of triglyceride in HDL resulting in the circulation of HDL particles almost exclusively corresponding to the size of HDL2 (Carlson et al., 1986). Affected individuals may have an increased risk of developing premature atherosclerosis.

(c) Lecithin-cholesterol acyl transferase

LCAT is synthesised in hepatocytes (McLean et al., 1986) and circulates in plasma in association with HDL (Albers et al., 1981). Cholesterol esters are generated by LCAT by the transfer of a fatty acid, usually linoleate, from the *sn*-2 position of lecithin to the 3-ß

hydroxyl position of cholesterol to produce esterified cholesterol and lysolecithin (Glomset, 1968). The reaction occurs in two stages, the first stage being the formation of lysolecithin and acylation of LCAT by a phospholipase A_2 reaction (Aron et al., 1978) followed by the transfer of the acyl group to cholesterol (Yokoyama et al., 1980). The esterification reaction probably takes place on the surface of HDL where the reaction is activated by apoA-I (Fielding et al., 1972). LCAT can be activated by several other apolipoproteins including apoA-IV, apoE and apoC-I (Soutar et al., 1975) although co-factors other than apoA-I are probably only of physiological importance in disorders such as Tangier disease when apoA-I levels in plasma are reduced (Chen and Albers, 1985).

LCAT enriches nascent discoidal and small spherical HDL particles with cholesterol ester, converting these particles into mature spherical HDL (Norum et al., 1975). Some of the cholesterol ester is retained within the non-polar cores of HDL leading to the production of HDL particles with increasing size and decreasing density, while a proportion of cholesterol ester is transferred to VLDL and chylomicrons by the action of CETP.

LCAT may also catalyse the partial reversal of the reaction by acylating LDL-associated lysolecithin to produce lecithin and acylated enzyme (Subbaiah, 1986), the acyl group coming from another lecithin molecule. The physiological significance of this partial reversal of the LCAT reaction is unclear as net production of free cholesterol does not occur (Sorci-Thomas et al., 1990). However such a reaction may have a role in completing the repair of oxidatively damaged LDL phospholipids after phospholipase A_2 has removed oxidized acyl chains from phospholipids (Parthasarathy and Barnett, 1990).

1.5 Lipid transfer proteins

There are at least two proteins in plasma which transfer lipids between lipoprotein particles (Morton and Zilversmit, 1982; Tall et al., 1983a; Hesler et al., 1987).

(a) Cholesterol ester transfer protein

CETP, also known as lipid transfer protein I or LTP-I, is a glycoprotein which promotes the transfer of cholesterol esters from HDL to VLDL and chylomicrons and the reciprocal exchange of triglyceride from triglyceride-rich particles to HDL (Marcel et al., 1980). CETP may additionally promote the exchange of cholesterol esters, with no net transfer, between HDL and LDL (Sniderman et al., 1978). Little is known about the sites of synthesis of CETP although cultured human monocyte-derived macrophages appear to synthesise and secrete a lipid transfer protein with properties similar to CETP (Tollefson et al., 1985). CETP circulates in association with HDL, mainly in the HDL particles that contain apoA-I but not apoA-II (Cheung et al., 1986), although it is not clear if CETP is secreted in association with HDL or if free CETP is secreted.

(b) Lipid transfer protein-II

Lipid transfer protein-II (LTP-II) transfers phosphatidylcholine from triglyceride-rich lipoproteins to HDL but is unable to promote the transfer of cholesterol esters (Albers et al., 1984). LTP-II has been shown, using ultracentrifugation, to associate in plasma with small HDL particles with very high density (density 1.20-1.26 g/ml) (Tall et al., 1983b). Studies of the association of CETP and LTP-II with lipoprotein particles in plasma have been difficult due to the effects

of prolonged ultracentrifugation which is known to disrupt lipoprotein particles (Kunitake and Kane, 1982) and which results in at least some CETP and LTP-II being recovered in the lipoprotein free fraction (Pattnaik et al., 1979; Tall et al., 1983b).

1.6 Lipoprotein receptors

(a) The apoB,E (LDL) receptor

The apoB,E or LDL receptor, associates with a protein called clathrin on the cell surface and then clusters together to form clathrin coated pits. The receptors are quickly re-internalised by invagination of the coated pit and any bound LDL is dissociated from the receptor before the receptor itself is recycled to the cell surface (Brown and Goldstein, 1986). ApoB-100 from internalised LDL is degraded to amino acids. LDL-associated cholesterol internalised by this mechanism suppresses the transcription of 3-hydroxy, 3-methyl-glutaryl coenzyme A (HMGCoA) reductase, the enzyme catalysing the rate limiting step of endogenous cholesterol synthesis (Goldstein and Brown, 1990). Secondly, it activates acyl CoA-cholesterol acyl transferase which re-esterifies free cholesterol, enabling excess cholesterol to be stored within the cell as cholesterol ester droplets (Goldstein et al., 1974) and finally internalised cholesterol reduces receptor mRNA concentrations causing suppression of further apoB,E receptor synthesis (Brown and Goldstein, 1975). Defects in LDL receptor synthesis, transport, ligand binding or internalisation of the receptor-LDL complex cause increased concentrations of plasma LDL which may accumulate in connective tissue and scavenger cells producing xanthomas and atheromas respectively.

(b) The apoE (remnant) receptor

This receptor is synthesised and expressed on the surface of hepatic parenchymal cells but unlike the apoB,E receptor does not appear to be expressed by extra-hepatic tissues. The receptor binds chylomicron remnants, VLDL remnants and HDL1, all of which contain apoE. Binding of newly secreted chylomicrons and VLDL does not occur probably because the binding sites on apoE are only unmasked in the remnant particles. Unlike the apoB,E receptor, apoE receptor synthesis is not down regulated by internalisation of cholesterol and therefore chylomicron remnants are internalised irrespective of the size of the intracellular cholesterol pool. The remnant receptor may account for the observation that apoE-containing lipoproteins are usually not elevated in individuals with defective apoB,E receptors (Brown and Goldstein, 1983; Rubinstein et al., 1990).

Little is known about the molecular structure of the apoE receptor or the gene controlling its synthesis. However a receptor, known as the LDL-receptor related protein (LRP), binds chylomicron remnants and has close structural and biochemical similarities to the LDL receptor but is much larger than the LDL receptor and is the largest membrane protein yet identified (Herz et al., 1988). There is some evidence that the LRP may be the remnant receptor as it binds apoE-rich lipoproteins (Kowal et al., 1989), undergoes endocytosis (Herz et al., 1990) and is found in intracellular compartments associated with endocytosis (Lund et al., 1989). Studies of the LRP in fibroblasts, using ß-migrating VLDL consisting of a mixture of chylomicron remnants and VLDL remnants that accumulate in the plasma of cholesterol fed rabbits, suggests that chylomicron remnant-like particles are taken up only after enrichment by apoE (Kowal et

al., 1989). The reason why ß-VLDL must be enriched with apoE before binding to the LRP is unknown but it may be due to inhibition of apoE binding by apoC-I and apoC-II which can displace and/or complex apoE rendering it inactive (Weisgraber et al., 1990).
LRP also appears to act as a receptor for α_2-macroglobulin and may have evolved as a receptor for this protein rather than for apoE (Strickland et al., 1990). Clearly further work is required on this receptor to determine whether or not LRP acts *in vivo* as a dual function receptor for chylomicron remnants and α_2-macroglobulin.

(c) The scavenger (acetyl LDL) receptor

Native LDL is not taken up to any great extent by macrophages *in vitro* but if LDL is modified by oxidation or acetylation, then it is rapidly taken up by a receptor termed the scavenger or acetyl-LDL receptor. This receptor is not down regulated as the intracellular cholesterol concentration increases, so that the macrophages can accumulate large amounts of cholesterol and form foam cells. The pathogenesis of atheroma is discussed later, but uptake of modified LDL may be one of the mechanisms of atheroma formation.

(d) ß-VLDL receptor

This receptor, present on macrophages, will bind and internalise ß-VLDL (lipoprotein particles with density <1.006g/ml but with ß rather than pre-ß electrophoretic mobility) but as in the scavenger receptor, is not down regulated as the intracellular cholesterol concentration increases. In Type III hyperlipoproteinaemia, ß-VLDL accumulates in the plasma and is taken up by macrophages via this receptor.

(e) The proposed HDL receptor

HDL and HDL3 both stimulate efflux of unesterified cholesterol from many types of cells in culture including fibroblasts (Stein et al., 1976; Daniels et al., 1981; Oram et al., 1981), arterial smooth muscle cells (Stein et al., 1976) and endothelial cells (Graham and Oram 1987). The nature of the interaction between HDL and the cell membranes remains in some doubt. Efflux of free cholesterol may not necessarily require a specific receptor for HDL. Other mechanisms by which HDL could be removed from cell membranes include simple desorption of free cholesterol into the unstirred water layer from cholesterol-rich plasma membrane domains or alternatively collisions between acceptor molecules and plasma membrane free cholesterol within cholesterol-poor plasma membrane domains could result in net cholesterol efflux from the cell (Rothblat et al., 1992).

Nevertheless there is some evidence which supports the existence of a HDL receptor. A protein (MW 110,000) that binds HDL3 has been isolated from a variety of cell types (Graham and Oram, 1987). This proposed receptor fulfils some of the requirements of a HDL receptor in that binding is specific and of high affinity for HDL3, the binding activity is increased when cells are loaded with cholesterol and trypsin treatment of cell monolayers abolishes binding of HDL3. The binding of HDL to the cell surface receptor is reversible and endocytosis does not occur indicating that efflux of cholesterol from cells can occur without internalisation of HDL3 (Oram et al., 1987). Further studies have shown that the interaction of the receptor with HDL involves the binding of HDL apoA-I to the proposed cell surface receptor (Slotte et al., 1987; Aviram et al., 1989).

However HDL will associate with more than one class of binding site

on fibroblast membranes. The average molecular weight (16,000) of these sites is much lower than that of other classical receptor proteins (Mendel et al., 1988b). Others have suggested that binding is mediated by membrane lipid and is directly related to the cellular cholesterol content (Tabas and Tall, 1984; Mendel et al., 1988a).

The two opposing views of the existence of the HDL receptor could be somewhat reconciled by the observations that translocation of cholesterol to the cell membrane is an energy dependant mechanism (Kaplan and Simoni, 1985) and that binding of HDL may activate transport of cholesterol from intracellular compartments to the cell surface. The signal promoting the intracellular translocation of cholesterol is thought to involve protein kinase C (PKC) as activation of PKC by phorbol esters stimulates translocation of intracellular sterols and cholesterol efflux from adipose cells (Theret et al., 1990), while sphingosine, a PKC inhibitor, blocks intracellular translocation of sterol to the plasma membrane but has no effect on the efflux of plasma membrane cholesterol (Mendez et al., 1991). In this model, after binding by apoA-I, the HDL particle interacts with cell surface receptors stimulating the translocation of unesterified cholesterol to the cell surface where it is picked up by particles separate from those which originally bound to the cell surface receptor and involving the lipid components of HDL rather than the apolipoproteins. The rate limiting step would therefore be the triggering of intracellular sterol translocation by means of a PKC-dependent pathway.

Essentially, a specific receptor for HDL has not yet been clearly identified although there is increasing circumstantial evidence that a receptor may exist.

1.7 Coronary heart disease

CHD is the commonest single cause of death and a major cause of morbidity in the United Kingdom (Shaper et al., 1981). Clinical features of CHD, which are consequent to the formation of atheromatous plaques and the formation of thrombi on the surface of those plaques, include angina, acute myocardial infarction, stroke and sudden death (McGill, 1988).

(a) Atherogenesis

Atherosclerosis begins in early childhood with the formation of fatty streaks consisting of lipid, principally cholesterol and cholesterol esters, in the intima of medium and large arteries. These fatty streaks may regress, at least in animals (Eggen at al., 1974), or may progress to form fibrous plaques consisting of a lipid core surrounded by capsules of smooth muscle and connective tissue. Complicated lesions are fibrous plaques with calcified, necrotic cores that continue to grow until the arterial lumen is stenosed. Ulceration and haemorrhage into the plaque predisposes to thrombus formation on the surface of the plaque.

The initial event leading to the formation of fatty streaks is unknown but may involve injury to the endothelium causing increased permeability of the cells or exposure of the sub-endothelial surface. Increased infiltration of lipoproteins into the arterial subintima can occur through these sites of injury. Lipoproteins are then ingested by monocytes forming lipid-rich macrophages, also known as foam cells. Growth factors secreted by macrophages, endothelial cells or platelets associated with any thrombus attached to the plaque, cause migration and proliferation of smooth muscle cells and collagen

formation which traps the foam cells. This cycle is perpetuated leading to the formation of fibrous plaques and complicated lesions.

(b) Thrombogenesis

Formation of thrombus on an atherosclerotic plaque may cause occlusion of coronary arteries resulting in myocardial infarction and sudden death. Platelets adhere to fibrin-rich material in the plaque resulting in release of adenosine triphosphate and thromboxanes which cause aggregation of platelets. Thrombin and fibrin formed stabilise the thrombus and promote further platelet aggregation causing the thrombus to increase in size. Thrombus formation is affected by many blood components including the coagulation factors, fibrinolytic activity and platelet aggregation.

(c) Risk factors

A number of factors, termed risk associations or risk factors, are associated with CHD. Much of the controversy about CHD has centred on the relative importance of each individual risk factor and the mechanisms through which those factors operate. The identified risk factors for coronary heart disease may be more involved in the progression of fatty streaks to fibrous plaques and complicated lesions than in initiating the intimal deposition of lipid (Solberg and Strong, 1983). One survey reported 246 suggested coronary risk factors which included serum lipoproteins, blood pressure, cigarette smoking, family history, age, gender, diet and physical activity (Hopkins and Williams, 1981).

(i) Serum lipoproteins

The evidence that lipoproteins are risk factors for the development of CHD is derived from a number of sources including human epidemiology, animal experimentation, cellular biology, clinical observations and intervention trials. Epidemiological evidence accumulated so far is mainly based on measurements of serum total cholesterol, triglycerides and high density lipoprotein cholesterol, although the exact cellular and molecular processes by which these lipoproteins are linked to atherogenesis is unclear. Concern has been expressed about the wisdom of lowering serum cholesterol concentrations because of the association between low blood cholesterol and non-cardiovascular deaths due to a variety of causes such as lung cancer, other non-colon cancers and digestive disease (Hulley et al., 1992)

(ii) Blood pressure

Hypertension has been shown to be associated with accelerated progression of atherosclerosis (Solberg and Strong, 1983), possibly due to the direct effect of increased pressure on the arterial wall. While lowering blood pressure by various anti-hypertensive drugs has reduced the incidence of strokes, there has been less impact on coronary morbidity (Collins et al., 1990).

(iii) Cigarette smoking

Cigarette smoking increases risk of CHD, cerebrovascular disease, peripheral vascular disease and aortic aneurysms (Doll et al., 1990). Although the precise mechanisms through which smoking affects the cardiovascular and haemostatic system are not clear, the increased

fibrinogen concentration observed in smokers (Ogston et al., 1970; Meade et al., 1987) is likely to be one of the major mechanisms through which smoking mediates its harmful effects on the cardiovascular system (Bain, 1992).

(iv) Family history

A family history of premature CHD may be expressed through genetic defects such as familial hypercholesterolaemia or may be due to interaction of multiple genetic and environmental risk factors as in Type III hyperlipoproteinaemia.

(v) Age, gender and ethnic background

The incidence of CHD increases with age in both sexes and men are at greater risk of developing CHD than pre-menopausal women although the incidence of CHD in post-menopausal women is equal to or greater than that of men (Shaper et al., 1985). The incidence of CHD varies between native populations although the incidence rises in migrants who move from nations with a low incidence to nations with a high incidence of CHD (Marmot et al., 1975).

(vi) Diet

High intakes of saturated fat are associated with increased incidence of CHD (Keys, 1975), probably due to increased serum cholesterol and compositional changes induced in lipoprotein particles. However, a review of the long term efficacy of diets in lowering serum cholesterol reported that the response to the step 1 diet (total fat less than 30% of total calories, polyunsaturated fat:saturated fat ratio of 1 and cholesterol less than 300mg daily) was clinically insignificant

in the management of adults with serum cholesterol concentrations greater than 6.5mmol/L (Ramsey et al., 1991).

Moderate alcohol intakes probably protect against CHD (Marmot et al., 1981) possibly by dilating coronary arteries and/or increasing HDL cholesterol (Belfrage et al., 1977) although chronic alcohol abuse is a common cause of hyperlipidaemia.

(vii) Physical activity

Regular aerobic exercise seems to protect against CHD. The evidence and mechanisms for the protective effect of exercise are reviewed later.

1.8 Possible protective mechanisms mediated by lipoproteins

The clinical implications and some of the evidence from epidemiological studies that has shown that serum HDL cholesterol concentrations are inversely correlated with the risk of CHD, have recently been reviewed (Gordon and Rifkind, 1989; Gordon et al., 1989). Overall, an increase in HDL cholesterol of 0.03mmol/L was associated with a decrease of 1% in CHD risk. Furthermore, while progression of atherosclerosis may be associated with high serum concentrations of LDL cholesterol, Lp(a) and other atherogenic lipoproteins, regression of atherosclerosis or protection against further progression, is associated with high serum HDL cholesterol concentrations (Barth and Arntzenius, 1991). There are at least two proposed mechanisms by which HDL, or a subfraction of HDL, may protect against CHD. One mechanism involves rapid interconversion and recycling of HDL but there are other mechanisms involving HDL which may not require rapid cycling between subfractions.

(a) High density lipoprotein

The most attractive of the rapid recycling hypotheses is the process, called reverse cholesterol transport, by which cholesterol is removed from peripheral cells and returned to the liver (Glomset, 1968). Unesterified cholesterol, picked up by HDL from cell membranes is re-esterified by LCAT. Some of the cholesterol ester so produced is retained within the core of the HDL particle and some is transferred, by CETP, into the core of other lipoproteins, usually VLDL. Cholesterol removed from cells therefore can be transported back to the liver either directly in association with HDL or by transfer to other lipoproteins which may be eventually taken up by the liver. An alternative mechanism, again involving interconversion of HDL subfractions and known as the remnant hypothesis, states that HDL acts as a marker for the rate of lipolysis of triglyceride-rich lipoproteins but does not itself protect against CHD. Impaired lipolysis of triglyceride-rich lipoproteins would lead to accumulation of atherogenic remnant particles and low levels of HDL as release of surface components normally acquired by HDL is impaired (Zilversmit, 1979). Evidence from clinical studies supports this hypothesis as it has been shown that there is a curvilinear inverse relationship between HDL cholesterol and VLDL triglyceride (Phillips et al., 1981). Lipid transfer proteins transfer cholesterol from HDL to VLDL and would be important in both the reverse cholesterol transport hypothesis and the remnant particle hypothesis. In reverse cholesterol transport, LTPs provide a mechanism to transfer cholesterol to particles which will be ultimately taken up by the liver but in the remnant particle hypothesis LTPs provide a mechanism to transfer cholesterol into atherogenic remnants.

Other mechanisms, not necessarily requiring rapid recycling between HDL subfractions but which may protect against CHD, include competition by HDL for non-receptor mediated LDL uptake, protection of LDL from oxidation (Parathasarathy et al., 1990) and aggregation (Khoo et al., 1990) or prevention of thrombi formation on atheromatous plaques by HDL stabilisation of prostacyclin in the vessel walls (Yui et al., 1988).

Any or all of these mechanisms may be important in protecting against cardiovascular disease. Clinical observations in certain diseases which are associated with altered levels of HDL suggest that turnover rather than the absolute level of HDL is important. For example in Tangier disease, when HDL is very low, probably due to rapid turnover and catabolism rather than due to reduced synthesis of HDL, the risk of coronary heart disease is only slightly increased while in conditions with low HDL due to defective synthesis of HDL such as familial hypoalphalipoproteinaemia, there is a marked increase in the risk of coronary heart disease.

Although the reverse cholesterol transport hypothesis and the remnant particle theory are both attractive, it is still unclear how HDL exerts the protective effect shown in epidemiological studies and the evidence supporting these hypotheses is still mostly circumstantial.

(b) Lipoproteins containing apoA-I but not apoA-II

Removal of cholesterol from cells is the first step in any proposed mechanism of reverse cholesterol transport and there have been intensive investigations to discover the particles that are the most efficient acceptors of cholesterol. One candidate for the acceptor

particle is LpAI. There are at least two sub-populations of HDL which contain apoA-I, LpAI and LpAI:AII (Norfeldt et al., 1981; Cheung and Albers, 1982; Nestruk et al., 1983). Neither LpAI or LpAI:AII correspond exactly to HDL subfractions classified by density, as HDL3 and HDL2 when separated by density floatation contain a mixture of both LpAI and LpAI:AII (Cheung and Albers, 1982), although similarities exist between LpAI and HDL2 in that both have relatively low protein content and relatively high total cholesterol content (Kilsdonk et al., 1990). Molar ratios ranging from 1.4 to 1.6 for the ratio of apoA-I to apoA-II have been reported in LpAI:AII particles (Ohta et al., 1988; James et al., 1988; Kilsdonk et al., 1990), with LpAI particles reported to contain from 2 to 8 molecules of apoA-I (Nichols et al 1985; Cheung et al., 1987; Kilsdonk et al., 1990).

The two particles are affected differently by alcohol (Puchois et al., 1990) and by lipid lowering drugs, nicotinic acid increasing LpAI and decreasing LpAI:AII while probucol decreases LpAI but has no effect on LpAI:AII (Atmeh et al., 1983). Approximately 80% of plasma CETP and 70% of plasma LCAT is associated with LpAI particles (Cheung et al., 1985) and kinetic studies indicate that LpAI and LpAI:AII probably have different metabolic roles (Rader et al., 1991).

LpAI but not LpAI:AII particles have been reported to promote cholesterol efflux from cholesterol-enriched OB 1771 mouse adipose cells (Barbaras et al., 1987). Clinical evidence has shown that male subjects with angiographically defined CHD have lower levels of LpAI particles when compared with individuals who do not have CHD while the concentrations of LpAI:AII particles are similar between the two groups (Puchois et al., 1987). This clinical evidence along with *in vitro* studies of LpAI particles and apoA-I have led to the proposal

that apoA-I is an agonist and apoA-II an antagonist of cholesterol efflux (Barbaras et al., 1990).

Within this group of apoA-I only containing particles, there is a subset of HDL particles which contain apoA-I but not apoA-II and have pre-beta rather that alpha electrophoretic mobility (Kunitake et al., 1985). This subset of particles may be important in promoting cholesterol efflux from cells as these particles, which are enriched in both phospholipid and protein but relatively depleted in cholesterol, have been shown to be the primary cholesterol acceptors from fibroblasts in culture (Castro and Fielding, 1988). These particles may have the postulated hinged domain of apoA-I in the "open" position (Segrest et al., 1992), enabling this extended hinged region to interact with plasma membranes (Rothblat et al., 1992). Pre-ß HDL and α-HDL probably undergo interconversion and HDL particle size is regulated by lipoprotein processing enzymes and transfer proteins including LPL, HL, LCAT and CETP (Kunitake et al., 1992).

1.9 Lipids and apolipoproteins as discriminators for atherosclerosis

Serum cholesterol is now generally accepted as a risk factor for CHD, although serum cholesterol concentrations are relatively poor at discriminating between those individuals who will develop premature CHD and those who are unlikely to develop premature CHD. The distributions of serum cholesterol concentrations in men in the Framingham Study shows considerable overlap between those individuals who develop CHD and in those who do not (Kannel et al., 1964).

While it is clearly very important to be able to identify those

patients who are at high risk of developing premature CHD, this overlap of distributions and the poor predictive value of serum cholesterol concentrations even at high values, means that it cannot be predicted using serum cholesterol concentrations alone which individuals will develop CHD and those who will not. Other lipid risk factors such as LDL cholesterol and HDL cholesterol have been found to be only slightly better discriminators than total cholesterol (Wilson et al., 1980). Other risk factors such as apoA-I (Maciejko et al., 1983), apoB (Sniderman et al., 1980; Durrington et al., 1986) or both apoA-I and apoB (Avogaro et al., 1979) and Lp(a) (Dahlen et al., 1986; Seed et al., 1990; Wiklund et al., 1990) have been proposed as discriminators of CHD risk.

Some individuals may develop premature CHD without any obvious involvement of the major risk factors. A register of families with premature coronary heart disease and without perceived risk factors was therefore established to facilitate identification of risk factors and to provide a store of genetic material for study with existing probes and so that any further advances in genetic analysis could be applied retrospectively. Furthermore, in those patients with premature coronary heart disease, it is important to investigate which lipoprotein particles are the best discriminators of CHD risk. Most studies have measured total apoA-I rather than selectively measuring those particles which contain apoA-I but not apoA-II. Similarly studies of HDL subfractions have classified HDL by density rather than apolipoprotein content. As LpAI particles may be the anti-atherogenic fraction of HDL, it is important to determine if individuals with premature CHD have reduced serum LpAI concentrations. Middle-aged men with early onset CHD were selected

from the coronary heart disease register. More detailed lipoprotein studies were undertaken to examine if any changes in total apoA-I are accompanied by similar changes in LpAI and to determine if LpAI is a better discriminator of CHD than total apoA-I or HDL cholesterol. A study of middle-aged men after myocardial infarction reported that Lp(a) concentrations were as good as family history at discriminating between patients and controls (Durrington et al., 1988) and others have reported that plasma Lp(a) levels above 30mg/dl are associated with a two fold increase in the risk of premature CHD (Hoefler et al., 1988). Furthermore in individuals with familial hypercholesterolaemia (FH) and increased LDL cholesterol levels, Lp(a) was the best predictor of angiographically documented CHD (Seed et al., 1990). These results were supported by a another study of FH patients (Wiklund et al., 1990), although others have shown that Lp(a) concentrations were not significantly higher in those with FH and CHD compared with those with FH but without CHD (Mbewu et al., 1991). Lp(a) concentrations will also be measured in the present study to determine if concentrations are raised in individuals with premature CHD and to determine if discrimination between those with CHD and those without can be improved by measuring Lp(a) in addition to apoA-I and LpAI.

1.10 The effects of exercise on serum lipids, lipoproteins and lipoprotein processing enzymes

In view of the association of serum lipoprotein concentrations and CHD risk, many studies have been undertaken to modify lipoprotein profiles using procedures, such as exercise, which are acceptable to the general population.

(a) Lipoproteins

A number of cross sectional studies have shown that individuals who regularly participate in physical activity such as running, ski-ing or tennis have higher serum HDL cholesterol concentrations when compared with inactive control groups (Wood et al., 1977; Lehtonen and Viikari, 1978; Vodak et al., 1980), even when factors such as differences in diet between athletes and inactive controls are taken into account (Hartung et al., 1980). Physical activity may therefore be an effective and physiological method of increasing serum HDL cholesterol concentrations. However most longitudinal studies conducted using relatively inactive but healthy individuals have been relatively brief and have been terminated after two to four months (Huttunen et al., 1979; Stubbe et al., 1983; Sopko et al., 1985; Raz et al., 1988) with one short term study lasting less than three weeks (Despres et al., 1988).

In addition to the problems of the differences in duration of the studies, there may be a threshold limit for exercise which must be exceeded before any beneficial effects on plasma lipoproteins are observed. A year long study reported a strong correlation between HDL cholesterol and the average distance run per week, once a distance threshold of 8 miles per week was exceeded (Wood et al., 1983). This implies that it might be possible to observe a relationship between fitness and serum HDL cholesterol concentration. However indices of physical fitness such as maximum oxygen uptake (VO_2 max), do not appear to be correlated with HDL cholesterol (Brownell et al., 1982; Haskell, 1986), possibly because VO_2 max is largely genetically pre-determined (Klissouras et al., 1973) and while it does increase somewhat with increased physical activity, it does not fully reflect

improvements in stamina and endurance. However while some studies using intense physical exercise, which would be expected to produce the most benefit in terms of increased stamina and endurance, serum HDL cholesterol has increased (Thompson et al., 1988), other studies using comparable exercise routines have been unable to demonstrate any increase in HDL cholesterol (Stubbe et al., 1983; Findlay et al., 1987) and conversely those using less intense exercise regimes have been able to demonstrate favourable changes in serum HDL cholesterol concentrations (Sopko et al., 1985; Baker et al., 1986).

Quite clearly there is some doubt about the intensity of exercise and the duration of the exercise program that is required to produce favourable changes in lipoprotein profiles. A study of long term, low intensity exercise showed that it was associated with improved longevity and reduced risk of death due to cardiovascular or respiratory causes (Paffenbarger et al., 1986). Another study reported that there was a relationship between miles walked per day by postal workers and serum HDL2 cholesterol concentrations (Cook et al., 1986). These studies indicate that frequent low intensity exercise may have favourable effects both in increasing life expectancy and in modifying serum lipoprotein profiles.

Many previously inactive people find high intensity exercise programs difficult to adhere to and are more likely to sustain injuries than in low intensity programs (reviewed by Martin and Dudert, 1982), it therefore seems reasonable to study the effects of frequent low intensity exercise, such as brisk walking, on serum lipoprotein concentrations. In determining the amount of exercise needed to provoke changes in serum lipoprotein concentrations, three studies were taken into account. A study of Harvard alumni reported

that men expending more than 8.4 MJ per week on low intensity exercise had a lower incidence of coronary artery disease when compared with less active individuals (Paffenbarger et al., 1978) and a second study showed that men walking more than 9 miles per week had a 21% lower risk of death from all causes than those who walked less than 3 miles per week (Paffenbarger et al., 1986). Using the approximation that individuals expend 420 KJ of energy per mile during brisk walking, an individual would need to walk approximately 20 miles per week to expend 8.4 MJ of energy. Finally it has been suggested that an exercise program leading to an increase in energy expenditure in excess of 5 MJ per week will provoke increases in serum HDL cholesterol concentrations in previously sedentary individuals (Haskell, 1986). Taking the findings of these studies together, it might be expected that some benefit in terms of cardiovascular risk and favourable changes in serum lipoprotein profiles might be expected in individuals walking between 9 and 20 miles per week.

Most studies of exercise training have been concluded after weeks or months and all the longer term trials have involved high intensity exercise (Wood et al., 1983; Wood et al., 1988; Thompson et al., 1988). It therefore seems justified to evaluate the potential of socially acceptable, moderate exercise to modify serum lipoprotein profiles as it is more likely that individuals will continue with a moderate exercise programs that may accrue long term benefits in terms of CHD risk. Serum Lp(a) concentrations will also be measured as no studies so far have examined the effect of exercise on this lipoprotein particle. Although serum Lp(a) concentrations are largely genetically determined and commonly used lipid lowering drugs and

diets have not produced substantial changes in Lp(a) concentrations, it is important to determine if exercise can modify serum Lp(a) concentrations.

(b) Apolipoproteins

Most studies have reported the effects of exercise on lipoproteins but relatively few have measured apolipoproteins and when apolipoproteins have been measured, conflicting results have been reported. Thus some studies reporting a significant increase in serum HDL cholesterol concentrations during training programs have found that serum apoA-I is unchanged (Marti et al., 1990; Huttunen et al., 1979) while others have found that both HDL cholesterol and apoA-I are increased (Schwartz, 1987). This apparent discrepancy could be explained if one effect of exercise is to redistribute apoA-I between HDL subfractions. Reports of the effect of exercise on HDL subfractions differ to some extent, some finding serum HDL2 cholesterol increased with (Nye et al., 1981; Wood et al., 1983) and without (Stubbe et al., 1983) a decrease in serum HDL3 cholesterol, while others have found that both serum HDL2 cholesterol and HDL3 cholesterol increase (Thompson et al., 1988; Wood et al., 1988; Marti et al., 1990). However, in general if any effect is observed, there tends to be a redistribution of HDL subfractions in favour of HDL2. Any changes in serum apoA-I concentrations may therefore be masked by a redistribution of apoA-I between HDL subfractions, HDL2-associated apoA-I increasing and HDL3-associated apoA-I decreasing. It is therefore important to examine if any exercise-induced changes in HDL subclass distribution towards a potentially anti-atherogenic pattern, with or without a change in total apoA-I

concentration, is accompanied by any change in LpAI concentrations. As these particles are thought to be the anti-atherogenic fraction of HDL, any redistribution of apoA-I into these LpAI particles is potentially a major benefit of exercise programs.

(c) Lipoprotein lipase and hepatic lipase

One of the mechanisms by which regular exercise may produce favourable changes in serum lipoprotein profiles is by improved processing of triglyceride-rich particles due to increases in skeletal and adipose tissue LPL activity (Nikkila et al., 1978b). The higher concentrations of serum HDL cholesterol concentrations in endurance athletes compared with sedentary controls and the increases in serum HDL cholesterol concentrations associated with exercise programs may partly be due to increased lipolysis of triglyceride-rich lipoproteins by LPL, with the surface components of these particles being picked up by HDL3, leading to an increase in HDL mass and the formation of HDL2.

1.11 The effects of lipoprotein lipase and hepatic lipase on HDL composition and metabolism

Serum concentrations of HDL cholesterol depend on the balance between the synthesis, interconversion of HDL subfractions and catabolism of HDL. LPL and HL are located primarily on the endothelial surfaces and influence interconversion of HDL subclasses and the catabolism of HDL rather than the synthesis. Experimental evidence has shown that HDL2-like particles are produced *in vitro* from HDL3 particles when lipids and apoproteins are released during lipolysis of human VLDL by bovine LPL (Patsch et al., 1978). The

formation of true HDL2 particles requires enrichment of HDL with cholesterol which may either be acquired from cell membranes or transferred from other lipoprotein particles by the action of CETP. Cholesterol acquired by HDL is esterified by LCAT and moves into the core of the particle. The formation of HDL2 from HDL3 therefore depends on the activities of three key enzymes, LPL, CETP and LCAT (Eisenberg et al., 1984). The catabolism of HDL2 is mainly dependent on the activity of hepatic lipase which hydrolyses HDL phospholipids and triglycerides and probably creates suitable conditions for the transfer of cholesterol from HDL2 to the liver. The two lipolytic enzymes therefore appear to have opposite effects on HDL metabolism, lipoprotein lipase influencing the formation of HDL2 and hepatic lipase influencing the degradation of HDL2.

HDL and HDL2 levels also depend on the apolipoproteins present in the particle. However HDL particles undergo continuous re-modelling by uptake and removal of lipid components before the complete HDL particle including HDL apolipoproteins are removed from the circulation. Nevertheless, apolipoprotein metabolism probably regulates the concentration and subfraction distribution of HDL particles as the conversion of HDL3 to HDL2 requires the incorporation of one extra apoA-I molecule (Eisenberg et al., 1984). Clinical evidence has shown that apoA-I concentrations are negatively correlated with plasma HL activities and positively correlated with LPL activities (Kuusi et al., 1989). If the activities of LPL and HL influence the levels of HDL3 cholesterol, HDL2 cholesterol and serum apoA-I, it is reasonable to assume that these enzymes may also affect the distribution of apoA-I within the HDL particle.

Kuusi *et al* (1989) showed that the apoA-I/apoA-II ratio was elevated

in women, but not in men, with hyperalphalipoproteinaemia and suggested that LPL and HL may modulate the metabolism of LpAI and LpAI:AII particles although neither particle was measured directly. A method was therefore developed and validated to measure LPL and HL activity in post heparin plasma so that a study could be undertaken to determine if activities of these enzymes were related to HDL cholesterol, apoA-I, apoA-II and the distribution of apoA-I within the HDL particles.

LPL and HL can both be released from intravascular binding sites by intravenous injection of heparin. The recommended sampling time for measurement of LPL and HL is ten minutes after injection of heparin (Mount et al., 1982), although HL activity has been reported to reach maximum at between 2 and 5 minutes after heparin administration (100U/kg body weight), with the maximum activity for LPL being approximately 20 minutes later (Huttunen et al., 1975). The release of lipolytic enzymes into the plasma will be monitored in normolipaemic individuals for 30 minutes following administration of heparin to determine the optimum sampling time.

1.12 High density lipoprotein isolation procedures

It is appropriate to review the methods used to isolate and measure HDL cholesterol as much of the work presented involves the measurement of HDL cholesterol. Various techniques have been used to isolate HDL including ultracentrifugation (Havel et al., 1955), electrophoresis (Conlon et al, 1979), gel filtration, immuno-affinity column chromatography (Cheung and Wolf, 1989) and specific precipitation techniques (Burstein and Samille, 1960; Burstein and Scholnick, 1973; Warnick et al., 1982; Grove, 1979).

(a) Ultracentrifugation

Ultracentrifugation, although used as a reference method, suffers from incomplete recovery of HDL (Warnick et al., 1979a), contamination of HDL fraction (density < 1.063 g/ml) by apoB associated cholesterol, mostly Lp(a) (Albers et al., 1972) and loss of apoA-I from HDL (Kunitake and Kane, 1982). Ultracentrifuges are not widely available and are unsuitable for analysing large numbers of samples.

(b) Electrophoresis

Lipoprotein electrophoresis, using paper, cellulose acetate, agarose and polyacrylamide gels, may be used to quantitate lipoproteins using scanning densitometry rather than to prepare lipoprotein fractions for further analysis. Although results for ß and pre-ß lipoproteins, using electrophoresis followed by scanning densitometry compare well with those obtained by ultracentrifugation, results for HDL and chylomicrons compare less favourably (McIntyre and Harry, 1991) and additionally, electrophoretic and densitometric techniques are unsuitable for processing large numbers of samples.

(c) Gel filtration and immuno affinity column chromatography

Gel filtration and immuno affinity column chromatography cause less disruption to lipoprotein particles and are useful when undertaking compositional and structural studies of intact lipoproteins. Both methods are rather time consuming and are only applicable to small scale separation of lipoproteins.

(d) Precipitation procedures

Precipitation techniques are more often used than the other techniques, especially in large scale studies, as the procedures are rapid, inexpensive and more applicable to analysing large numbers of samples. Precipitation may however alter the structure and biological properties of precipitated lipoproteins.

Burstein and Samille (1960) first described precipitation methods for isolating lipoprotein fractions and Cornwell and Kruger (1961) showed that high molecular mass polysaccharides at neutral pH formed complexes with serum LDL. Lower molecular mass polysaccharides, for example heparin and dextran sulphate, will also precipitate lipoproteins but require the presence of a divalent cation such as manganese, magnesium or calcium. Other polyanions such as sodium phosphotungstate (with magnesium chloride) and anionic detergents such as polyethylene glycol have also been used to precipitate lipoproteins.

The exact nature of the interaction of the precipitating reagents with lipoproteins is unknown. For polyanion-divalent cation procedures, the precipitation is probably facilitated by interaction of divalent cations with negatively charged groups, such as phospholipids, on lipoproteins or by interaction of polyanions with positively charged domains of apoB (Kim and Nishida, 1979). Furthermore, the specificity of the precipitation appears to depend on the lipid:protein ratio of the lipoprotein (Burstein et al., 1970). Thus complex formation is more likely, under given conditions, to occur most easily with chylomicrons, than with the other lipoproteins VLDL, LDL or HDL which are progressively more difficult to precipitate from serum. Other factors which influence the specificity of the precipitation

include the precipitants used, ionic strength and reagent concentrations. Thus heparin and manganese chloride will precipitate all apoB-containing lipoproteins but if magnesium replaces manganese as the divalent cation, then chylomicrons and VLDL but not LDL are precipitated from serum (Burstein et al., 1970).

(i) Heparin-manganese chloride

The heparin-manganese chloride method of isolating HDL has been extensively studied (Bachorik et al., 1976; Warnick and Albers, 1978b) and results by this method are in good agreement with ultracentrifugal techniques (Bachorik et al., 1976). Furthermore, the heparin-manganese chloride method has been widely used in epidemiological studies (reviewed by Heiss et al., 1980).

In the original descriptions of the method (Burstein et al., 1970), manganese chloride at a final concentration of 0.046 mol/L was used. This manganese chloride concentration was later shown to be optimal for serum but borderline when plasma, using EDTA as anticoagulant, is used. Chelation of manganese by EDTA, resulting in a free manganese concentration below that which is required for complete precipitation of apoB-containing lipoproteins, causes small amounts of these lipoproteins to remain in the supernatant (Warnick and Albers, 1978b). Using manganese chloride at a final concentration 0.092 mol/L ensures complete precipitation of apoB-containing lipoproteins from EDTA-plasma while causing negligible precipitation of HDL.

(ii) Dextran sulphate-magnesium chloride

The molecular weight of dextran sulphate, a synthetic heparin analogue, determines the specificity of lipoprotein precipitation.

Dextran sulphate (MW 15,000) used in early studies (Burstein et al., 1970), was later shown to incompletely precipitate apoB-containing lipoproteins (Warnick et al., 1982). Conversely, higher molecular weight (MW 500,000) dextran sulphate (Finlay et al., 1978) tends to precipitate HDL as well as apoB-containing lipoproteins, thereby underestimating HDL cholesterol concentration (Warnick et al., 1979a). Magnesium chloride is used in combination with dextran sulphate as magnesium, unlike manganese, does not interfere with enzymic cholesterol methods using phosphate buffers.

(iii) Phosphotungstic acid-magnesium chloride

The concentrations of phosphotungstic acid and magnesium chloride originally recommended (Burstein et al., 1970) were later shown to precipitate some HDL (Warnick et al., 1979a). Others have shown that the pH of the phosphotungstic acid-magnesium chloride reagent is as important as the reagent concentrations as incomplete precipitation of apoB-containing lipoproteins occurs when the phosphotungstate solution pH is greater than 7.6 (Grove et al., 1979). Phosphotungstic acid, adjusted to pH 6.15 using 1 mol/L sodium hydroxide was found to completely precipitate apoB-containing lipoproteins, although others have described methods using reagents at pH ranging from pH 2.5 (Assmann et al., 1983) to pH 7.4 (Kostner et al., 1979). Prolonged storage of phosphotungstic acid may result in formation of isophosphotungstic acid, effectively reducing the phosphotungstic acid concentration. Stability of the reagents can be improved by using phosphotungstic acid at pH 2.5 and concentration 1.06 g/L (Draeger et al., 1982).

(iv) Polyethylene glycol

Polyethylene glycol (PEG) causes large, relatively lipid-rich lipoproteins such as VLDL and LDL to precipitate due to steric exclusion while leaving smaller lipoprotein particles such as HDL in solution. The selectivity of the precipitation reaction is determined by both the final pH of the PEG-serum mixture (Polson et al., 1964) and the final concentration of the PEG solution (Demacker et al., 1980). PEG 6000 at a concentration of 120g/L was originally recommended (Viikari et al., 1976), but this was shown to precipitate HDL as well as apoB-containing lipoproteins (Warnick et al., 1979a). This concentration was subsequently reduced to 100g/L to reduce precipitation of HDL (Allen et al., 1979) and even this lower PEG concentration was shown to precipitate some HDL unless PEG solution adjusted to pH 10 was used (Izzo et al., 1981).

Others, using a final PEG 6000 concentration of 75g/L, have shown that the final pH of the PEG-serum solution depends upon the initial serum pH rather than the initial pH of the PEG solution, aged serum with high pH causing small but significant increases in HDL cholesterol probably due to incomplete precipitation of apoB-containing lipoproteins (Demacker et al., 1980). However adjustment of the pH of the PEG solution is not necessary provided that fresh samples are analysed. PEG 6000 from different sources resulted in the pH of the PEG solution varying from pH 5.1-7.05 although again the final pH of the PEG-serum solution depended on the initial serum pH rather than the source of PEG 6000 (Demacker et al., 1980).

(e) Sedimentation of insoluble aggregates

Incomplete sedimentation of precipitated apoB-containing lipoproteins, resulting in suspension of the aggregates in solution or floatation of the aggregates over a clear infranatant, is a major problem with precipitation procedures. Incomplete sedimentation usually occurs when serum triglyceride concentrations exceed 4.5mmol/L but incomplete sedimentation of the precipitate may occur at lower concentrations and conversely some samples with high triglyceride concentrations yield clear supernatants (Warnick and Albers, 1978b). Sedimentation of the insoluble aggregates depends on the density of the aggregates compared with the density of the supernatant. Suspension of aggregates in solution occurs when the densities of the VLDL/LDL aggregates are approximately the same as or less than that of the supernatant and although usually seen at high triglyceride concentrations, it can occur at any serum triglyceride concentration, probably due to the large variation in hydrated densities of the VLDL particle.

Techniques which can be used to overcome the problem of incomplete sedimentation of insoluble aggregates include pre-treatment of the sample before precipitation and treatment of the supernatant after the precipitation stage. In the pre-treatment technique, triglyceride-rich lipoproteins are removed from serum by ultracentrifugation at density 1.006g/ml and the precipitating reagents added to the infranatant. In the post-precipitation techniques, the turbid supernatant can either be diluted with 0.15mol/L sodium chloride solution to reduce the density of the supernatant and appropriate volumes of precipitating reagents added to give the recommended final reagent concentrations or the turbid

supernatant is cleared by ultrafiltration through a 0.22μm filter. However, incomplete recovery of HDL-containing fractions can occur following ultracentrifugation and ultrafiltration can result in the removal of both HDL and insoluble aggregates if the filter becomes blocked while dilution of turbid supernatants by sodium chloride reduces low concentrations of HDL cholesterol even further. Clearly no technique satisfactorily deals with the problem of incomplete sedimentation of insoluble aggregates.

To ensure that the comparison of the precipitation techniques was not unduly influenced by turbidity produced by incomplete sedimentation of insoluble aggregates, samples with serum triglyceride concentrations >4.5mmol/L were excluded. All supernatants were closely inspected and any turbid supernatants were rejected.

(f) Precipitation of apoE-containing HDL

HDL1 is a minor subfraction of HDL that contains apoE in addition to the other apoproteins normally found in HDL. ApoE associated with apoB-containing lipoproteins is precipitated from serum by heparin-manganese chloride while HDL1 remains in solution but dextran sulphate-magnesium chloride precipitates both apoE associated with apoB-containing lipoproteins and HDL1 (Gibson et al., 1984). Phosphotungstic acid procedures precipitate negligible amounts of HDL1 when radiolabelled ^{125}I-HDL1 is added to normo and hyperlipidaemic sera (Assmann et al., 1983). No reports have appeared concerning the effect of PEG 6000 on HDL1.

(g) Analytical interference with cholesterol assays

The heparin-manganese chloride procedure can cause interference in the analytical phase as manganese precipitates with phosphate present in cholesterol methods using phosphate based reagent buffers and by direct interference with the colour development by manganese chloride (Steele et al., 1976). Both of these effects cause overestimation of HDL cholesterol. Although interference by manganese chloride can be reduced by adding EDTA (Steele et al., 1976) or sodium bicarbonate (Bachorik et al., 1984) to the HDL supernatant to remove excess manganese, interference by manganese chloride in enzymatic cholesterol methods is one of the factors why other precipitating reagents, such as dextran sulphate-magnesium chloride, phosphotungstic acid-magnesium chloride and PEG 6000 which do not interfere with enzymatic cholesterol methods, are being increasingly used.

(h) Comparisons of precipitation procedures

A number of comparisons of precipitation techniques for isolating HDL have been published, some of the more comprehensive comparisons using apolipoprotein measurement to assess precipitation (Warnick et al., 1979a; Demacker et al., 1980; Izzo et al., 1981). Most evaluations show a high degree of intercorrelation between methods and although there is bias between methods, the magnitude and direction of the bias is inconsistent. Furthermore, despite the apparent simplicity of the techniques, interlaboratory surveys have shown poor agreement between laboratories measuring HDL cholesterol (Warnick et al., 1983), with between laboratory coefficients of variation ranging from 8-48%

for different samples (McMillain and Warnick, 1988).

A comparison of HDL isolation procedures, incorporating recent modifications to the procedures, seems justified. The effectiveness of precipitation will be monitored by measuring apoA-I and apoB in the supernatants and in addition the effect of precipitation procedures on LpAI particles will be assessed as no studies have been made so far on the effects of precipitation procedures on these particles. No comparisons were made between precipitation methods and methods using ultracentrifugation as HDL isolated by ultracentrifugation is often contaminated by non-HDL lipoproteins (Albers et al., 1972) and the high ionic strength solutions used to form density gradients alter the composition of HDL (Kunitake and Kane, 1982). The aim of this study is to determine the relative relationships between different precipitation techniques and to determine if any differences between the isolation procedures are due to selective precipitation of LpAI particles.

Previous studies have used correlation coefficients and regression equations even though such statistical methods may be misleading and conceal differences between analytical methods (Bland and Altman, 1986). For example the slope of the line, calculated using least squares regression analysis, indicates the degree of agreement between the two methods but the slope and the correlation coefficient may be affected by the range of values studied. A wide range of values in many cases may produce a better correlation and different slope to that which would be produced by a narrower range of values. Also a high degree of correlation is often taken as evidence of good agreement between methods but it would be very unusual if results from two methods measuring the same analyte were not

related. Difference plots, rather than correlation coefficients and regression equations, will be used to highlight differences between HDL isolation methods.

CHAPTER 2. Methods

2.1 Chemicals, diagnostic kits, materials and equipment

(a) Chemicals and suppliers

Chemical	Supplier
Acrylamide	BDH
Agarose IEF	Pharmacia Ltd
Albumin, fatty acid free	Sigma Chemical Co. Ltd
Ammonium persulphate	BDH
Barbitone buffer	Ciba Corning Diagnostics
Boric acid	BDH
Bromophenol blue	BDH
Chloroform	BDH
Coomassie brilliant blue R-250	BDH
Dextran sulphate	Sochibo
Diethyl ether	BDH
Dithiothreitol	BDH
Ethanol (95%)	BDH
Fat Red 7B	Ciba Corning Diagnostics
Glycerol	BDH
Glycerol tri[9,10(n)-^{3}H]oleate	Amersham International plc
Heptane	BDH
Liquiscint	National Diagnostics
Lysophosphatidylcholine, type I	Sigma Chemical Co. Ltd
Manganese (II) chloride 4-hydrate	BDH
Methanol	BDH
Neuraminidase, Type V	Sigma
[9,10(n)-^{3}H] Oleic acid	Amersham International plc

Chemical	Supplier
Pharmalyte pH 4-6.5	Pharmacia Ltd
Phosphoric acid	BDH
Polyethylene glycol 6000	BDH
Repel-Silane	Pharmacia Ltd
Sodium acetate	BDH
Sodium chloride	BDH
Sodium dodecyl sulphate	BDH
Sodium hydroxide	BDH
Sulphosalicylic acid	BDH
Tetramethylethylene diamine	BDH
Trichloroacetic acid	BDH
Trizma-hydrochloride	Sigma Chemical Co. Ltd
Urea (Aristar)	BDH

(b) Diagnostic kits and suppliers

Diagnostic Kit	Supplier
Apolipoprotein A-I & B	Incstar Ltd.
Cholesterol CHOD-PAP	Boehringer Mannheim Ltd.
Cholesterol, unesterified CHOD-PAP	Boehringer Mannheim Ltd.
HDL cholesterol (PTA/$MgCl_2$)	Sigma Diagnostics
Lp(a), Immunozym	Immuno Ltd
Phospholipids	Boehringer Mannheim Ltd.
Triglyceride GPO-PAP	Boehringer Mannheim Ltd.

(c) Materials and suppliers

Material	Supplier
Agarose gels	Ciba-Corning Diagnostics Ltd.
LpA-I hydragel	Labmedics Ltd.
Multi-level QC sera	Randox Laboratories Ltd.
Precinorm L	Boehringer Mannheim Ltd.
Precilip EL	Boehringer Mannheim Ltd.
Wellcome One	Wellcome Diagnostics
Ultracentrifuge tubes	Beckman Instruments Ltd.

(d) Equipment and suppliers

Equipment	Supplier
ß-counter, LKB 1219	Pharmacia-LKB Ltd
Centrifuge, IEC Centra 7R	Damon/IEC Ltd.
Cobas Mira	Roche Products Ltd.
Electrophoresis unit, vertical	Pharmacia Ltd
Electrophoresis unit, horizontal	Labmedics Ltd.
Microplate reader, MCC/340	ICN Flow
Microplate shaker, Titertek	ICN Flow
Microplate washer, Titertek	ICN Flow
Pipettes, Gilson	Anachem Ltd.
Pipettes, pasteur	Alpha Labs
Power pack, Multidrive XL	Pharmacia Ltd.
Spectrophotometer	Perkin-Elmer Ltd.
Ultracentrifuge L8-60M	Beckman Instruments Ltd.
Ultracentrifuge rotor SW 41Ti	Beckman Instruments Ltd.
Ultrasonic disintegrator, Soniprep	Fisons Scientific Equipment

(e) Names and addresses of suppliers

Name of Supplier	Address
Anachem Ltd.	Luton, Beds
Alpha Labs	Eastleigh, Hampshire
Amersham International plc	Aylesbury, Bucks
BDH / R & L Slaughter	Upminster, Essex
Beckman Instruments Ltd.	High Wycombe, Bucks
Boehringer Mannheim Ltd.	Lewes, East Sussex
Ciba Corning Diagnostics Ltd.	Halstead, Essex
Damon/IEC Ltd.	Dunstable, Bedfordshire
Fisons Scientific Equipment	Loughborough, Leics
ICN Flow	High Wycombe, Bucks
Incstar Ltd.	Wokingham, Berkshire
Immuno Ltd.	Sevenoaks, Kent
Labmedics Ltd.	Stockport, Cheshire
National Diagnostics	Aylesbury, Bucks
Pharmacia	Milton Keynes, Bucks
Perkin-Elmer Ltd.	Beaconsfield, Bucks
Randox Laboratories Ltd.	Crumlin, Co. Antrim
Roche Products Ltd.	Welwyn Garden City, Herts
Sigma Chemical / Diagnostics Co.	Poole, Dorset
Sochibo	Boulonge, France
Wellcome Diagnostics	Dartford, Kent

All suppliers based in UK unless stated otherwise

2.2 Subjects and sample collection

(a) Comparison of precipitation procedures used to isolate HDL

Blood samples were collected into plain tubes from 30 fasting normolipaemic subjects (mean cholesterol 5.2mmol/L, range 4.0-5.8mmol/L; median triglyceride 0.9mmol/L, range 0.5-1.6 mmol/L) and from 10 dyslipoproteinaemic subjects (mean cholesterol 8.4mmol/L, range 6.2-9.8mmol/L; median triglyceride 1.9mmol/L, range 0.5-3.8mmol/L). The subjects included some individuals with high Lp(a) concentrations (median Lp(a) 17mg/dl range <5-58mg/dl).

Subjects with triglyceride concentrations >4.5mmol/L were excluded as incomplete sedimentation of the polyanion-divalent cation-lipoprotein complex usually occurs at these triglyceride concentrations (Warnick et al., 1978b). Serum was removed after centrifugation, stored at 4°C and analysed the same day to ensure maximal precipitation of apoB-containing lipoproteins while minimising precipitation of HDL (Bachorik et al., 1980).

(b) Dyslipoproteinaemia in UK families with severe expression of coronary heart disease

Probands with a personal and family background of premature CHD were recruited over a two year period, mainly through cardiological units in Southern England. Other family members were then followed up in the UK and abroad. The minimum criteria for inclusion were that probands must have had a myocardial infraction or documented angina below age 55 years and have either two living affected family members in two generations, or a minimum of one living affected plus two living unaffected members in families for which only one

generation was accessible. The referring units regarded the probands as having no perceived risk associations and thus welcomed an interest in the "unexplained" premature CHD.

Twenty-six families comprising 20 male and 6 female probands were recruited with blood samples and clinical data obtained from 276 adults from the 366 adult family members aged 15 years and above. Failure to recruit all family members was mainly due to the unwillingness of the very young or very elderly to participate in the study and due to some family members living abroad. Only one (large) single generation family was studied, with data being mostly collected from two generation families (21/26 families studied), three generation (3/26) or four generation families (1/26). Probands with known familial hypercholesterolaemia, type III hyperlipoproteinaemia, diabetes, thyroid, renal or hepatic disorders were excluded from the register.

Participants completed a questionnaire covering general health, known risk associations such as tobacco habits and alcohol consumption and any previous dietary advice. A full physical examination and review of lipid stigmata was performed by a cardiology nursing sister. Resting blood pressure, electro-cardiogram and body mass index were recorded. Venous blood was collected, either after an overnight fast or pre-prandially, into tubes containing EDTA as anticoagulant and plasma separated after centrifugation. Samples for cholesterol, triglycerides and HDL cholesterol were stored at 4°C and analysed within two days. Samples for apoE phenotyping were ultracentrifuged and the floated lipoproteins delipidated. The protein pellet was stored under nitrogen at -70°C and phenotyped within one month.

(c) Lipoproteins and apoA-I only containing particles in early onset coronary heart disease

Men (age range 41-55 years) with early onset CHD were selected from the coronary heart disease register described above. Sixteen male probands and ten male affected male family members who had a coronary artery bypass graft (CABG) below age 55 years were selected for inclusion in this study. Four men had previously had a myocardial infarction prior to CABG and all men had coronary angiography prior to CABG. Myocardial infarction was diagnosed using electrocardiographic criteria and characteristic serum cardiac enzyme changes. Patients with known familial hypercholesterolaemia, type III hyperlipoproteinaemia, diabetes, thyroid, renal or hepatic disorders or who were taking anti-hypertensive or lipid lowering drugs were excluded from the study. Venous blood samples were taken at least six months after CABG to exclude any temporary effects of the surgery on lipoprotein profiles. Fasting venous blood samples from both subjects and controls for cholesterol, triglycerides and HDL cholesterol were stored at 4°C and analysed within two days. Samples for apolipoproteins, Lp(a) and LpA-I particles were stored at -70°C for up to six months and defrosted at 37°C immediately before analysis. Controls were recruited from similar social groups and matched with the CHD group for age and body mass index. All controls underwent medical screening before acceptance into the study. Controls with clinical CHD were excluded: other exclusion criteria were as for the CHD group. Details of dietary intake and alcohol consumption were recorded for both groups. Any patients or controls whose declared alcohol consumption exceeded 20 units of alcohol per week were excluded from the study.

(d) The effects of moderate exercise on serum lipids and apolipoproteins in previously sedentary middle-aged men.

Men (age range 41-61 years), who did not participate in regular exercise, nor who were employed in a strenuous job, were recruited and underwent medical screening before acceptance into the study. Individuals with total serum cholesterol concentrations greater than 6.5mmol/L and those who smoked or consumed in excess of 20 units of alcohol per week were excluded from the study. Additionally, any individuals with clinical CHD, resting arterial blood pressure >160/95mm Hg, diabetes, thyroid, renal or hepatic disorders or who were taking anti-hypertensive or lipid lowering drugs were excluded from the study. Men were randomly allocated into control or exercise groups, with slightly more men in the exercise group to compensate for any dropouts during the twelve months. All participants subsequently completed the twelve month study, although one individual in the exercise group was excluded due to poor adherence to the exercise program. All men were asked to keep their dietary and other living habits, with the exception of following the set exercise program, as constant as possible during the twelve months of the study.

All participants were asked to complete a questionnaire concerning exercise habits over the four weeks prior to the start of the study. Walkers were initially supervised during a timed 1.6km walk around an athletics track and were then asked to start with three 20 minute brisk walks per week, gradually increasing the amount of brisk walking until at three months they were walking two miles per day in approximately thirty minutes and at six months, three miles per day in approximately forty-five minutes. Exercise diaries detailing

time taken and distance walked were maintained by the participants and collected at monthly intervals. Diaries were studied and all participants contacted regularly to offer encouragement and advice. Changes in endurance fitness were assessed by Drs Hardman and Stencil at Loughborough University using an incremental uphill walking test on a motorised treadmill. Each subject walked at a constant speed (either 1.34 or 1.56 m/s) for 4 minutes up each of 4 increasingly steep gradients. The treadmill speed and grades were selected to elicit approximately 50, 60, 70 and 80% of each man's predicted maximal oxygen uptake. Fingerprick samples of capillary blood were taken before exercise and at the end of each 4 minute test stage. These samples were immediately deproteinised and stored at -70°C prior to lactate analysis. Body weight (and density by hydrostatic weighing) was recorded and blood samples were taken at 0, 3, 6, 9, 12 months. Serum for cholesterol, triglyceride, HDL cholesterol, apolipoproteins, Lp(a) and LpAI particles was stored at -70°C for up to twelve months and defrosted at 37°C immediately before analysis. Dietary intakes were assessed by Drs Hardman and Stencil from 7-day weighed food inventories (Bingham et al., 1987).

(e) Effects of lipoprotein lipase and hepatic lipase on HDL composition and metabolism

To determine the reference ranges for LPL and HL, normolipaemic adults (11 males and 12 females) were recruited from laboratory staff. Individuals (13 males and 13 females) with low HDL cholesterol (serum HDL cholesterol concentration <5th percentile) and those (12 males and 13 females) with high HDL cholesterol (serum HDL cholesterol concentration >50th percentile) were recruited from normal volunteers

and through lipid clinics. Individuals on lipid lowering medication or with familial hypertriglyceridaemia, diabetes, renal disease and those with declared alcohol intakes which exceeded 20 units of alcohol per week were excluded from the study. Following an overnight fast, blood samples where taken into tubes containing EDTA from controls and subjects before and 5, 10 20 and 30 minutes after an intravenous injection of heparin (100U/kg body weight). Samples were kept on ice and plasma separated within 10 minutes of sample collection. Plasma was stored at -70°C before measurement of lipolytic activity, apolipoproteins and LpAI concentrations. Samples for cholesterol, triglyceride and HDL cholesterol were stored at 4°C and analysed within 2 days of collection. ApoE phenotypes were performed using delipidated lipoproteins separated by ultracentrifugation. The protein pellet was stored under nitrogen at -70°C and phenotyped within one month.

2.3 Analytical methods

Assays for total cholesterol, HDL cholesterol, triglycerides, Lp(a), apoA-I, apoB and LpAI was monitored using commercial quality control sera, human serum frozen at -70°C and by participation in the appropriate quality assessment schemes when available.

(a) Total cholesterol assay

Cholesterol was measured using the cholesterol oxidase-peroxidase-4-aminophenazone method (CHOD-PAP) in which cholesterol esters are hydrolysed by the enzyme cholesterol esterase, then free cholesterol is oxidised and the hydrogen peroxide generated is monitored using a chromogenic oxygen acceptor. Reagents were supplied in a

commercial kit by Boehringer Mannheim UK and the analyses performed on a Cobas Mira. Quality control sera with Center for Disease Control (CDC) assigned values were regularly assayed, in addition to monitoring the performance by standard internal and external quality control procedures. Between batch imprecision, expressed as percentage coefficient of variation (CV%), was 2.8, 2.4 and 2.0 at concentrations of 3.4, 6.8 and 10.3mmol/L respectively.

Cholesterol ester + H_2O $\xrightarrow{\text{cholesterol esterase}}$ cholesterol + RCOOH

Cholesterol + O_2 $\xrightarrow{\text{cholesterol oxidase}}$ Δ^4-cholestenone + H_2O_2

H_2O_2 + 4-aminophenazone + phenol $\xrightarrow{\text{peroxidase}}$ 4-(p-benzoquinone-monoimino)-phenazone + $4H_2O$

Formation of the coloured product, 4-(p-benzoquinone-monoimino)-phenazone is monitored at 500 nm against a reagent blank.

(b) Free cholesterol assay

Free cholesterol was measured in the HDL supernatants using the same assay as that used for total cholesterol except that in this assay, cholesterol esterase is omitted so that cholesterol esters are not hydrolysed and only free cholesterol is oxidised by cholesterol oxidase. Between batch imprecision, expressed as percentage coefficient of variation (CV%), was 4.8 at mean concentration of 0.25mmol/L.

Cholesterol + O_2 $\xrightarrow{\text{cholesterol oxidase}}$ Δ^4-cholestenone + H_2O_2

H_2O_2 + 4-aminophenazone + phenol $\xrightarrow{\text{peroxidase}}$ 4-(p-benzoquinone-monoimino)-phenazone + $4H_2O$

Formation of the coloured product, 4-(p-benzoquinone-monoimino)-phenazone is monitored at 500nm against a reagent blank.

(c) Calculation of LDL cholesterol

LDL cholesterol was calculated using the Friedewald formula (Friedewald et al., 1972).

$$\text{LDL-cholesterol} = TC - HDL\text{-}C - \frac{TTG}{2.19}$$

where: TC: Total cholesterol (mmol/L)

HDL-C: HDL cholesterol (mmol/L)

TTG: Total triglycerides (mmol/L)

LDL cholesterol was not calculated for those with type III hyperlipoproteinaemia, for those with serum triglyceride concentrations greater than 4.5mmol/L or for those samples taken from non-fasting subjects.

(d) HDL cholesterol isolation procedures

The five procedures were used to isolate HDL. The initial and final reaction concentrations (table 1) and the temperature and time of the incubation and centrifugation stages (table 2) varied for the different lipoprotein precipitation methods. After precipitation and centrifugation, clear supernatants were recovered, further reagents added to remove interfering substances where indicated and analysed. Appropriate correction factors, for dilution of the samples by the precipitating reagents, were applied as necessary. HDL cholesterol was measured in the supernatants using the total cholesterol method previously described but using a sample volume of 15µl instead of 5µl to improve precision and linearity at HDL cholesterol concentrations below 1mmol/L.

Table 1. Initial and final concentrations of precipitants used for HDL isolation

Method	Initial conc.	Final conc.
Heparin (U/ml) Manganese chloride 1 (mol/L)	5000 0.5	175 0.044
Heparin (U/ml) Manganese chloride 2 (mol/L)	5000 1	175 0.088
Dextran sulphate (g/L) Magnesium chloride (mol/L)	10 0.5	1.67 0.08
Phosphotungstic acid (mol/L) Magnesium chloride (mol/L)	0.03 0.10	0.005 0.017
PEG 6000 (g/L)	450	75

Table 2. Incubation and centrifugation conditions for HDL isolation procedures (Temp = temperature; RCF = relative centrifugal force; see text for method abbreviations)

	Incubation		Centrifugation		
Method	Time (min)	Temp. (°C)	Time (min)	Temp. (°C)	RCF (g)
HM1	30	4	30	4	2000
HM2	30	4	30	4	2000
DS	30	Ambient	30	Ambient	2000
PTA	5	Ambient	30	Ambient	2000
PEG	15	Ambient	30	Ambient	2000

(i) Heparin-manganese chloride 1 (HM1)

The procedure is essentially that of Burstein and Samille (1960) with some minor modifications. To 1ml of serum, 0.04mL sodium heparin solution (5000 U/ml) was added and vortex mixed. Then 0.1ml of 0.5mol/L manganese chloride was added and vortex mixed again. Samples were incubated, centrifuged (table 2) and clear supernatants recovered. To remove excess manganese, 0.02ml of 0.4mmol/L EDTA solution was added to 0.5ml supernatant. This HDL isolation procedure was routinely used for all subsequent investigations using serum unless otherwise stated. Between batch imprecision, expressed as CV%, was 2.2 at mean concentration of 1.3mmol/L.

(ii) Heparin-manganese chloride 2 (HM2)

The procedure was carried out as detailed for the heparin manganese method 1 but with an initial manganese chloride concentration of 1mol/L. This procedure was routinely used for all subsequent investigations using plasma unless otherwise stated. Between batch imprecision (CV%) was 2.4 at mean concentration of 1.1mmol/L.

(iii) Dextran sulphate-magnesium chloride (DS)

The procedure, proposed as a reference procedure, was used (Warnick et al., 1982). A stock solution (20g/L) of dextran sulphate (MW 50,000), pH 7.0, was prepared and mixed in equal volumes with magnesium chloride solution (1mol/L) to produce the working solution. To 1ml of serum, 0.2ml dextran sulphate-magnesium chloride solution was added and vortex mixed. Samples were incubated, centrifuged (table 2) and clear supernatants recovered. CV% was 2.7 at mean concentration of 1.2mmol/L.

(iv) Phosphotungstic acid-magnesium chloride (PTA)

A pre-mixed working solution containing phosphotungstic acid (0.03mol/L) and magnesium chloride (0.1mol/L) was used. To 1ml of serum, 0.2ml of phosphotungstic acid-magnesium chloride reagent was added and the tubes vortex mixed. Samples were incubated, centrifuged (table 2) and clear supernatants recovered. CV% was 1.2 at mean concentration of 1.2mmol/L.

(v) Polyethylene glycol 6000 (PEG 6000)

The method described by Demacker (1980a) was used. To 1ml of serum, 0.2ml of PEG solution (450g/L) was added and the tubes vortex mixed. Samples were incubated, centrifuged (table 2) and clear supernatants recovered. CV% was 2.6 at mean concentration of 1.2mmol/L.

(e) Phospholipid assay

Phospholipids were measured in the HDL supernatants using coupled reactions in which choline and phosphatidic acids are produced by hydrolysis of phospholipid using phospholipase. Choline is oxidised by choline oxidase and the hydrogen peroxide generated is monitored using a chromogenic oxygen acceptor. Reagents were supplied in a commercial kit by Boehringer Mannheim UK. CV% was 2.1 at mean concentration of 1.5mmol/L.

$$\text{Phospholipids} + H_2O \xrightarrow{\text{phospholipase D}} \text{choline} + \text{phosphatidic acids}$$

$$\text{Choline} + 2O_2 + H_2O \xrightarrow{\text{choline oxidase}} \text{betaine} + 2H_2O_2$$

$$2H_2O_2 + \text{4-aminophenazone} + \text{phenol} \xrightarrow{\text{peroxidase}} \text{4-(p-benzoquinone monoimino)-phenazone} + 4H_2O$$

(f) Triglyceride assay

Triglycerides were measured using the glycerophosphate oxidase-peroxidase-4-aminophenol method in which glycerol-3-phosphate, produced from triglycerides by two coupled enzymic reactions, is oxidised and the hydrogen peroxide generated is monitored using a chromogenic oxygen acceptor. Reagents were supplied in a commercial kit by Boehringer Mannheim UK and the analyses performed on a Cobas Mira. Between batch imprecision (CV%) was 3.9, 3.7 and 2.4 at mean concentrations of 0.58, 1.3 and 3.9mmol/L respectively.

$$\text{Triglycerides} + 3H_2O \xrightarrow{\text{esterase}} \text{glycerol} + 3\text{RCOOH}$$

$$\text{Glycerol} + \text{ATP} \xrightarrow{\text{glycerol kinase}} \text{glycerol-3-phosphate} + \text{ADP}$$

$$\text{Glycerol-3-phosphate} + O_2 \xrightarrow{\text{GPO}} \text{dihydroxyacetone-phosphate} + H_2O_2$$

$$H_2O_2 + \text{4-aminophenol} + \text{4-CP} \xrightarrow{\text{peroxidase}} \text{4-(p-benzoquinone-monoimino)-phenazone} + 2H_2O + HCl$$

Where: GPO: glycerophosphate oxidase

4-CP: 4-chlorophenol

Formation of the coloured product, 4-(p-benzoquinone-monoimino)-phenazone is monitored at 500nm against a reagent blank.

(g) ApoA-I, apoA-II and apoB assay

ApoA-I, apoA-II and apoB were measured by immunoturbidimetry using specific antisera supplied by Incstar Limited. Samples, calibrators and quality control sera were diluted in 0.15mol/L sodium chloride solution (1:20 v/v) and mixed with specific antisera diluted in 4% PEG 6000 (1:20 v/v). Turbidity produced by the formation of insoluble antigen-antibody complexes was monitored at 340nm.

Between batch imprecision for apoA-I (CV%) was 2.2 and 2.4 at mean concentrations of 1.3 and 2.1g/L respectively, for apoA-II was 2.8 and 2.5 at mean concentrations of 0.35 and 0.45g/L respectively and for apoB was 1.8 and 1.4 at mean concentrations of 0.77 and 1.3g/L respectively.

(h) LpAI assay

LpAI particles were measured by electroimmunoassay using pre-prepared agarose plates supplied by Labmedics Limited. Samples were diluted 1:50 (v/v) in 0.15mol/L sodium chloride solution and applied to the agarose plate. Electrophoresis continued for 4 hour at 50V after which the plates were pressed, washed and then stained using coomassie blue. Antibody to apoA-II was incorporated in excess into the gel and retarded those particles containing apoA-II while those particles containing apoA-I but not apoA-II migrated further and reacted with antibody to apoAI thus forming the rockets. The height of the rocket was measured and the concentration calculated using a standard curve produced by running calibrators supplied by Labmedics Limited. Between assay imprecision, expressed as CV%, was 6.1 and 5.5 at mean LpAI concentrations of 0.35 and 0.70g/L respectively.

(i) Lp(a) assay

Lp(a) was measured by an enzyme linked immuno-sorbent assay supplied by Immuno Limited. Samples, diluted 1:500 (v/v) in the diluent provided, were pipetted into the wells of a microtitre plate coated with a polyclonal antibody against apo(a) and simultaneously incubated with a second monovalent anti-apo(a) antibody conjugated

to peroxidase. After incubation, diluted serum and unbound antibody-enzyme conjugate were removed by washing and the substrate for peroxidase, tetra-methyl-benzidine, added to the wells. The reaction was stopped by addition of sulphuric acid and the absorbance measured at 450nm. Between assay imprecision, expressed as CV% was 6.8 and 6.5 at mean Lp(a) concentrations of 20 and 45mg/dl respectively.

(j) ApoE phenotyping

ApoE phenotypes were determined by isoelectric focusing of delipidated VLDL in polyacrylamide rods (Warnick *et al.*, 1979b) with some modifications (Bouthillier et al., 1983). The major isoforms E-2, E-3 and E-4 focus at pH 5.9, 6.0 and 6.1 respectively.

(i) Isolation of VLDL

VLDL was isolated using ultracentrifugation by layering 5ml of plasma under 8ml of 0.15mol/L sodium chloride (density=1.006g/ml) in a cellulose nitrate ultracentrifugation tube. Tubes were loaded into a SW41 swing out rotor and ultracentrifuged at 10°C for 20 hours at 105,000g. Lipoproteins were recovered by aspiration in approximately 2ml of saline and 10µl reserved for lipoprotein electrophoresis. The remainder was transferred to a cellulose nitrate tube, topped up with 0.15mol/L sodium chloride and ultracentrifuged again using the same conditions as before. Lipoproteins were recovered from the top of the tube, stored at -20°C for up to seven days and then delipidated.

(ii) Electrophoresis of VLDL

Plasma, VLDL isolated from the plasma and a reference plasma were electrophoresed in agarose gels using 0.05mol/L barbitone buffer, pH 8.6. Samples were electrophoresed at room temperature for 35 minutes at a constant voltage of 90V. After electrophoresis the gel was dried using hot air, then stained using 0.225g/L Fat Red 7B dissolved in methanol and destained using methylated spirit and water (1:1 v/v). The electrophoretic pattern of the plasma was examined for a broad ß pattern and mobility of isolated VLDL was examined for the presence of ß-migrating particles.

(iii) Delipidation of VLDL

VLDL triglycerides were measured using the triglyceride method previously described. An aliquot containing 0.9mg of triglyceride (equivalent to approximately 150µg of protein) was mixed with an equal volume of neuraminidase solution (5kU/L in 0.1mol/L acetate buffer) and incubated for 2 hours at 37°C. Lipoproteins were then delipidated by slowly adding 3ml of ice-cold methanol in a 12ml centrifuge tube. Diethyl ether was cooled to 0°C and 7ml added while vortexing the tube. Tubes were kept in an ice bath for 20 minutes and then centrifuged at 0°C for 20 minutes at 2000g after which the solvent was aspirated and the pellet re-suspended in 10ml of ice cold diethyl ether. The tubes were placed in an ice bath for 15 minutes and re-centrifuged using the same conditions as before. Solvent was aspirated and the protein pellet dried under nitrogen. The protein was solubilised by adding 0.2ml 10mmol/L tris buffer pH 8.6 containing 8mol/L urea and 10mmol/L dithiothreitol and left to dissolve at 4°C for 1 hour.

(iv) Isoelectric focusing

A stock gel reagent containing 500g/L acrylamide and 13g/L N,N' methylenebisacrylamide was prepared and 4.8g of ultrapure urea dissolved in 3ml of distilled water were added to 1.5ml of this stock solution, followed by 0.5ml of Ampholyte pH 4-6.5. The volume was adjusted to 10ml with distilled water, giving final concentrations of 75g/L and 1.95g/L for acrylamide and methylenebisacrylamide respectively. Ammonium persulphate solution (100g/L) was prepared just before use and 40µl added to the gel mixture, followed by 10µl of tetramethylene diamine. The resulting solution was de-aerated under vacuum, pipetted into Repel-Silane coated glass tubes (12.5cm long and 0.5cm internal diameter) leaving a 2cm gap at the top of the rods and left to polymerise for 1 hour.

Gels were prefocused at a constant voltage of 110V for 1 hour at 4°C using 10mmol/L phosphoric acid in the anode chamber and 20mmol/L sodium hydroxide in the upper, cathode chamber. Overlay solution was prepared by diluting tris buffer-urea-dithiothrietol sample diluent with distilled water (1:1 v/v). Fresh electrolyte solutions were added to the electrophoresis tank and 0.1ml of overlay solution was pipetted onto the top of each rod, followed by the samples which were carefully layered under the overlay solution. The proteins were focused at a constant voltage of 250V for 16 hours at 4°C. Samples of known apoE phenotype were included to aid interpretation.

(v) Fixing and staining of gels

Gels were fixed using 115g/L trichloroacetic acid solution containing 35g/L sulphosalicylic acid after which proteins were stained using 0.9g/L coomassie blue dissolved in the destain solution consisting of

methylated spirits, glacial acetic acid and distilled water (3.1:1:8.4 v/v/v). After staining the bands, gels were destained until the background was clear and then preserved using glacial acetic acid, glycerol and water (1:2:17 v/v/v).

(k) Lipoprotein lipase and hepatic lipase assays

Post heparin LPL and HL activities were measured using the method of Nilsson-Ehle and Ekman (1977) with some modifications (lower incubation temperature and sodium dodecyl sulphate used to inhibit HL). Labelled oleic acid, hydrolysed from glycerol tri{9,10(n)-^{3}H}oleate by LPL and HL, was counted after extraction using the method of Belfrage and Vaughn (1969). Activities were measured at 28°C rather than 37°C as LPL may be partially de-activated at 37°C and zero order kinetics are observed for a much longer time at 28°C rather than at 37°C (Greten et al., 1968). LPL activity was selectively measured using sodium dodecyl sulphate (SDS) to inhibit HL and HL activity was measured using 1mol/L sodium chloride to inhibit LPL and a substrate that did not include the activator of LPL, apoC-II (Baginsky and Brown, 1979).

(i) Preparation of LPL substrate

The substrate was prepared by evaporating 15µl of glycerol tri{9,10(n)-^{3}H}oleate in toluene (equivalent to 75µCi) to dryness under nitrogen in a glass vial and adding 33mg of unlabelled glycerol trioleate to give a specific activity of 2.0µCi/µmol, followed by 0.168mg of lysophosphatidyl choline. Twelve ml of 0.2mol/L tris buffer, pH 8.2, containing 0.15mol/L sodium chloride was added to the vial which was then vortexed mixed. The mixture, kept in an ice

bath, was sonicated for a total of 6 minutes by repeatedly sonicating for 30 seconds followed by a rest period of 30 seconds. Following sonication, 1.0ml of heat-inactivated serum (cholesterol 4.8mmol/L and triglyceride 1.0mmol/L) and 2.0mL of 0.2mol/L tris buffer, pH 8.2, containing 105mg of fatty acid free bovine serum albumin and 0.15mol/L sodium chloride, was added producing a total albumin concentration of approximately 10mg/ml, sodium chloride concentration of 0.14mmol/L and triolein concentration of 2.5 µmol/ml.

(ii) Inhibition of hepatic lipase

HL was inhibited by adding 50µl of post heparin plasma to 50µl of 70mmol/L SDS dissolved in tris buffer pH 8.2 and incubating in a shaking water bath for 30 minutes at 28°C.

(iii) Assay of lipoprotein lipase activity

After preparation the substrate was incubated at 37°C for 60 minutes and then kept at 4°C until use when 0.5ml of substrate was pipetted into glass tubes for pre-incubation at 28°C for 10 minutes. The reaction was started by adding 10µl of SDS-treated post heparin plasma to the substrate. At 60 minutes, the reaction was terminated by pipetting 3.25ml of extraction mixture containing methanol-chloroform-heptane (1.41:1.25:1 v/v/v) into the tube. After vortexing, 1.05ml of 0.1mol/L potassium carbonate-borate buffer, pH 10.5, was added and the mixture vortexed for 30 seconds. Phases were separated by centrifugation at 3000g for 30 minutes. Labelled oleic acid was measured by mixing 1ml of the upper methanol-water phase with 6ml of scintillant and counting on a liquid scintillation counter with automatic quench correction. All incubations were performed in

duplicate. The efficiency of the extraction procedure was measured by extracting [9,10 ^{3}H]-oleic acid from 0.5ml of substrate mixture containing all substrate components but with labelled oleic acid instead of labelled triolein. Extraction efficiency was typically approximately 76%. Blanks were run for each assay by adding the extraction mixture immediately after addition of serum to the substrate.

$$\text{Enzyme activity} = \frac{C \times F}{E \times S \times T \times P \times V} \quad \mu\text{mol/ml/hr}$$

where

C	radioactivity per sample	(cpm)
F	volume of water-methanol phase	(ml)
E	extraction efficiency	
S	specific activity	(cpm/µmol triolein)
T	incubation time	(hr)
P	volume of plasma used	(ml)
V	volume of water-methanol phase counted	(ml)

(iv) Preparation of hepatic lipase substrate

The substrate was prepared by evaporating 15µl of glycerol tri{9,10(n)-^{3}H}oleate in toluene (equivalent to 75µCi) to dryness under nitrogen in a glass vial and adding 33mg of unlabelled glycerol trioleate to give a specific activity of 2.0µCi/µmol, followed by 0.168mg of lysophosphatidyl choline. Fifteen ml of 0.2mol/L tris buffer, pH 8.6, containing 1mol/L sodium chloride and 150mg fatty acid free bovine serum albumin was added to the vial which was then vortexed mixed. The mixture was sonicated as for the LPL substrate preparation. Final reaction concentrations were 10mg/ml albumin and 1mol/L sodium chloride.

(v) Assay of hepatic lipase activity

The substrate was kept at 4°C until use when 0.5ml of substrate was pipetted into glass tubes for pre-incubation at 28°C for 10 minutes. The reaction was started by adding 10µL of post heparin plasma, diluted 1:1 v/v with 0.15mol/L sodium chloride, to the substrate. The reaction was terminated after 60 minutes by extracting and counting labelled fatty acids as described for the LPL assay.

(vi) Validation of the assays

Post heparin plasma with previously determined LPL and HL activities were included with each batch to monitor the performance of the assays. The between batch imprecision, expressed as CV%, for mean LPL activities of 1.5 and 3.8 µmol/ml/hr was 14.3 and 11.0 respectively and for mean HL activities of 11.8 and 17.5 µmol/ml/hr was 12.8 and 10.2 respectively. To ensure that full inhibition of the enzyme was occurring when using the appropriate inhibitor, a "zero activity tube" using 1ml of HL substrate, which cannot be hydrolysed by LPL, and 20µl of SDS-treated post-heparin plasma, which inhibits HL, was assayed for each of the patient's samples. Under these conditions, residual lipase activity was less than 0.3µmol/ml/hr.

The activity remaining after inhibition of HL by SDS was assessed by incubating SDS-treated post heparin plasma with various substrates with increasing volumes of heat-inactivated serum added. The albumin concentration was adjusted as the volume of heat-inactivated serum increased to maintain approximately the same concentration of albumin throughout. Linearity of some of the assays was checked by incubating larger volumes of post heparin plasma and substrate and removing 0.5ml of reaction mixture at 15, 30, 60 and 75 minutes.

2.4 Statistical analysis

Results from the studies of HDL isolation techniques were analysed using difference plots (Bland and Altman, 1986). Statistical analyses for other investigations were performed using Minitab version 6 after exploratory data analysis according to the Minitab handbook. Variables which did not conform to a Gaussian distribution were normalised by logarithmic transformation. The following statistical methods were used:-

(i) Paired T test

Tables 3, 4.

(ii) Unpaired T test

Tables 19, 20, 24-30, 33. Figures 29, 30.

(iii) One way analysis of variance

Tables 3, 4, 25-30. Figures 29, 30.

(iv) Spearman correlation coefficient

Tables 21, 22, 34.

(v) Discriminant function analysis

Table 23.

CHAPTER 3: Results

3.1 Comparison of precipitation procedures used to isolate HDL

(a) HDL composition by different isolation procedures

The mean value, interquartile range and range of values for cholesterol, apoA-I and LpAI in the supernatants after removal of apoB-containing lipoproteins by the different precipitation methods are plotted in figures 1, 2 and 3 respectively. The composition of HDL isolated by the different precipitation methods showed that there were no significant differences in total cholesterol and triglyceride concentrations between the precipitation methods but that free cholesterol and phospholipid concentrations were significantly lower for heparin-manganese chloride method 2 compared with the other methods (table 3, mean ± standard deviation, s).

Table 3. Lipid composition (mean ± s, except § median and range) of HDL isolated by different precipitation methods (‡ $p<0.001$)

	Total Cholesterol (mmol/L)	Free Cholesterol (mmol/L)	Triglyceride § (mmol/L)	Phospholipid (mmol/L)
HM1	1.5 ± 0.4	0.3 ± 0.1	0.3(0.1-0.5)	1.5 ± 0.2
HM2	1.4 ± 0.3	0.1 ± 0.1‡	0.2(0.1-0.4)	1.3 ± 0.2‡
DS	1.5 ± 0.3	0.3 ± 0.1	0.3(0.2-0.5)	1.5 ± 0.3
PEG	1.4 ± 0.3	0.3 ± 0.1	0.3(0.1-0.4)	1.5 ± 0.2
PTA	1.4 ± 0.3	0.3 ± 0.1	0.3(0.1-0.5)	1.5 ± 0.2

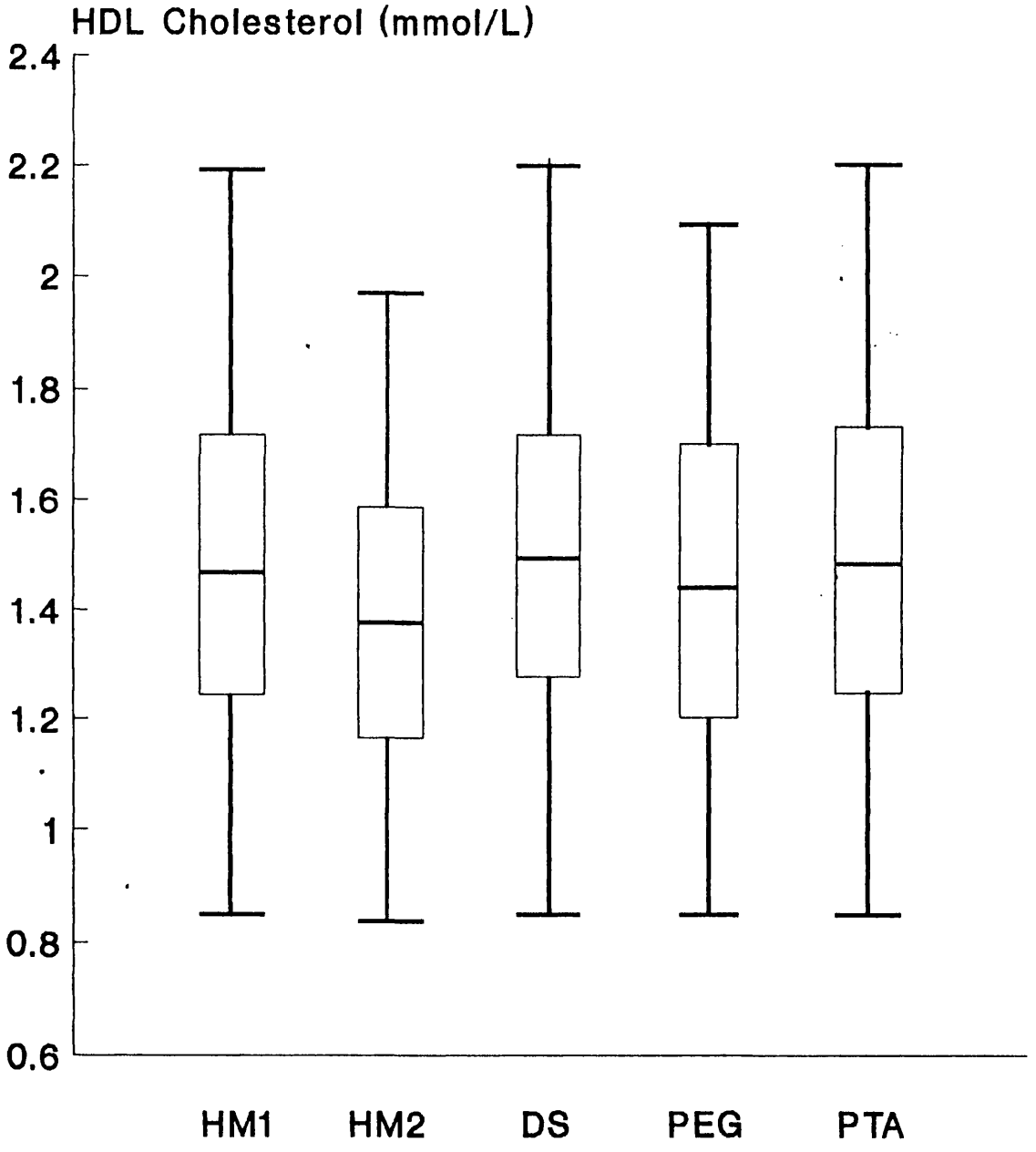


Figure 1. Plot of HDL cholesterol concentrations by five precipitation procedures showing median, interquartile range and range of values

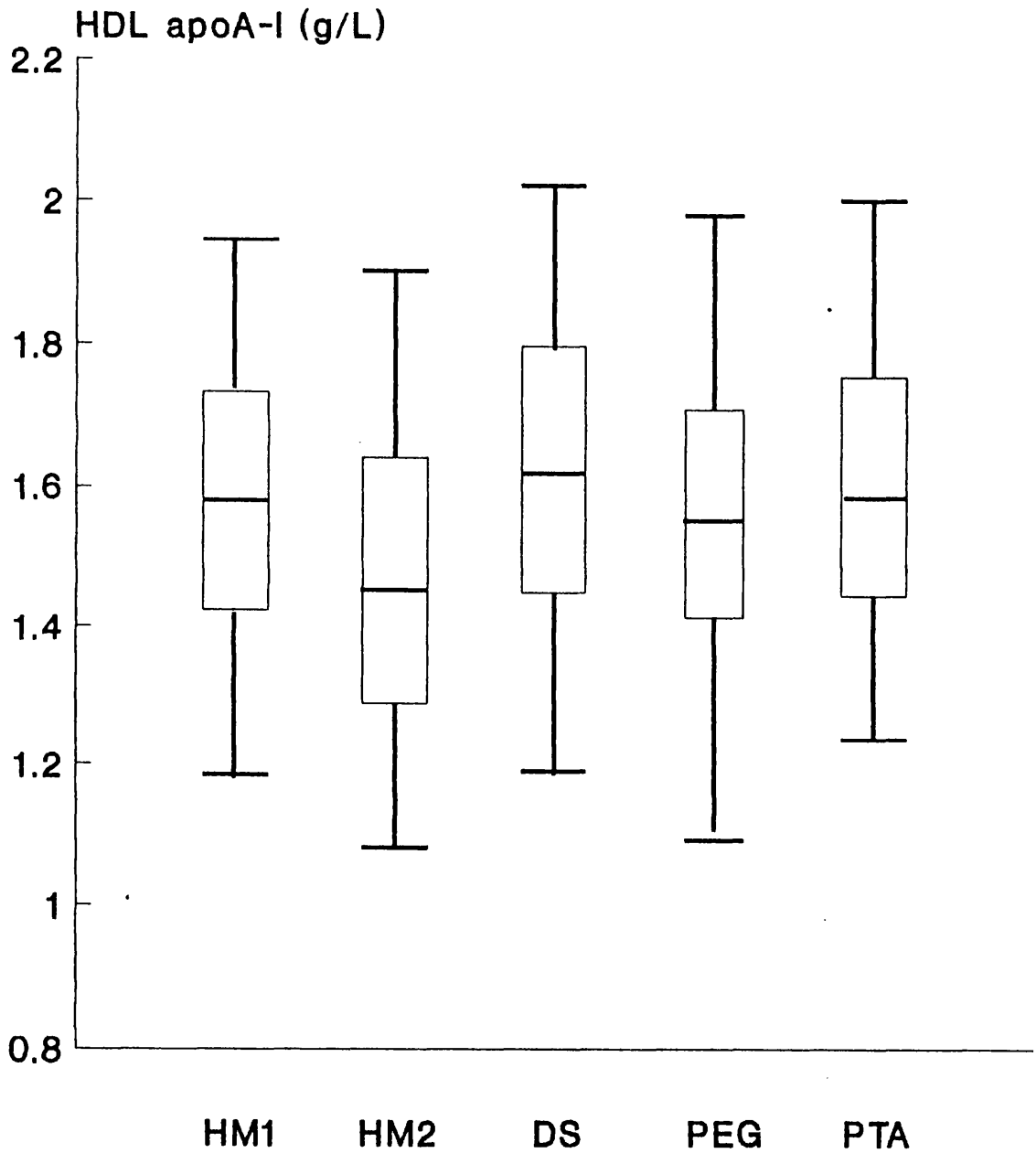


Figure 2. Plot of HDL apoA-I concentration by five precipitation procedures showing median, interquartile range and range of values.

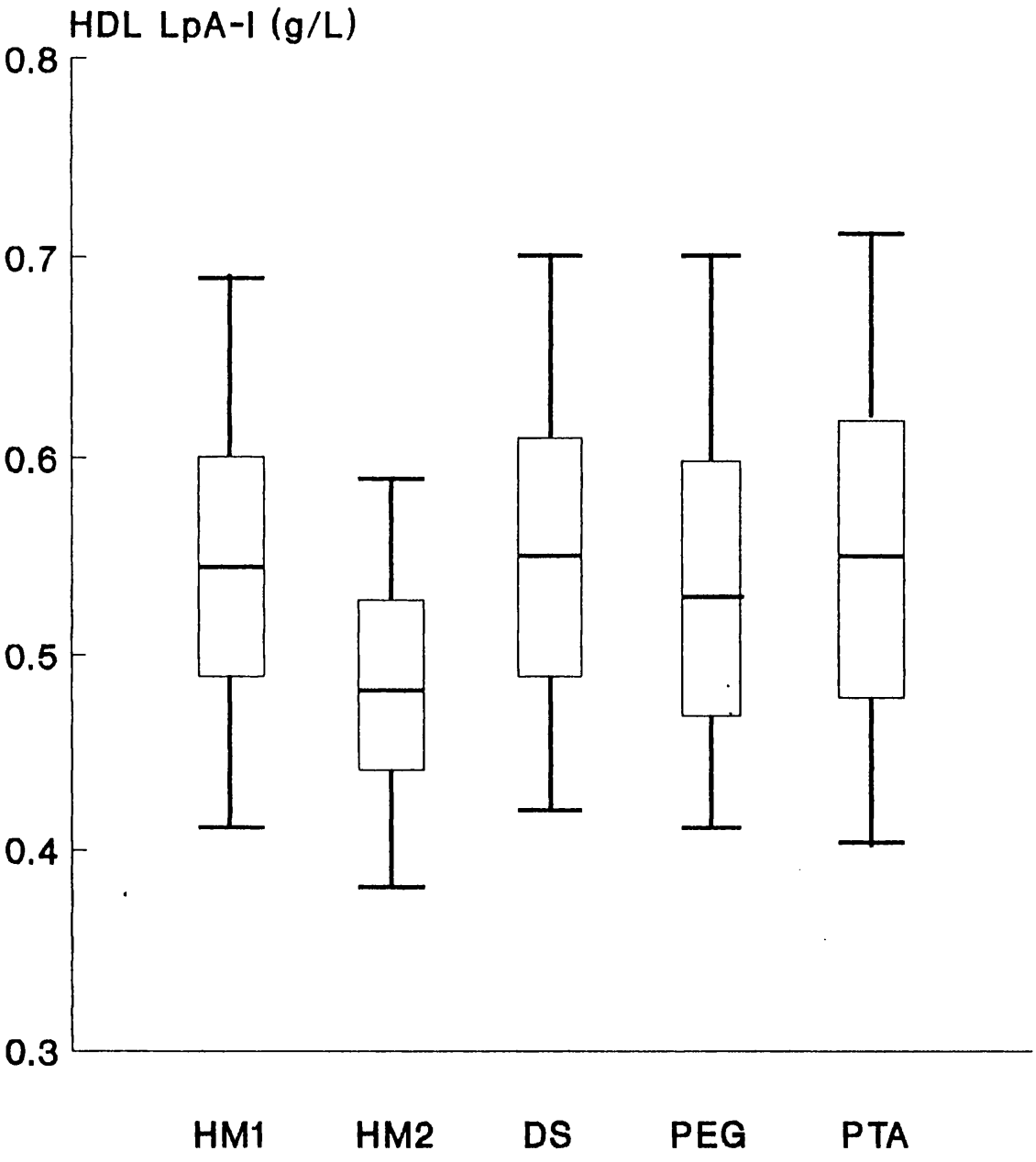


Figure 3. Plot of HDL LpA-I concentrations by five precipitation procedures showing median, interquartile range and range of values

Although ApoA-I ($p<0.05$) and LpAI ($p<0.001$) concentrations were lower for heparin-manganese chloride method 2, the percentage of total apoA-I circulating in apoA-I-containing particles (LpAI:apoA-I ratio) was not significantly different between the methods (table 4).

Table 4. Apoprotein and LpAI concentrations (mean ± s) of HDL isolated by different precipitation methods and significance when compared with heparin-manganese chloride method 1 (* $p<0.05$; ‡ $p<0.001$)

	ApoA-I (g/L)	LpAI (g/L)	LpAI/apoA-I ratio (%)
HM1	1.6 ± 0.2	0.54 ± 0.1	34.7 ± 4.8
HM2	1.5 ± 0.2*	0.49 ± 0.1‡	34.1 ± 5.6
DS	1.6 ± 0.2	0.55 ± 0.1	34.8 ± 4.9
PEG	1.6 ± 0.2	0.53 ± 0.1	34.8 ± 4.9
PTA	1.6 ± 0.2	0.55 ± 0.1	34.4 ± 5.5

Lp(a) and apoB were undetectable in the HDL supernatants, even after concentration by ultracentrifugation at density 1.21g/ml and 105,000g for 16 hours, indicating that apoB-containing lipoproteins were essentially completely removed by all the precipitation procedures.

(b) HDL cholesterol difference plots

Data from each comparison of different methods were analysed by plotting the difference between results against the mean value.

$$\text{Difference between results (\%)} = (a - b) \times \frac{2}{(a + b)} \times 100$$

where: a = result by precipitation method A

b = result by precipitation method B

The HDL cholesterol difference plots show that the differences were constant throughout the range of values studied (HM1 versus HM2 figure 4, HM1 versus DS figure 5, HM1 versus PEG figure 6 and HM1 versus PTA figure 7). The largest mean percentage differences occurred between heparin-manganese chloride method 1 and heparin-manganese chloride method 2 (6.7%, table 3) and between heparin-manganese chloride method 1 and PEG 6000 (4.1%, table 5). Differences between heparin-manganese chloride method 1 and dextran sulphate-magnesium chloride and between heparin-manganese chloride method 1 and phosphotungstic acid-magnesium chloride were modest.

Table 5. Percentage differences (mean ± s) between HDL isolation methods

	Cholesterol (percentage difference)	ApoA-I (percentage difference)	LpAI (percentage difference)
HM1-HM2	6.7 ± 5.4	8.6 ± 6.3	10.6 ± 5.2
HM1-DS	-1.3 ± 3.9	-2.7 ± 5.2	-2.7 ± 5.3
HM1-PEG	4.1 ± 3.7	2.5 ± 5.3	2.2 ± 7.6
HM1-PTA	0.9 ± 4.5	-2.9 ± 4.4	-1.8 ± 7.9

The limits of agreement between the methods, calculated as mean difference ± two standard deviations, show that the widest range of differences in measured HDL cholesterol occurred between heparin-manganese chloride method 1 and heparin-manganese chloride method 2 with 95% of the heparin-manganese chloride method 1 values being between 0.24 mmol/L higher and 0.06 mmol/L lower than values obtained by heparin-manganese chloride method 2 (table 6). The range of differences for heparin-manganese chloride method 1 varied between 0.16 mmol/L higher and 0.13 mmol/L lower than values by the phosphotungstic acid-magnesium chloride method.

Table 6. Limits of agreement, calculated as mean difference ± 2 standard deviations (d ± 2s), between HDL isolation methods

	Cholesterol (mmol/L)		ApoA-I (g/L)		LpAI (g/L)	
	d+2s	d-2s	d+2s	d-2s	d+2s	d-2s
HM1-HM2	0.24	-0.06	0.28	-0.04	0.10	0.01
HM1-DS	0.09	-0.13	0.12	-0.20	0.05	-0.07
HM1-PEG	0.18	-0.06	0.19	-0.13	0.09	-0.07
HM1-PTA	0.16	-0.13	0.09	-0.18	0.08	-0.10

(c) ApoA-I difference plots

Difference plots for apoA-I in the HDL supernatants show that the differences are constant throughout the range of values studied (HM1 versus HM2 figure 8, HM1 versus DS figure 9, HM1 versus PEG figure 10 and HM1 versus PTA figure 11). The largest percentage difference (8.6%) occurred between heparin-manganese chloride method 1 and heparin-manganese chloride method 2 (table 5). The heparin-manganese chloride method 2 underestimated apoA-I values compared with heparin-manganese chloride method 1.

Modest differences were observed between heparin-manganese chloride method 1 and the remaining precipitation methods (table 6).

(d) LpAI difference plots

Difference plots for HDL LpAI show that the differences are constant throughout the range of values studied (HM1 versus HM2 figure 12, HM1 versus DS figure 13, HM1 versus PEG figure 14 and HM1 versus PTA figure 15). The largest difference (10.6%) was between heparin-manganese chloride method 1 and heparin-manganese chloride method 2 (table 5). Differences between heparin-manganese chloride method 1 and the remaining precipitation techniques were modest (tables 5 and 6)

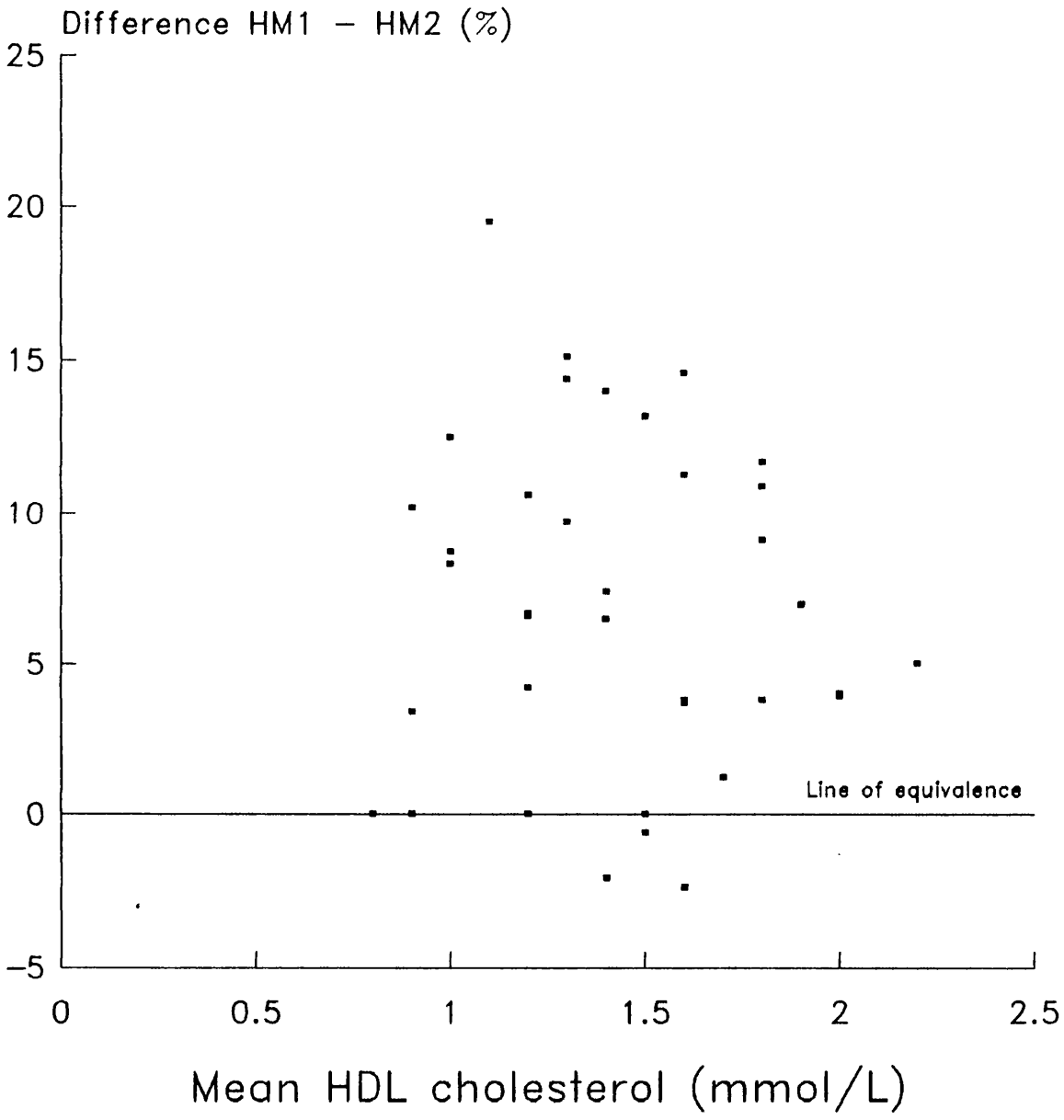


Figure 4. Difference plot for HDL cholesterol by heparin–manganese chloride 1 (HM1) and heparin–manganese 2 (HM2) precipitation procedures

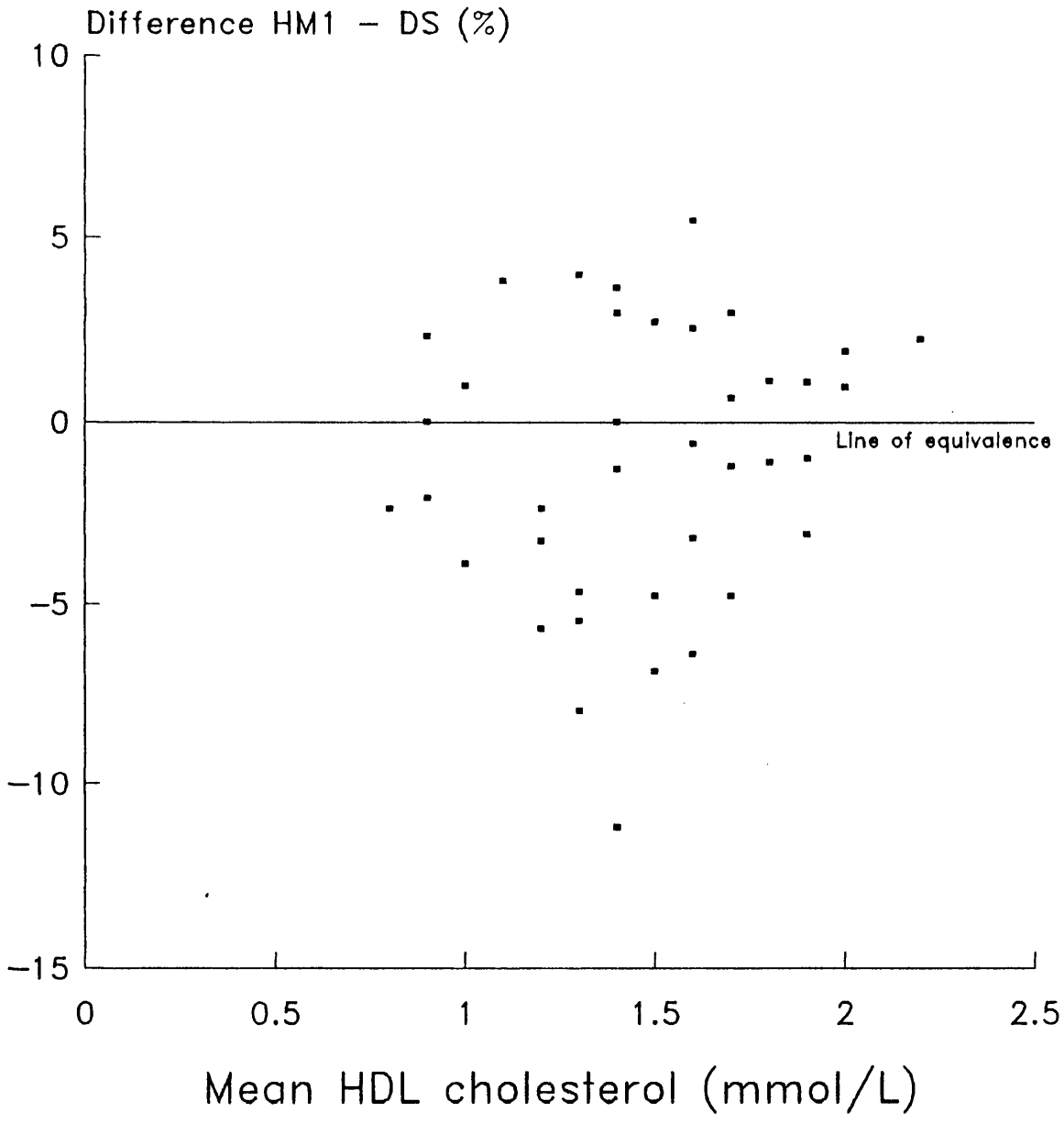


Figure 5. Difference plot for HDL cholesterol by heparin–manganese chloride 1 (HM1) and dextran sulphate–magnesium chloride (DS) precipitation procedures

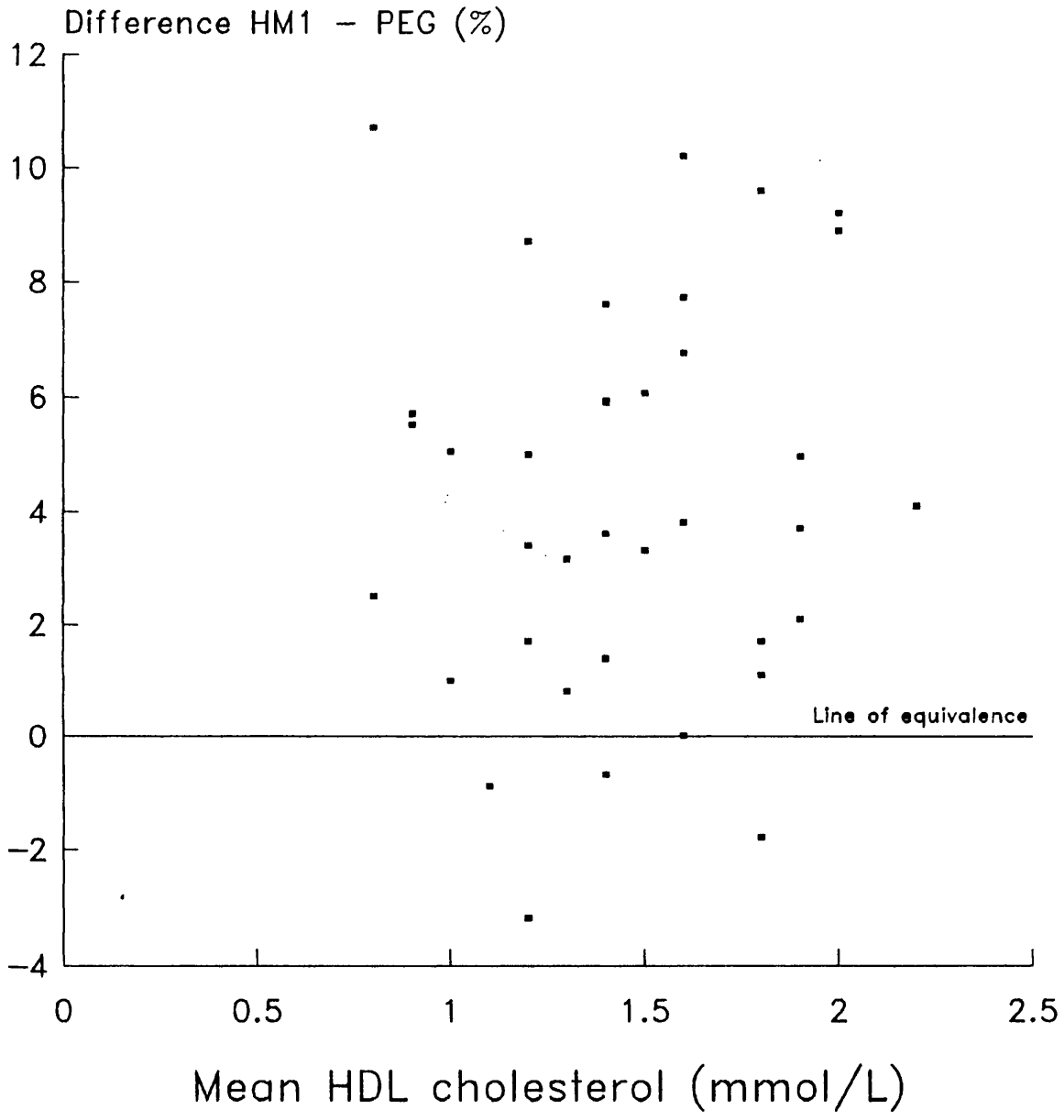


Figure 6. Difference plot for HDL cholesterol by heparin–manganese chloride 1 (HM1) and polyethylene glycol 6000 (PEG) precipitation procedures

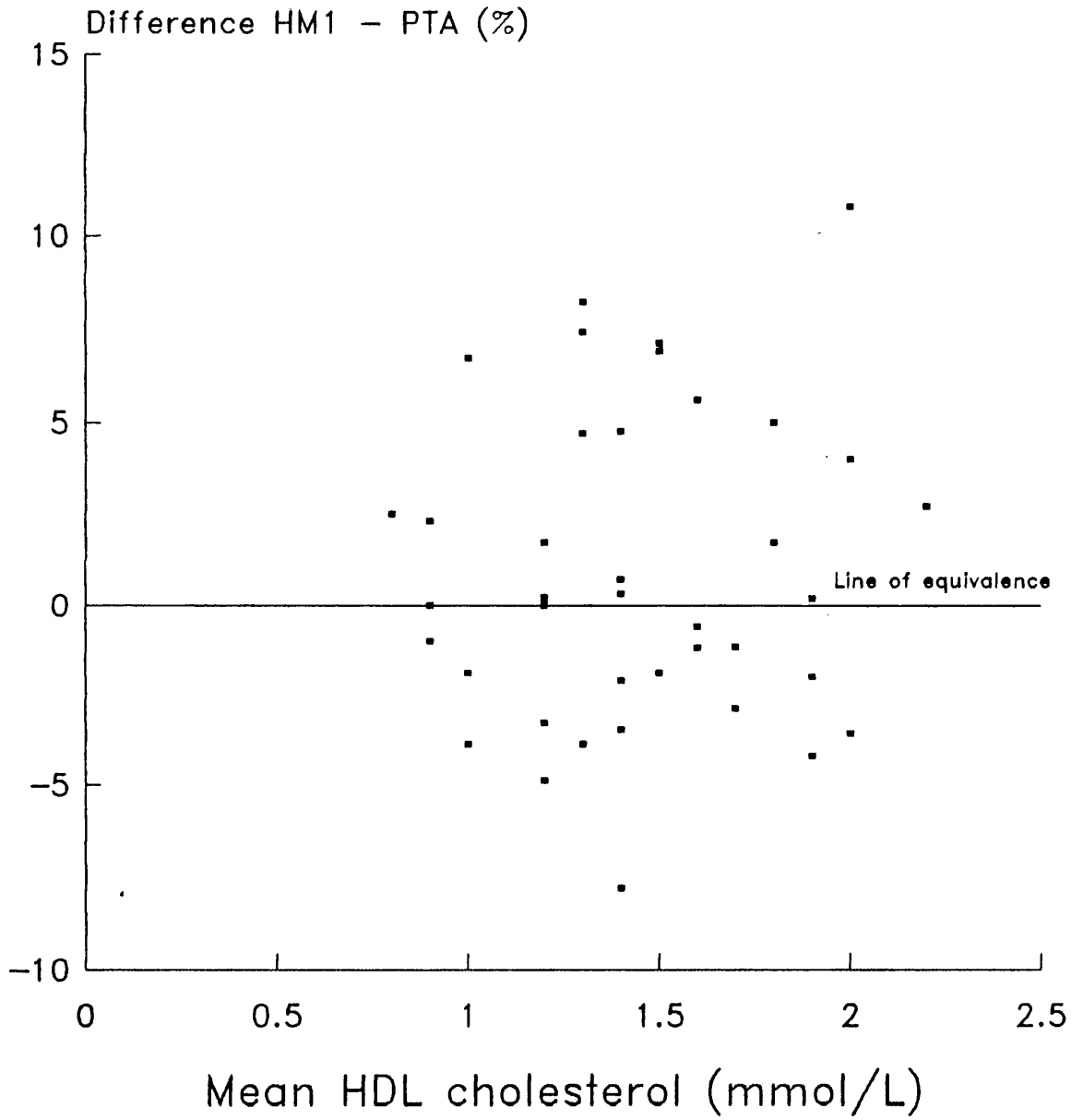


Figure 7. Difference plot for HDL cholesterol by heparin–manganese chloride 1 (HM1) and phosphotungstic acid–magnesium chloride (PTA) precipitation procedures

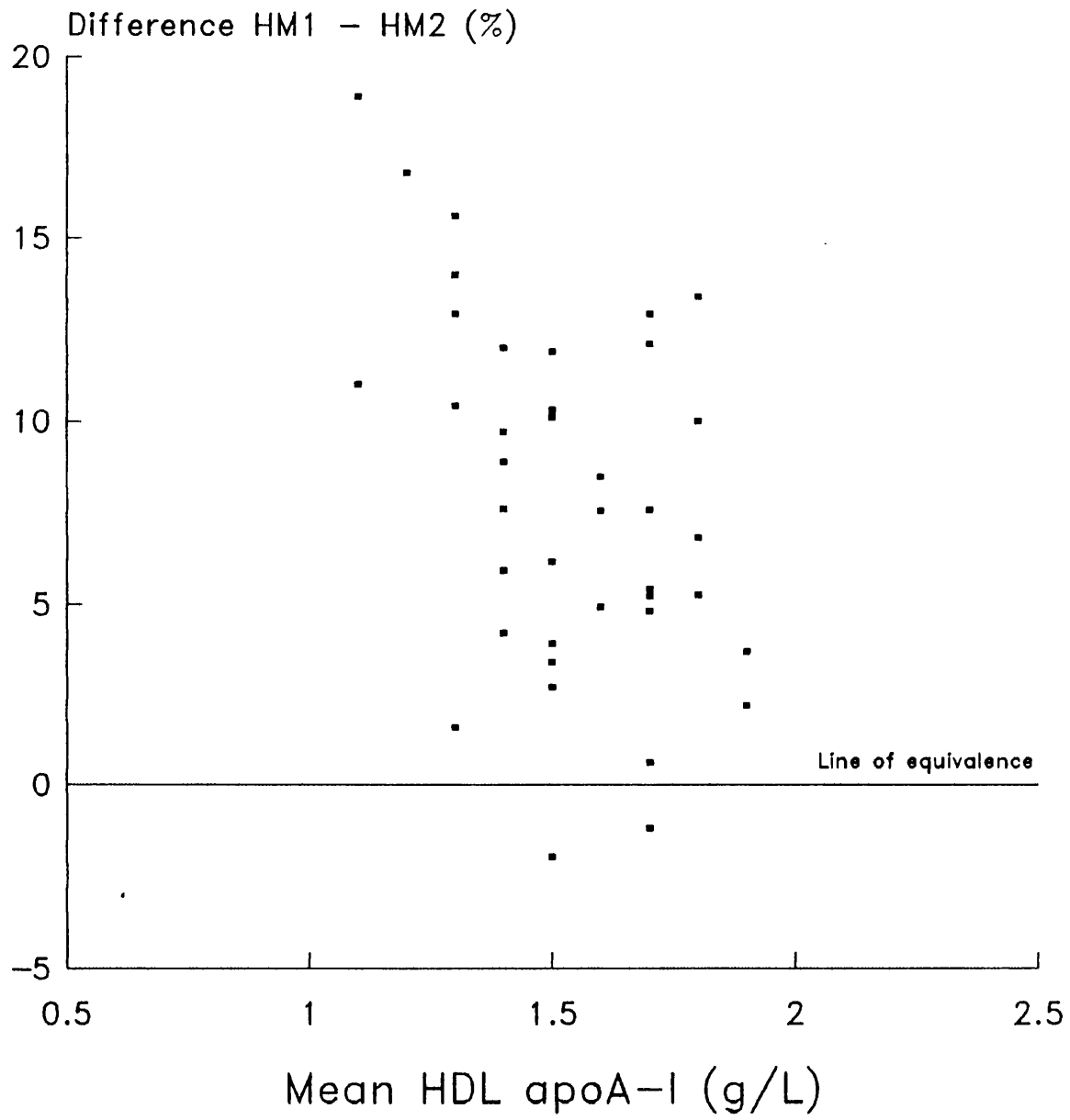


Figure 8. Difference plot for HDL apoA–I by heparin–manganese chloride (HM1) and heparin–manganese chloride 2 (HM2) precipitation procedures

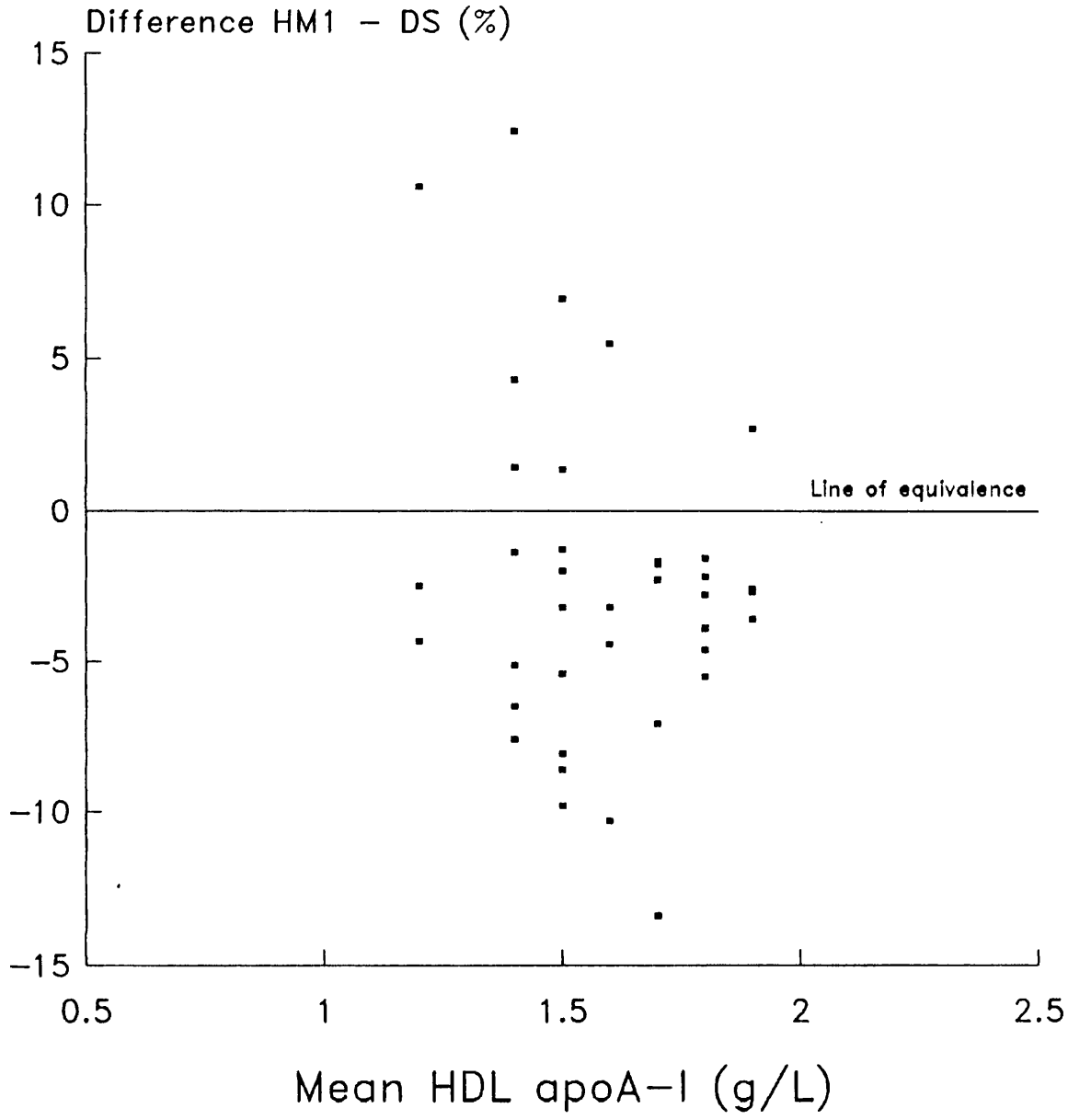


Figure 9. Difference plot for HDL apoA-I by heparin-manganese chloride 1 (HM1) and dextran sulphate-magnesium chloride (DS) precipitation techniques

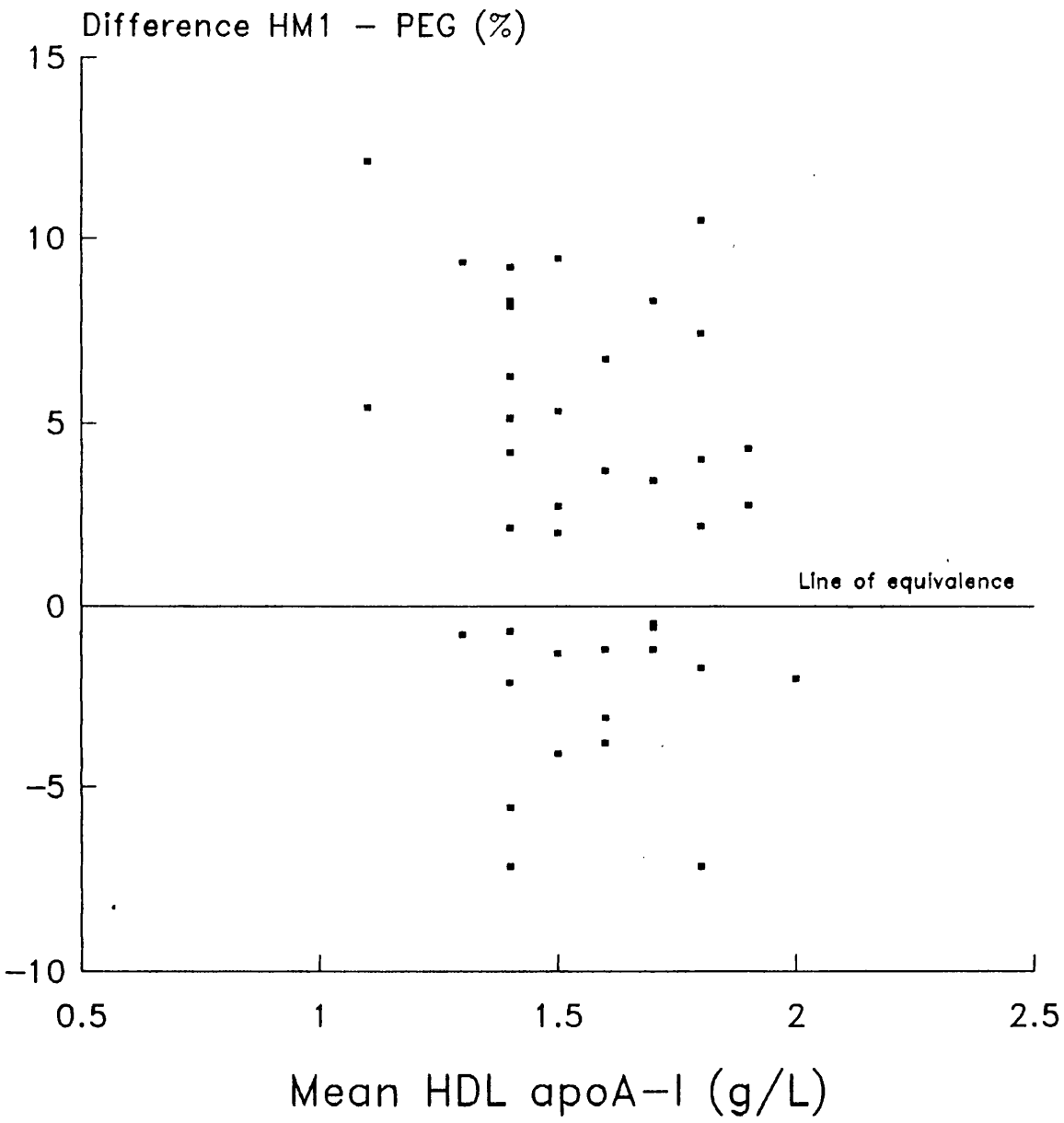


Figure 10. Difference plot for HDL apoA–I by heparin–manganese chloride 1 (HM1) and polyethylene glycol 6000 (PEG) precipitation procedures

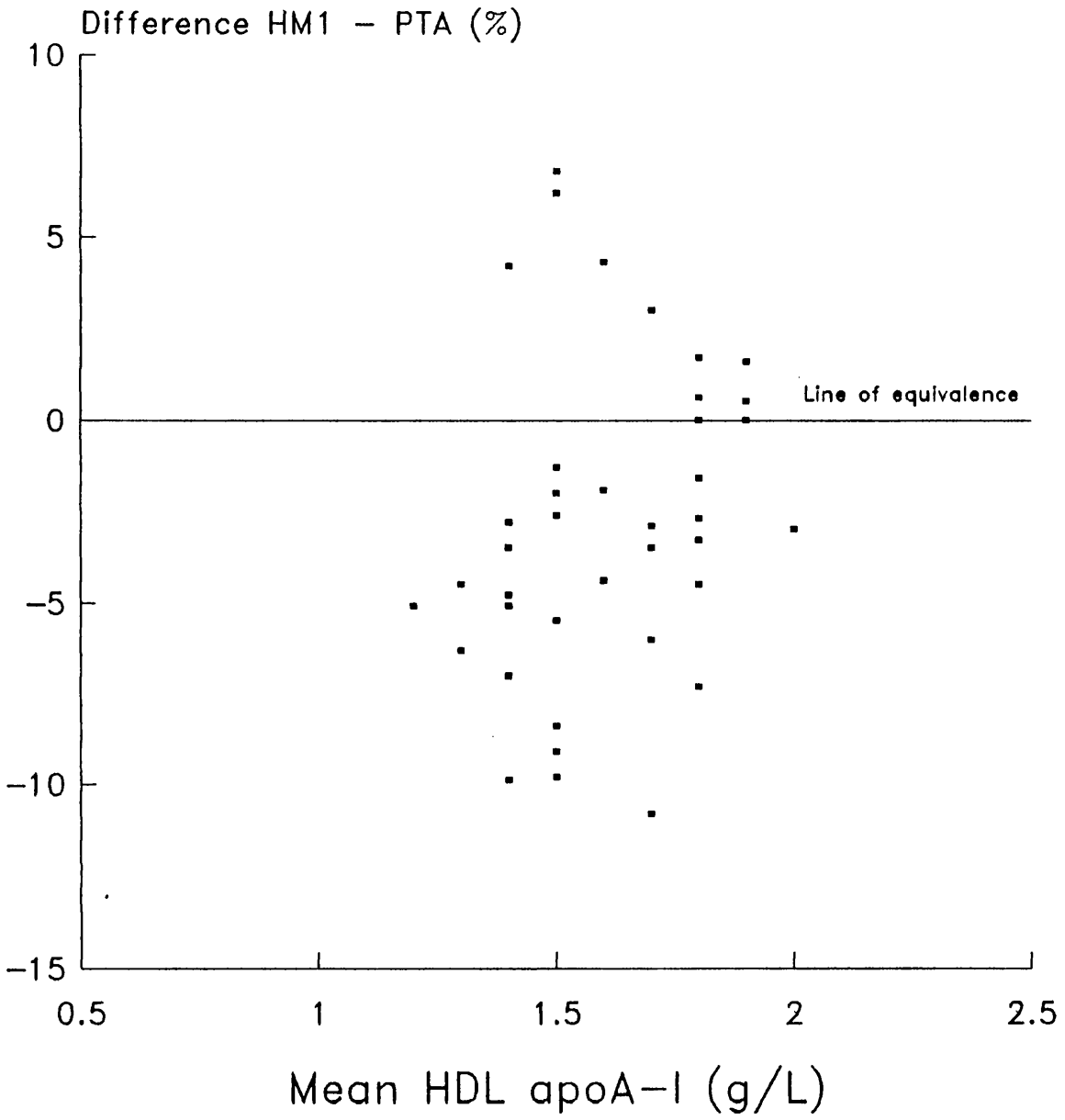


Figure 11. Difference plot for HDL apoA-I by heparin-manganese chloride 1 (HM1) and phosphotungstic acid-magnesium chloride (PTA) precipitation techniques

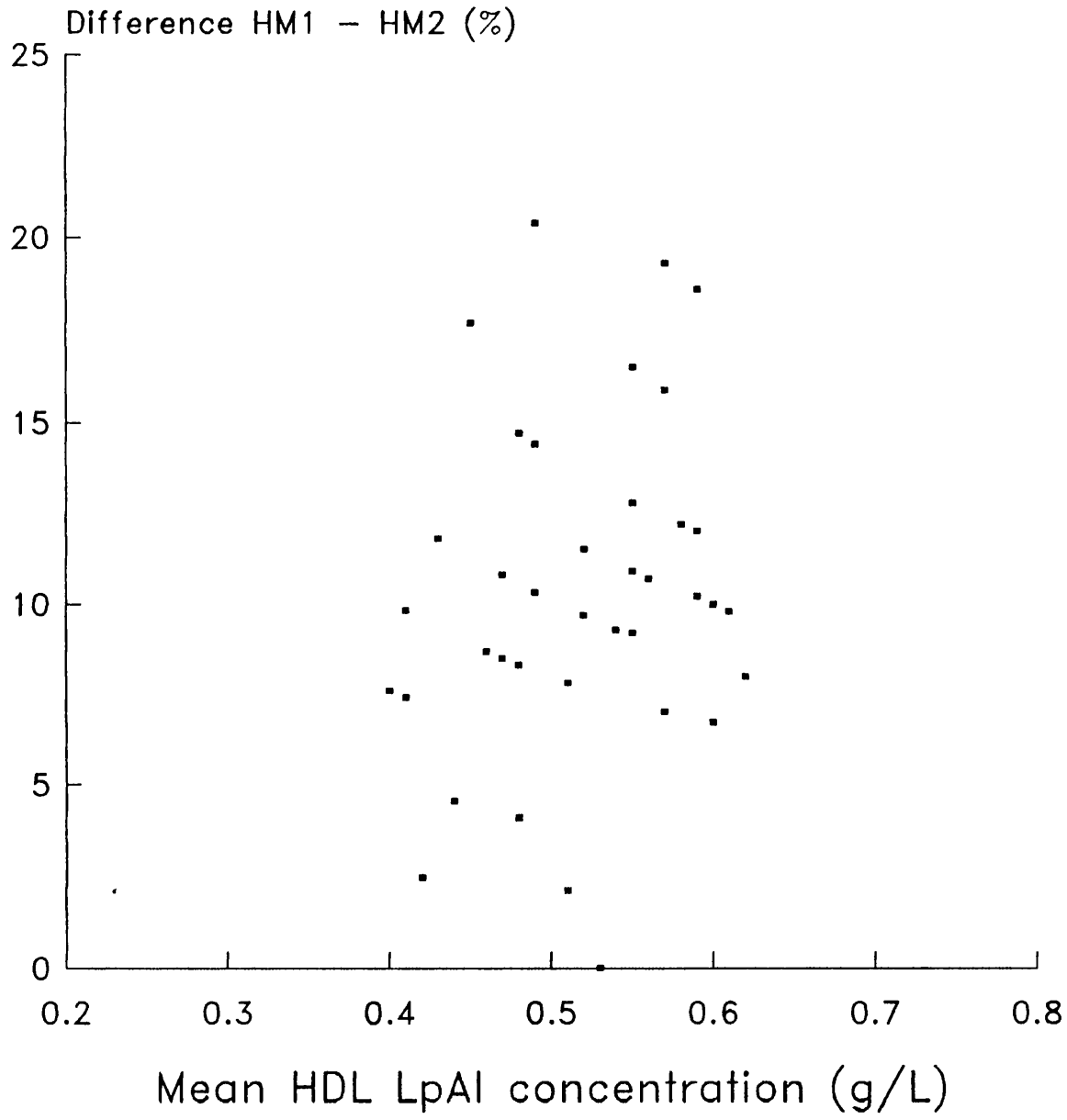


Figure 12. Difference plot for HDL LpAI by heparin–manganese chloride 1 (HM1) and heparin–manganese chloride 2 (HM2) precipitation techniques

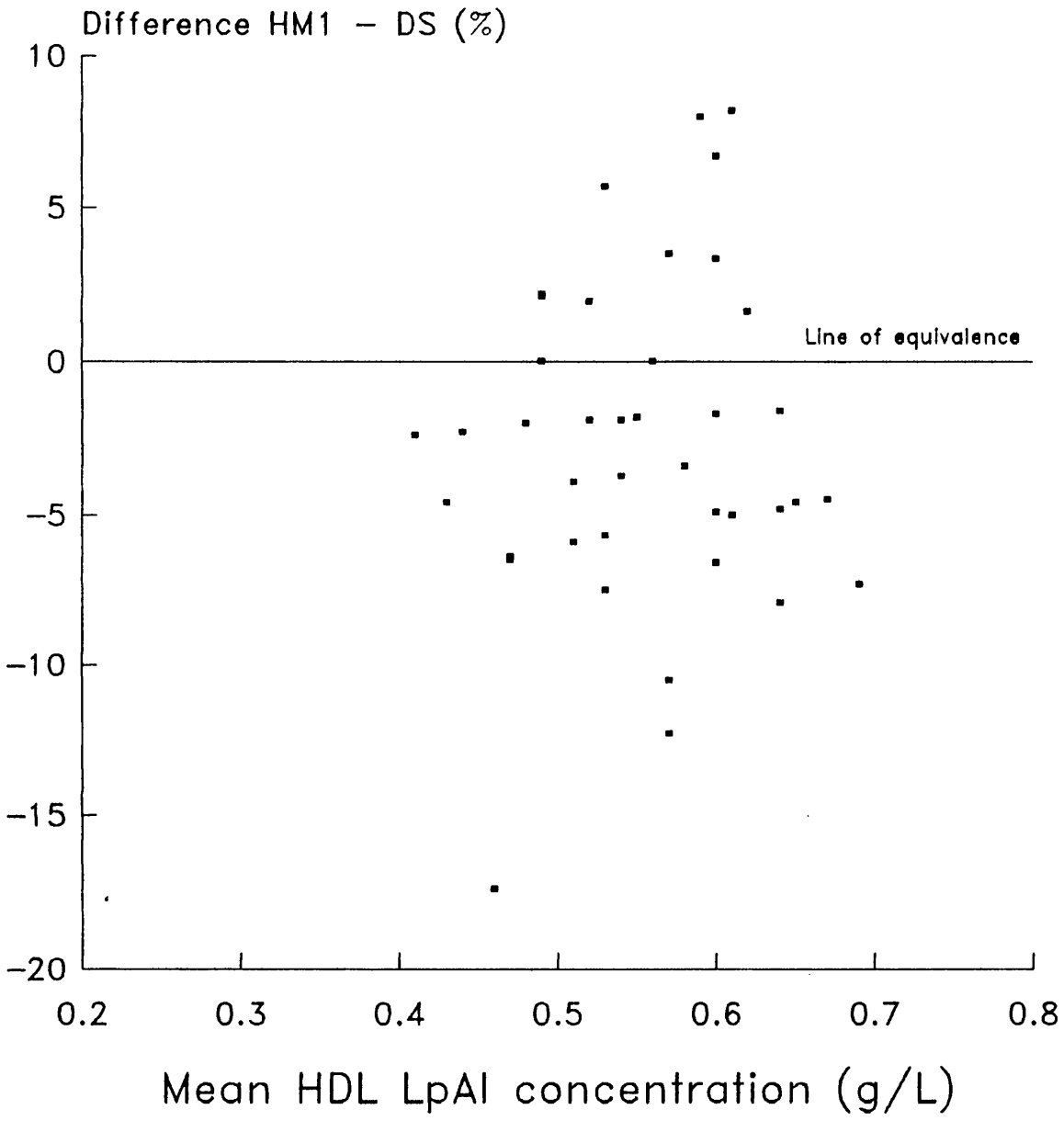


Figure 13. Difference plot for HDL LpAI by heparin–manganese chloride 1 (HM1) and dextran sulphate–magnesium chloride (DS) precipitation techniques

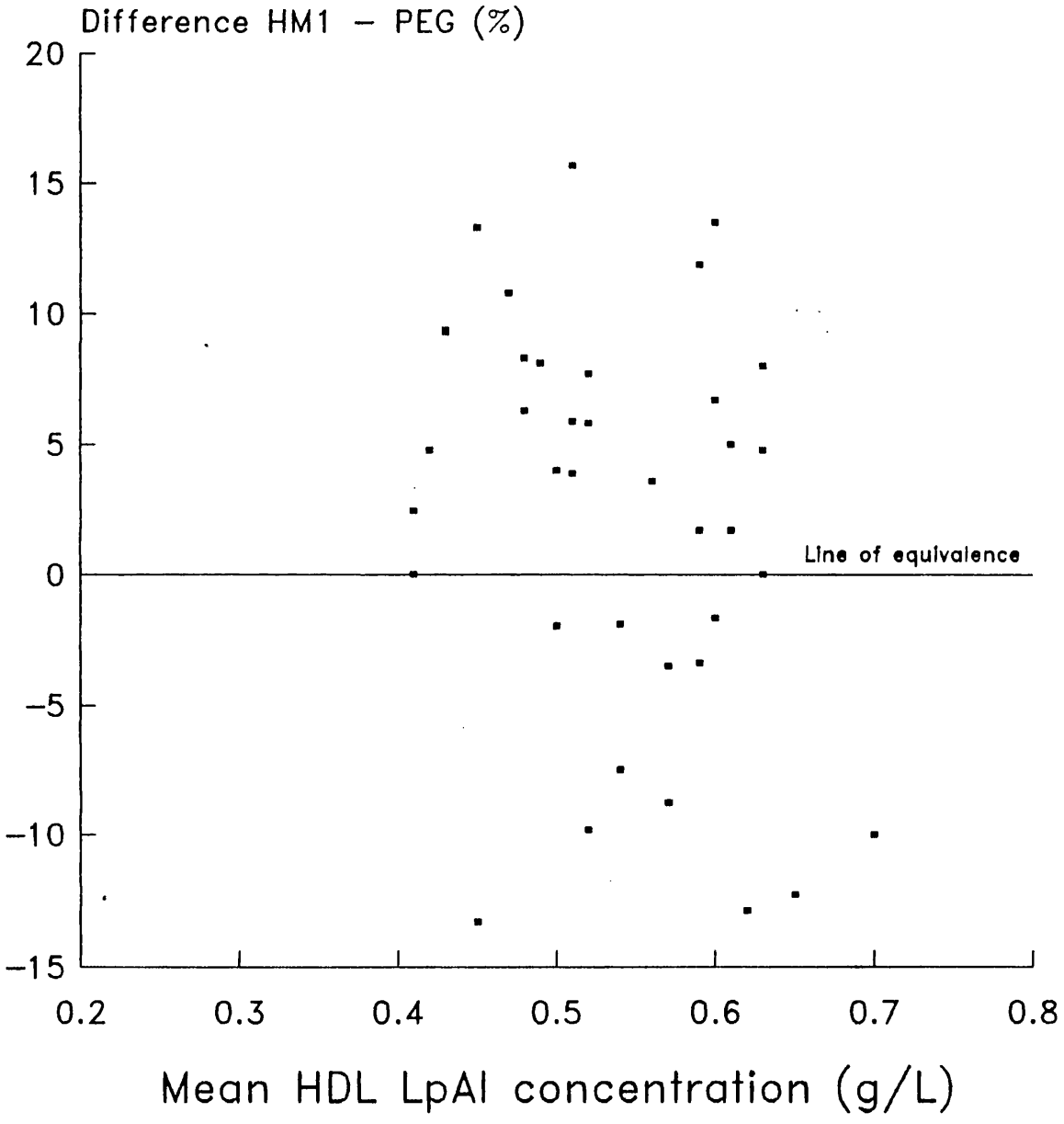


Figure 14. Difference plot for HDL LpAI by heparin–manganese chloride 1 (HM1) and polyethylene glycol 6000 (PEG) precipitation procedures

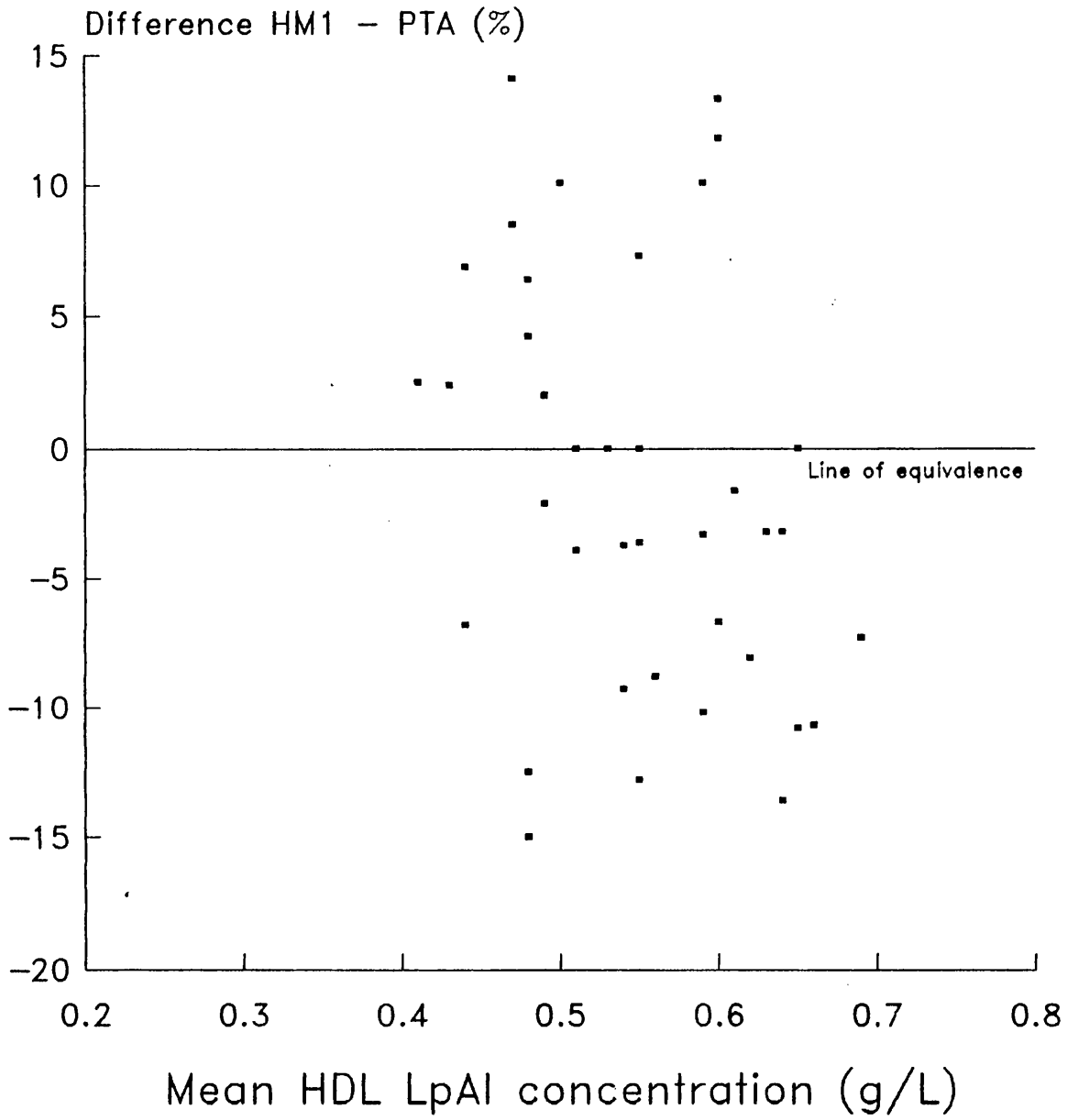


Figure 15. Difference plot for HDL LpAI by heparin–manganese chloride 1 (HM1) and phosphotungstate acid–magnesium chloride (PTA) precipitation techniques

3.2 Dyslipoproteinaemia in UK families with severe expression of coronary heart disease.

(a) Clinical characteristics in probands and other family members

Blood pressure and BMI for male (table 7) and female (table 8) non-probands were similar to those recorded for a matching UK population recruited opportunistically (Mann et al., 1988).

Table 7. Blood pressure measurements and body mass indices (mean ± s) in male probands (n=20) and non-probands (n=122)

	Blood pressure (mm Hg)		BMI, (kg/m^2)
	Systolic	Diastolic	
Probands	126 ± 16	81 ± 13	26.6 ± 2.5
Non-probands	133 ± 17	80 ± 12	25.2 ± 3.8
All	132 ± 17	81 ± 12	25.2 ± 3.7

Table 8. Blood pressure measurements and body mass indices (mean ± s) in female probands (n=6) and non-probands (n=128)

	Blood pressure (mm Hg)		BMI, (kg/m^2)
	Systolic	Diastolic	
Probands	134 ± 27	82 ± 10	26.0 ± 3.0
Non-probands	126 ± 19	79 ± 10	24.7 ± 4.6
All	126 ± 19	79 ± 11	24.7 ± 4.6

(b) Male probands and other family members

Data for male probands and other family members is summarised in figures for plasma concentrations of total cholesterol (figure 16), of calculated LDL cholesterol (figure 17), of HDL cholesterol (figure 18) and of triglycerides (figure 19).

The data is tabulated by age in male probands for total cholesterol and calculated LDL cholesterol concentrations (table 9) and for HDL cholesterol and triglyceride concentrations (table 10) and in male non-probands for total cholesterol and calculated LDL cholesterol concentrations (table 11) and for HDL cholesterol and triglyceride concentrations (table 12). LDL cholesterol was not calculated for probands and other family members with triglyceride concentrations greater than 4.5mmol/L.

Table 9. Plasma total cholesterol and calculated LDL cholesterol (mean ± s) concentrations by age in male probands. Numbers in brackets refer to numbers of individuals in each age group for which LDL cholesterol was calculated.

Age group (years)	No.	Total cholesterol (mmol/L)	LDL cholesterol (mmol/L)
35-39	1 (1)	7.7	5.8
40-44	4 (2)	9.2 ± 1.9	7.4 ± 2.6
45-49	3 (3)	6.5 ± 1.7	4.5 ± 1.5
50-54	7 (7)	7.0 ± 1.2	4.9 ± 1.1
55-59	5 (4)	7.2 ± 1.1	5.3 ± 0.8
ALL	20 (17)	7.4 ± 1.6	5.2 ± 1.5

Table 10. Plasma HDL cholesterol (mean ± s) and triglyceride (median and range) concentrations by age in male probands

Age group (years)	No.	HDL cholesterol (mmol/L)	Triglyceride (mmol/L)
35-39	1	0.9	2.2
40-44	4	1.1 ± 0.2	3.9 (0.9-7.6)
45-49	3	1.3 ± 0.2	1.4 (1.2-2.1)
50-54	7	1.2 ± 0.1	1.7 (1.2-3.3)
55-59	5	1.3 ± 0.6	2.2 (1.4-5.5)
ALL	20	1.2 ± 0.3	1.9 (0.9-7.6)

Table 11. Plasma total cholesterol and calculated LDL cholesterol concentrations (mean ± s) by age in male non-probands. Numbers in brackets refer to numbers of individuals in each age group for which LDL cholesterol was calculated.

Age group (years)	No.	Total cholesterol (mmol/L)	LDL cholesterol (mmol/L)
16-19	4	4.9 ± 0.7	3.0 ± 0.8
20-24	15	4.8 ± 1.0	2.8 ± 0.8
25-29	21	5.3 ± 1.3	3.2 ± 1.1
30-34	15	6.2 ± 1.0	3.9 ± 0.9
35-39	8	6.3 ± 1.0	4.3 ± 0.7
40-44	13 (12)	7.3 ± 1.7	5.0 ± 1.5
45-49	5 (4)	7.0 ± 2.0	4.3 ± 2.1
50-54	17 (14)	7.7 ± 1.6	5.4 ± 1.5
55-59	12 (11)	6.6 ± 1.3	4.0 ± 0.8
60-64	9	6.6 ± 1.5	4.6 ± 1.2
>65	3	6.8 ± 2.3	4.3 ± 1.5
ALL	122 (116)	6.2 ± 1.6	4.1 ± 1.4

Table 12. Plasma HDL cholesterol (mean ± s) and triglyceride (median and range) concentrations by age in male non-probands

Age group (years)	No.	HDL cholesterol (mmol/L)	Triglyceride (mmol/L)
16-19	4	1.5 ± 0.3	0.8 (0.5-1.4)
20-24	15	1.2 ± 0.3	1.5 (1.1-3.4)
25-29	21	1.4 ± 0.4	1.3 (0.5-3.5)
30-34	15	1.4 ± 0.2	1.8 (0.9-3.4)
35-39	8	1.4 ± 0.4	1.4 (0.6-3.8)
40-44	13	1.3 ± 0.4	1.8 (0.7-7.2)
45-49	5	1.2 ± 0.5	2.9 (0.9-5.1)
50-54	17	1.2 ± 0.3	2.7 (0.8-5.7)
55-59	12	1.3 ± 0.5	1.6 (0.8-6.2)
60-64	9	1.2 ± 0.3	2.0 (0.9-3.2)
>65	3	1.7 ± 0.6	1.9 (0.8-2.4)
ALL	122	1.3 ± 0.4	1.7 (0.5-7.2)

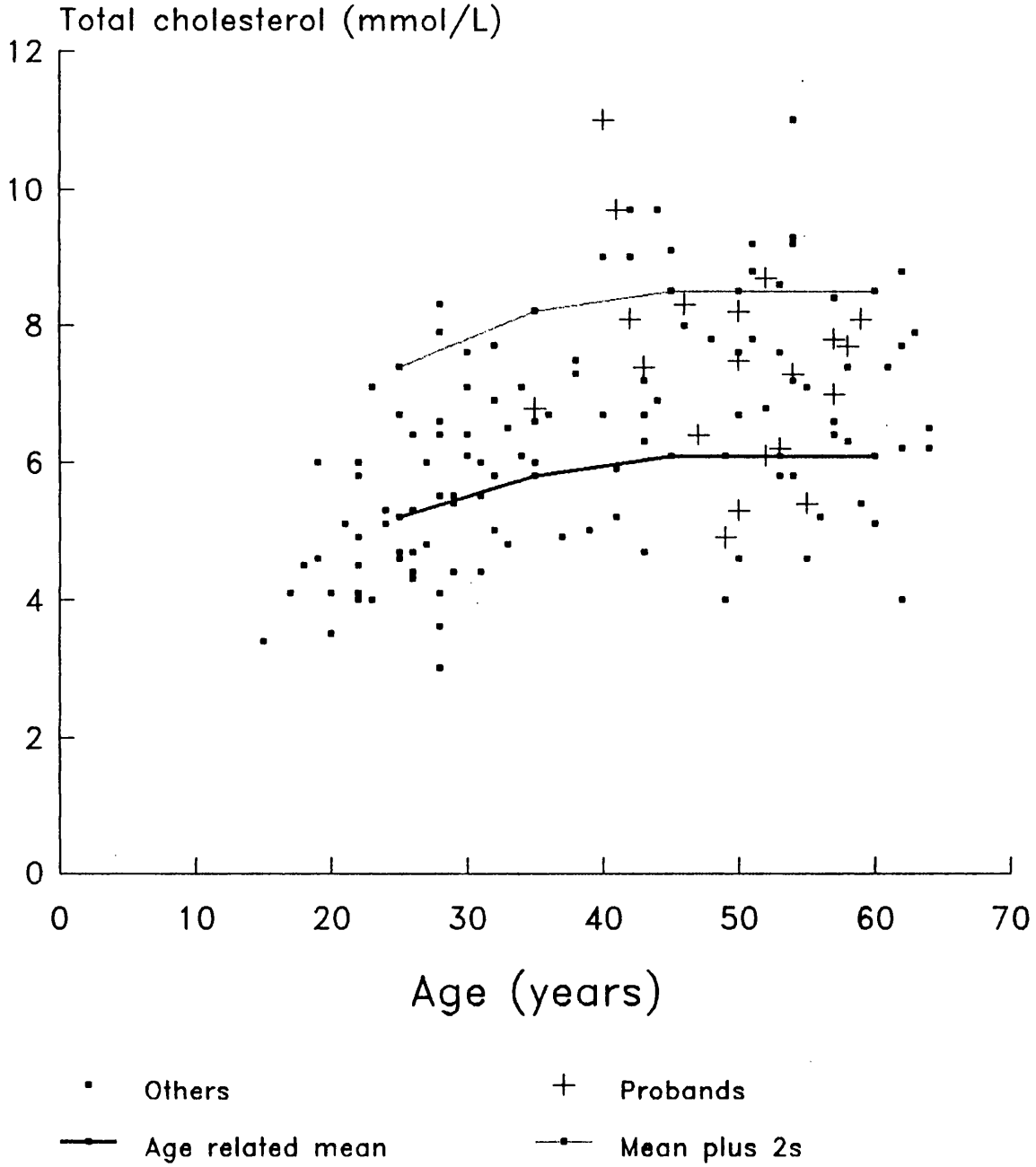


Figure 16. Plasma total cholesterol concentrations in male probands and male family members

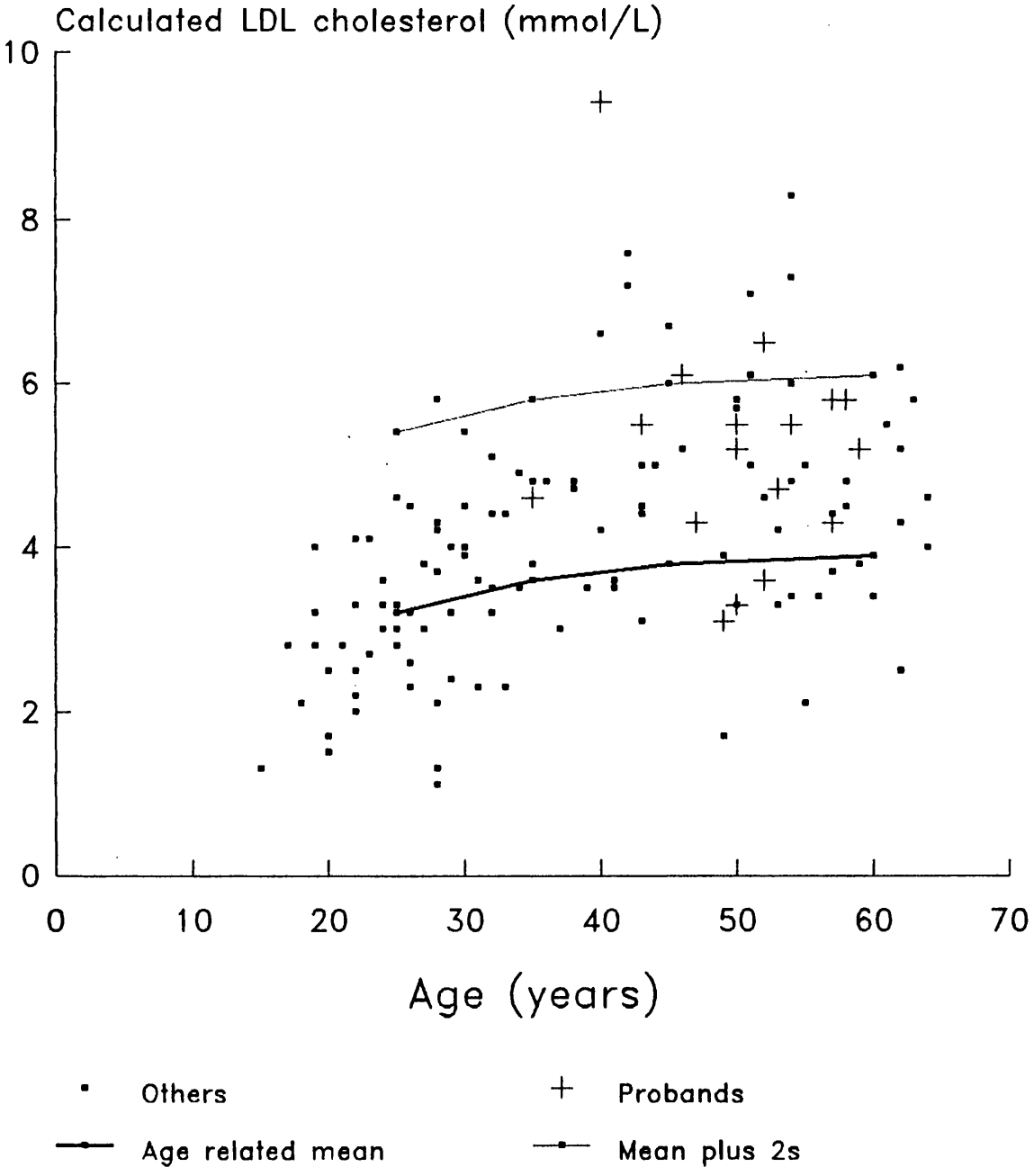


Figure 17. Calculated LDL cholesterol in male probands and male family members

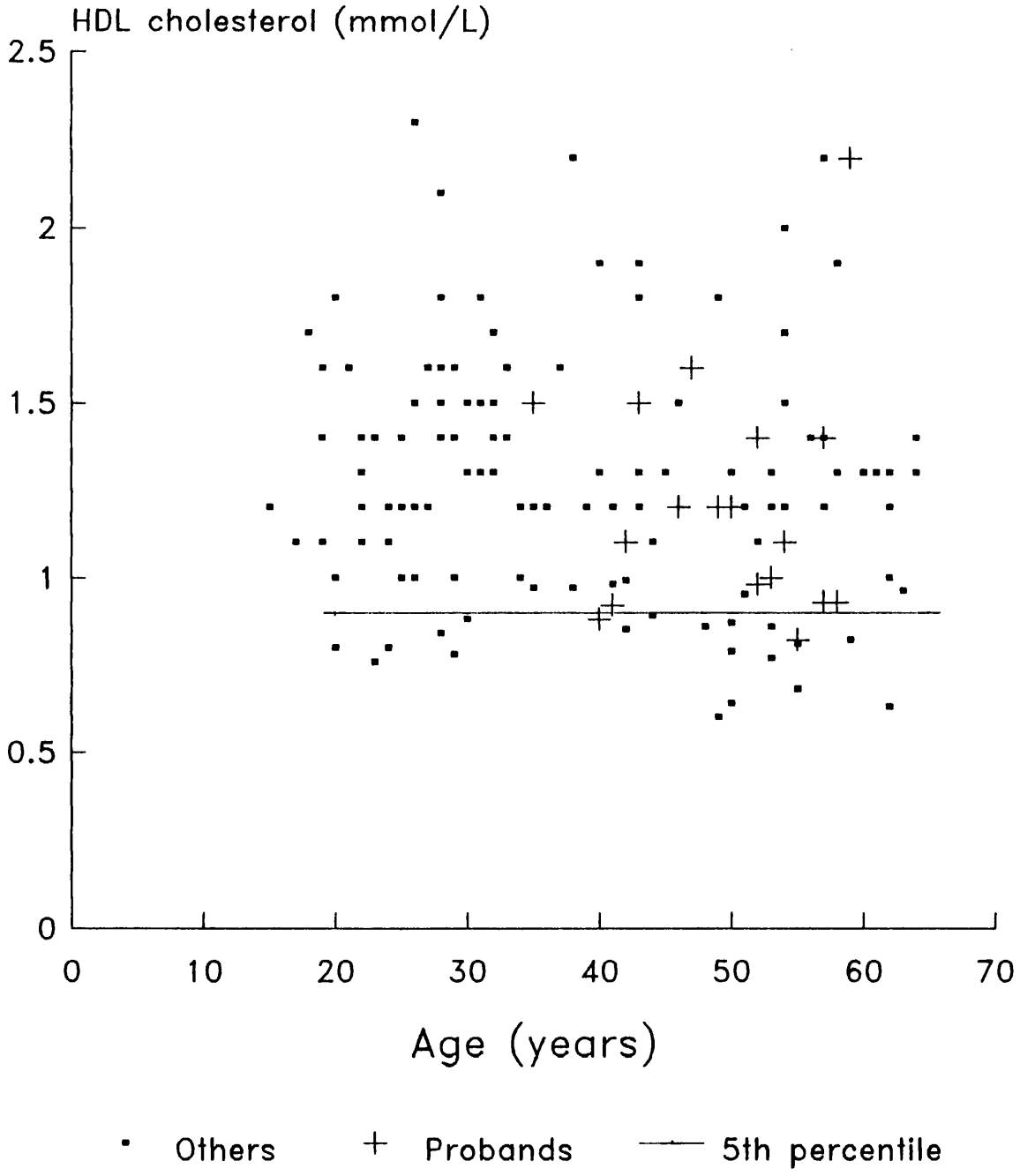


Figure 18. Plasma HDL cholesterol concentrations in male probands and male family members

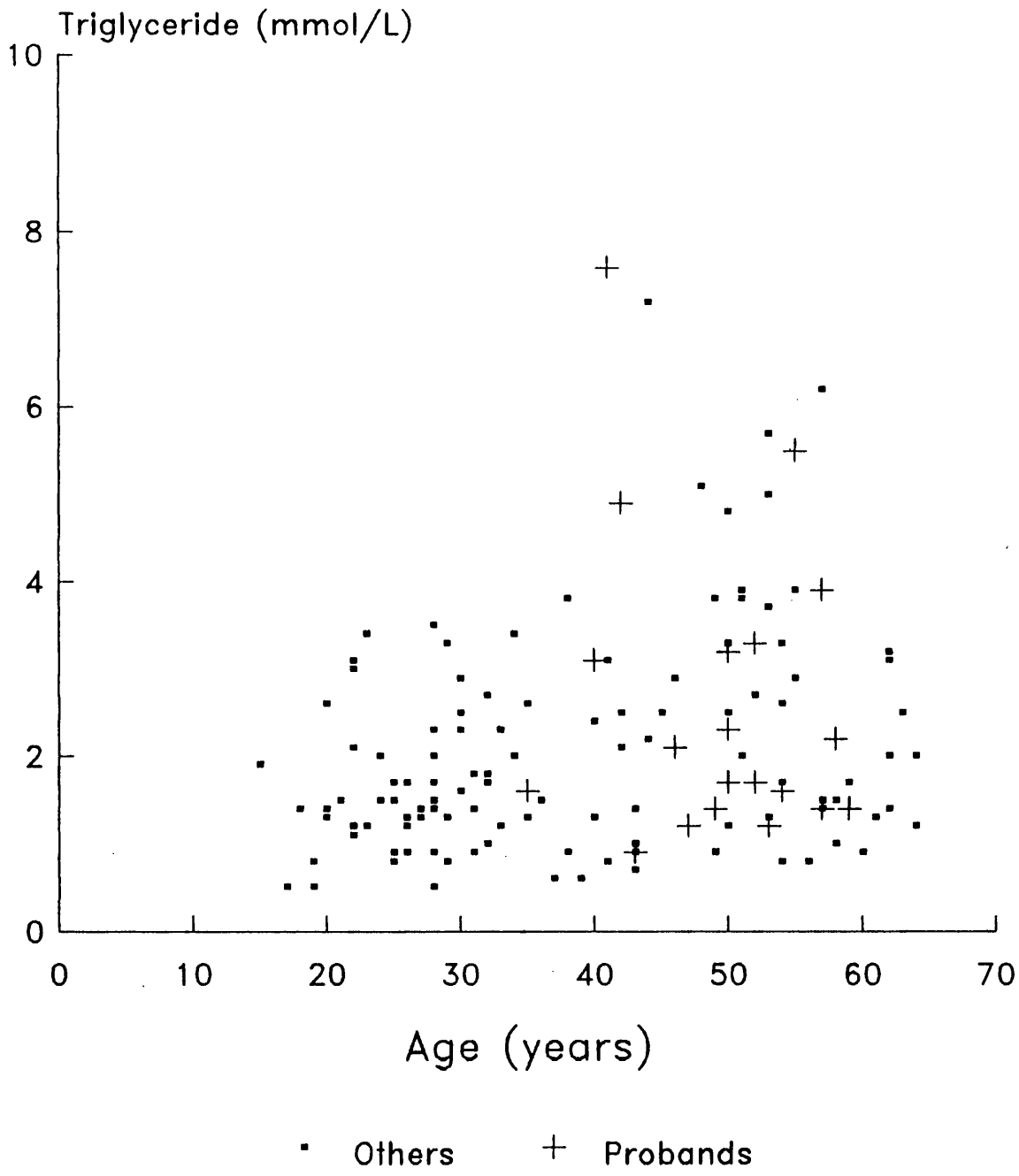


Figure 19. Plasma triglyceride concentrations in male probands and male family members

(c) Female probands and other family members

Data for female probands and other family members is summarised in figures for plasma concentrations of total cholesterol (figure 20), of calculated LDL cholesterol (figure 21), of HDL cholesterol (figure 22) and of triglycerides (figure 23). Data is tabulated in female probands for total cholesterol and calculated LDL cholesterol concentrations (table 13) and for HDL cholesterol and triglyceride concentrations (table 14) and in female non-probands for total cholesterol and LDL cholesterol concentrations (table 15) and for HDL cholesterol and triglyceride concentrations (table 16). LDL cholesterol was not calculated in those individuals with triglyceride concentrations greater than 4.5mmol/L.

Table 13. Plasma total cholesterol and calculated LDL cholesterol concentrations (mean ± s) by age in female probands

Age group (years)	No.	Total cholesterol (mmol/L)	LDL cholesterol (mmol/L)
40-44	2	7.4 ± 0.6	4.5 ± 0.5
45-49	1	9.1	6.7
50-54	2	6.2 ± 2.1	3.8 ± 1.8
55-59	1	10.8	7.8
ALL	6	7.8 ± 2.0	5.1 ± 1.9

Table 14. Plasma HDL cholesterol (mean ± s) and triglyceride (median and range) concentrations by age in female probands

Age group (years)	No.	HDL cholesterol (mmol/L)	Triglyceride (mmol/L)
40-44	2	1.4 ± 0.1	3.2 (3.0-3.4)
45-49	1	1.9	1.2
50-54	2	1.2 ± 0.3	2.8 (2.7-2.9)
55-59	1	1.7	2.8
ALL	6	1.5 ± 0.3	2.9 (1.2-3.4)

Table 15. Plasma total cholesterol and calculated LDL cholesterol (mean ± s) concentrations by age in female non-probands. Numbers in brackets refer to numbers of individuals in each age group for which LDL cholesterol was calculated.

Age group (years)	No.	Total cholesterol (mmol/L)	LDL cholesterol (mmol/L)
16-19	8	5.2 ± 1.3	3.2 ± 1.2
20-24	18	5.4 ± 0.8	3.4 ± 0.8
25-29	20	5.4 ± 0.8	3.3 ± 0.8
30-34	11	5.8 ± 1.0	3.8 ± 1.0
35-39	10	6.3 ± 1.5	4.1 ± 1.4
40-44	13	6.8 ± 1.4	4.2 ± 1.3
45-49	10 (9)	6.9 ± 1.0	4.3 ± 1.1
50-54	9 (8)	7.5 ± 1.7	5.3 ± 1.4
55-59	11 (8)	7.6 ± 1.8	4.9 ± 1.3
60-64	10 (9)	6.7 ± 1.3	4.2 ± 1.2
>65	8	8.3 ± 1.0	5.6 ± 0.8
ALL	128 (122)	6.3 ± 1.5	4.1 ± 1.4

Table 16. Plasma HDL cholesterol (mean ± s) and triglyceride (median and range) concentrations by age in female non-probands

Age group (years)	No.	HDL cholesterol (mmol/L)	Triglyceride (mmol/L)
16-19	8	1.3 ± 0.3	1.1 (0.7-4.1)
20-24	18	1.5 ± 0.2	1.0 (0.6-2.4)
25-29	20	1.7 ± 0.5	0.8 (0.5-2.1)
30-34	11	1.3 ± 0.4	1.4 (0.5-3.5)
35-39	10	1.8 ± 0.4	0.8 (0.6-1.6)
40-44	13	1.8 ± 0.5	1.4 (0.5-3.5)
45-49	10	1.7 ± 0.6	1.3 (0.6-5.0)
50-54	9	1.3 ± 0.5	1.9 (0.8-15.0)
55-59	11	1.6 ± 0.3	1.4 (0.8-6.6)
60-64	10	1.7 ± 0.5	1.4 (0.7-5.8)
>65	8	1.8 ± 0.6	1.7 (1.0-4.2)
ALL	128	1.6 ± 0.4	1.2 (0.5-15.0)

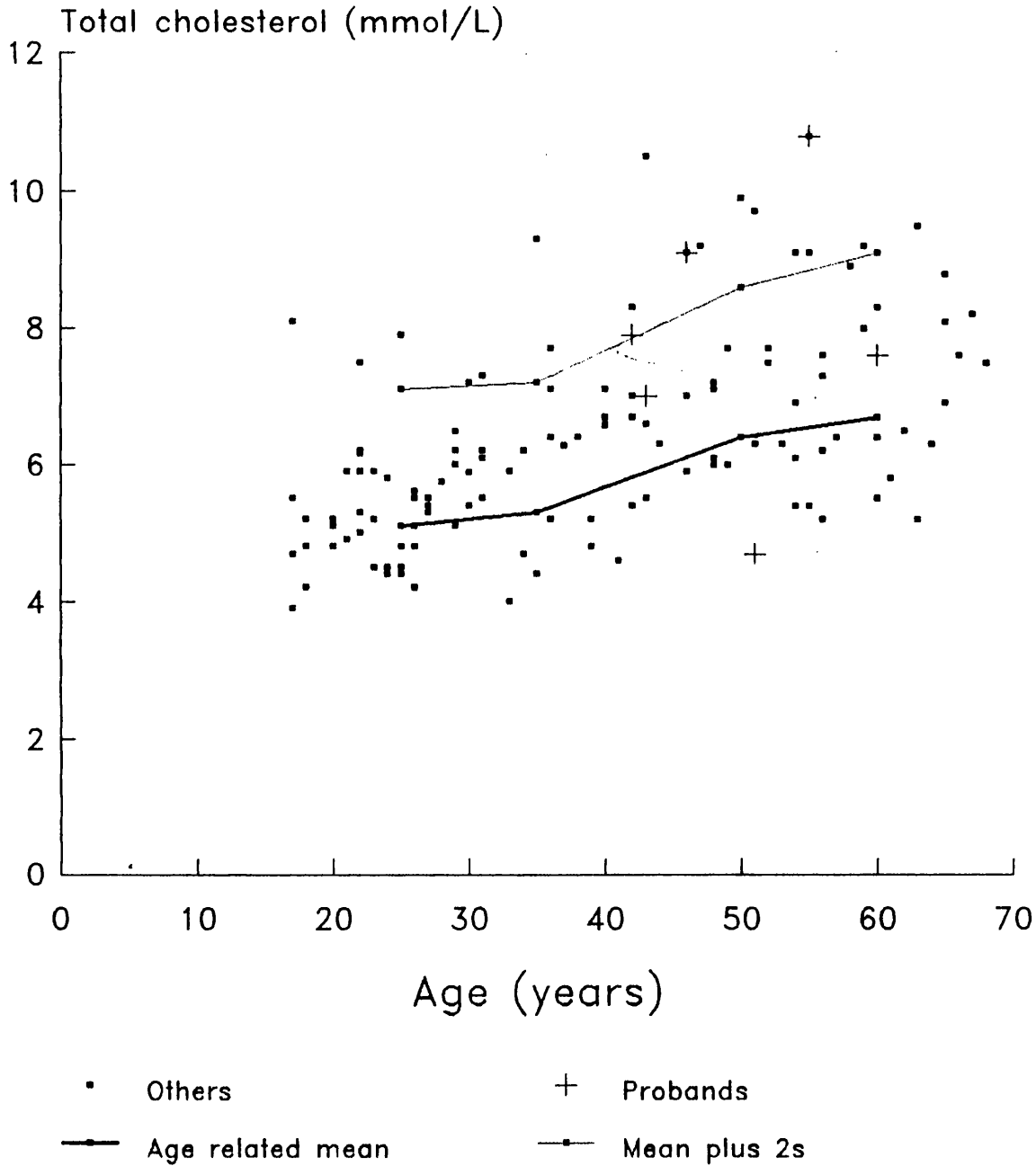


Figure 20. Plasma total cholesterol concentrations in female probands and female family members

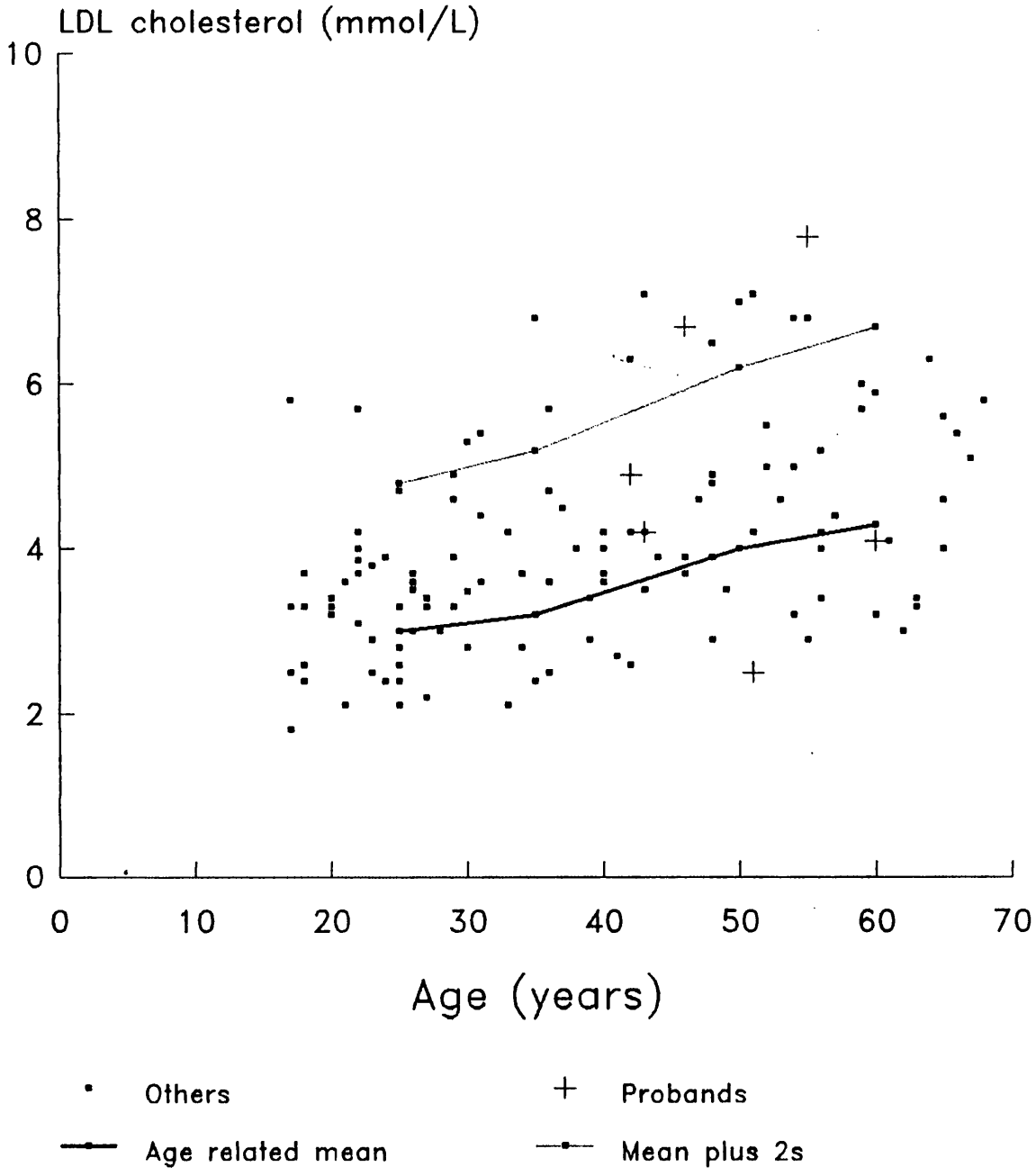


Figure 21. Calculated LDL cholesterol concentrations in female probands and female family members

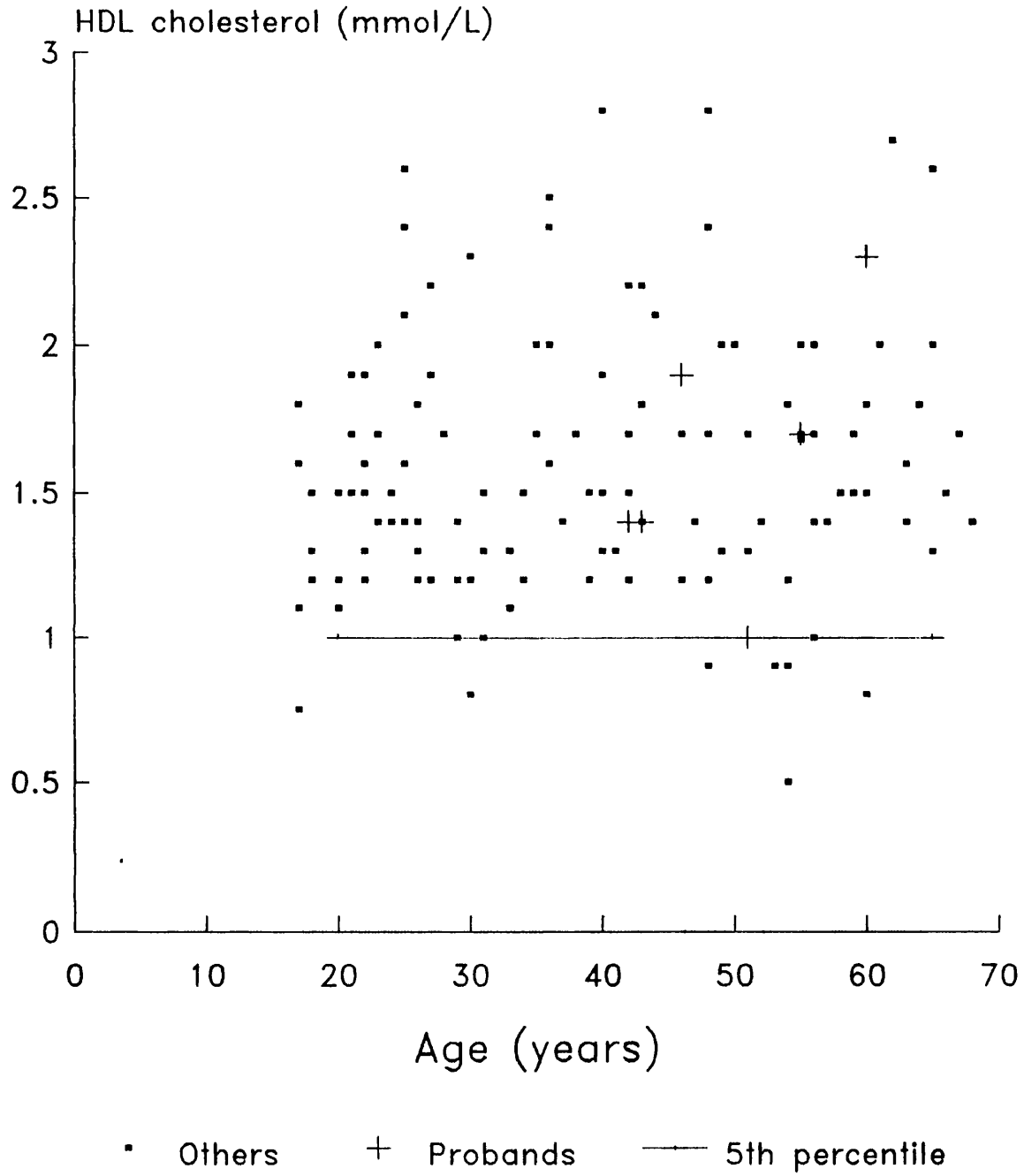


Figure 22. Plasma HDL cholesterol concentrations in female probands and female family members

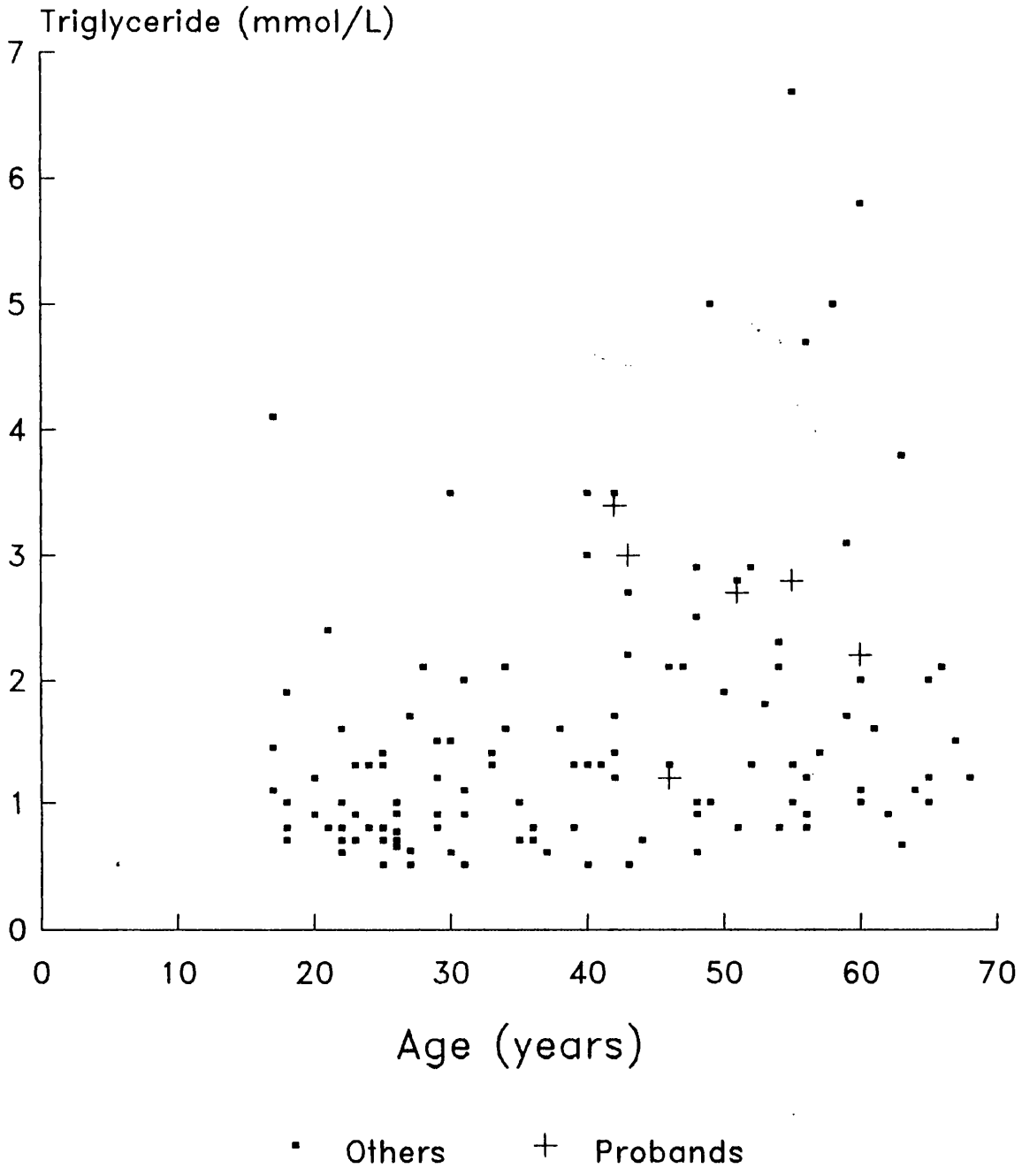


Figure 23. Plasma triglyceride concentrations in female probands and female family members

(d) Plasma lipids in probands and other family members

Although the mean total cholesterol level was similar in both male (mean=6.2mmol/L, s=1.6mmol/L) and female (mean=6.2mmol/L, s=1.6mmol/L) non-probands, the changes in cholesterol concentration with age were markedly different. Total cholesterol concentrations peaked in males at the 50-54 year old age group and thereafter declined, while in females values continued to increase with age with no discernable peak occurring. HDL cholesterol showed no discernible trends in either males or females although females had higher overall mean HDL cholesterol concentration (females 1.6mmol/L, males 1.3mmol/L) and higher HDL cholesterol concentrations throughout most age ranges. Triglyceride concentrations (after normalisation of the data using a log transformation) were higher in males than females and increased with age in males, peaking at between ages 45 and 54 years and thereafter declining. Triglyceride concentrations did not vary with age in females.

Lipoprotein profiles were determined by the same procedures, and in one of the participating laboratories, as those used for the National Lipid Screening Project (Mann et al., 1988). The results however were markedly different from those obtained in that and other control studies. For non-probands, 22/122 males (18%) and 16/128 females (12.5%) had levels of total cholesterol greater than two standard deviations above the age related means determined by the National Lipid Screening Project. For HDL cholesterol, 21/118 males (17.7%) and 8/128 females (6.3%) showed levels below the 5th. percentile (HDL cholesterol was not measured on 4 male patients with triglyceride concentrations exceeding 4.5mmol/L). In those individuals with clinical CHD (28 males and 12 females), 13 (32%) were hypercholesterolaemic

with levels greater than two standard deviations above their age and gender related means ($p<0.01$) and additionally in 5/28 males (17%), HDL cholesterol level was below the 5th percentile. From the clinical examinations and family data, probands did not appear to be affected with clinical familial hypercholesterolaemia.

(e) ApoE isoform patterns

ApoE isoform patterns (figure 24 and table 17) indicate that the relative allele frequencies in non-probands are similar to those reported for other Caucasian populations (Davignon et al., 1988). Numbers in the proband group were too small to reach a conclusion about the isoform pattern. No proband or other family member was homozygous for the ε-2 allele. Furthermore, electrophoresis of isolated VLDL from all individuals studied did not reveal VLDL of abnormal mobility (so called "floating ß-lipoprotein"), indicating that it is unlikely that there were any probands or other family members with clinically significant variant apoE isoforms (figure 25).

Table 17. Number (and frequency) of apoE isoforms in probands (n=26) and non-probands (n=250)

ApoE Isoform	Number (frequency) of apoE isoforms		
	Probands	Non-probands	All
E4/E4	0	2 (1%)	2 (1%)
E4/E3	10 (38%)	38 (15%)	48 (17%)
E4/E2	0	3 (1%)	3 (1%)
E3/E3	14 (54%)	163 (65%)	177 (64%)
E3/E2	2 (8%)	44 (18%)	46 (17%)
E2/E2	0	0	0

Figure 24. ApoE phenotyping using isoelectric focusing in polyacrylamide rods.

Apo E phenotypes are from left to right: lane 1 E4/E4, lane 2 E4/E3, lane 3 E3/E3, lane 4 E3/E2, lane 5 E2/E2.

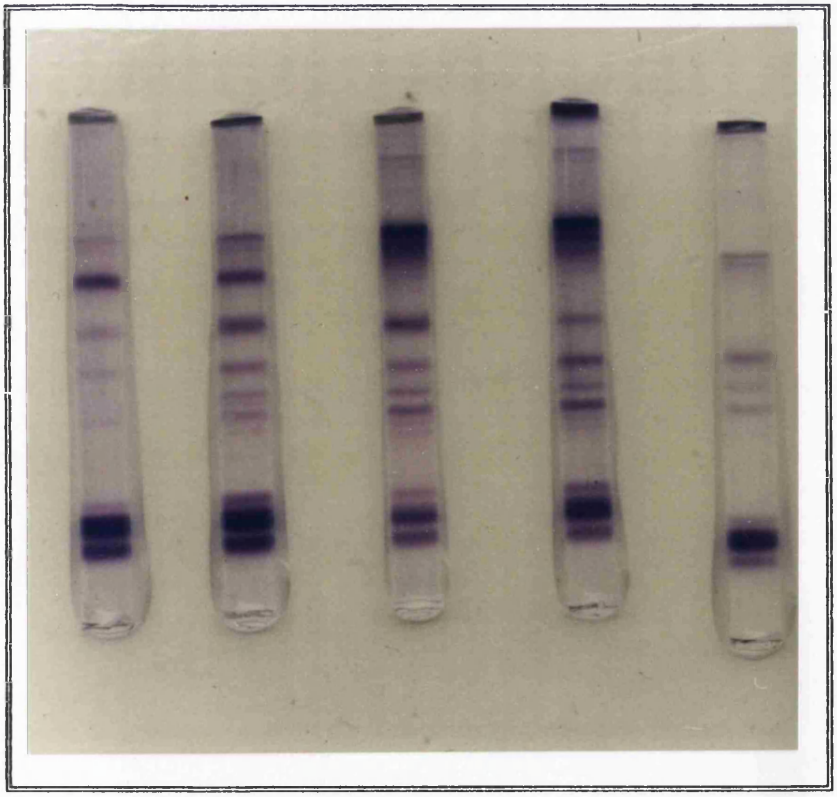
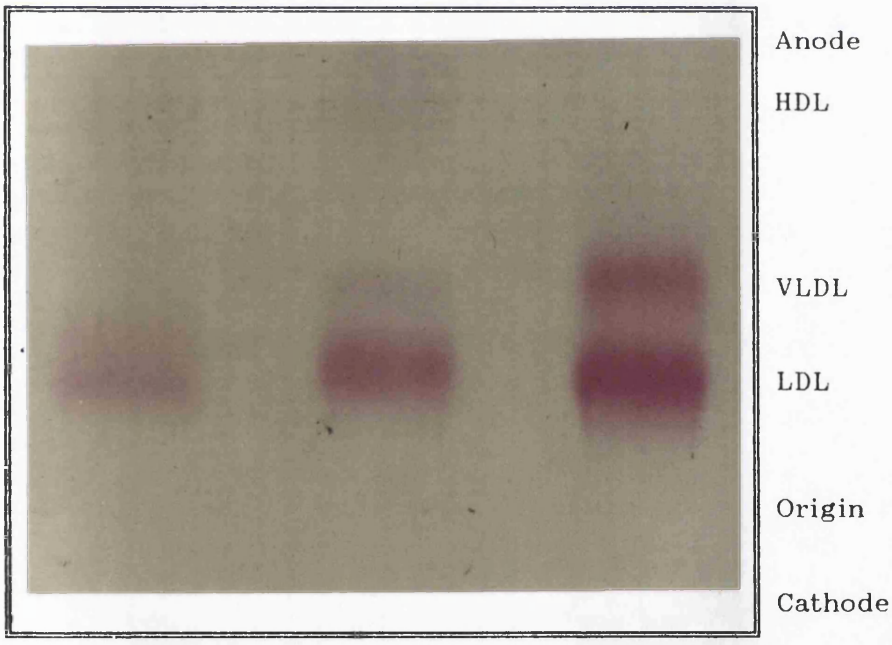


Figure 25. Agarose electrophoresis showing lipoprotein particles with ß-mobility but with density less than 1.006g/ml (floating ß-lipoprotein).

Electrophoretic strips are from left to right: lane 1 normal serum, lane 2 serum from patient with Type III, lane 3 VLDL with abnormal mobility from patient with type III hyperlipidaemia.



(f) Potential confounding effects of anti-hypertensive drugs prescribed for probands and other family members

Anti-hypertensive drugs prescribed for participants and which could affect lipoprotein metabolism, are listed in table 18. ß-blockers were taken by 22 individuals (7 probands and 15 non-probands), with atenolol being the most commonly prescribed ß-blocker (n=17). Angiotensin converting enzyme (ACE) inhibitors were taken by 7 individuals, calcium antagonists by 14 individuals and diuretics were taken by 11 individuals with 3 taking thiazide diuretics. Only 1 individual was taking both a ß-blocker and a thiazide diuretic, although 18 individuals (10 probands and 8 non-probands) were taking more than one anti-hypertensive, most commonly combinations of atenolol with a calcium antagonist.

Table 18. Probands and non-probands taking medications with possible effects on lipoprotein metabolism

Medication	Probands	Non-probands
Atenolol	5	12
Propanolol	1	2
Timolol	1	1
Nifedipine	4	3
Diltiazam	3	4
Enalapril	1	1
Captopril	3	2
Thiazide	0	3
Frusemide	5	1
Amiloride	1	1
Two or more drugs (total)	10	8

3.3 Lipoproteins and apoA-I only containing particles in early onset coronary heart disease

(a) Clinical characteristics in CHD and control groups

Clinical characteristics of patients with CHD and matched controls are given in table 19. All subjects in both CHD and control groups had either never smoked or had not smoked for at least one year prior to sampling.

Table 19. Clinical characteristics (mean ± s) of CHD and control groups

Variable	CHD	Controls
Age (yr)	50.3 ± 6.5	51.6 ± 6.1
BMI (kg/m^2)	26.1 ± 3.8	25.6 ± 3.4
Systolic BP (mm Hg)	132 ± 16	129 ± 12
Diastolic BP (mm Hg)	82 ± 10	80 ± 9

(b) Lipids and apolipoproteins in CHD and control groups

Subjects in the CHD group had higher triglyceride concentrations (p,0.05) than in controls while HDL cholesterol ($p<0.01$) and apoA-I ($p<0.05$) were both significantly lower in CHD than control group (table 20). Lp(a) concentrations were higher in the CHD group compared with controls ($p<0.05$). Statistical analysis was performed initially on the Lp(a) data by setting values of Lp(a)<5mg/dl to Lp(a)=1mg/dl, this being the approximate lower limit of detection as

defined by the manufacturers. The significance tests were repeated after deletion of those individuals with Lp(a)<5mg/dl (One individual in CHD group and four in control group had Lp(a) <5mg/dl). The difference in Lp(a) concentration between the CHD and control group was still significant even after removal of those individuals with Lp(a) concentrations less than 5mg/dl.

Table 20. Lipid and apolipoprotein concentrations (mean ± s, except § median and range) in CHD and control groups (* p<0.05; † p<0.01; ‡ p<0.001)

Variable	CHD	Controls
Total cholesterol (mmol/L)	7.1 ± 1.3	7.0 ± 0.8
LDL cholesterol (mmol/L)	4.9 ± 1.4	4.8 ± 0.7
HDL cholesterol (mmol/L)	1.1 ± 0.4	1.4 ± 0.3†
Triglyceride § (mmol/L)	2.2 (0.8-4.5)	1.5 (0.6-3.7)
Log(triglyceride)	0.32 ± 0.23	0.19 ± 0.18*
ApoA-I (g/L)	1.29 ± 0.24	1.47 ± 0.25*
ApoB (g/L)	0.92 ± 0.23	0.86 ± 0.16
LpAI (mg/dl)	0.41 ± 0.09	0.49 ± 0.11‡
Lp(a) (mg/dl) §	26 (5-57)	15 (5-67)
Log[Lp(a)]	1.37 ± 0.24	1.15 ± 0.09*

Lp(a) was not correlated with any other lipid or apolipoprotein measured in either the control or CHD group (table 21).

Table 21. Spearman correlation coefficients of Lp(a) with other risk factors

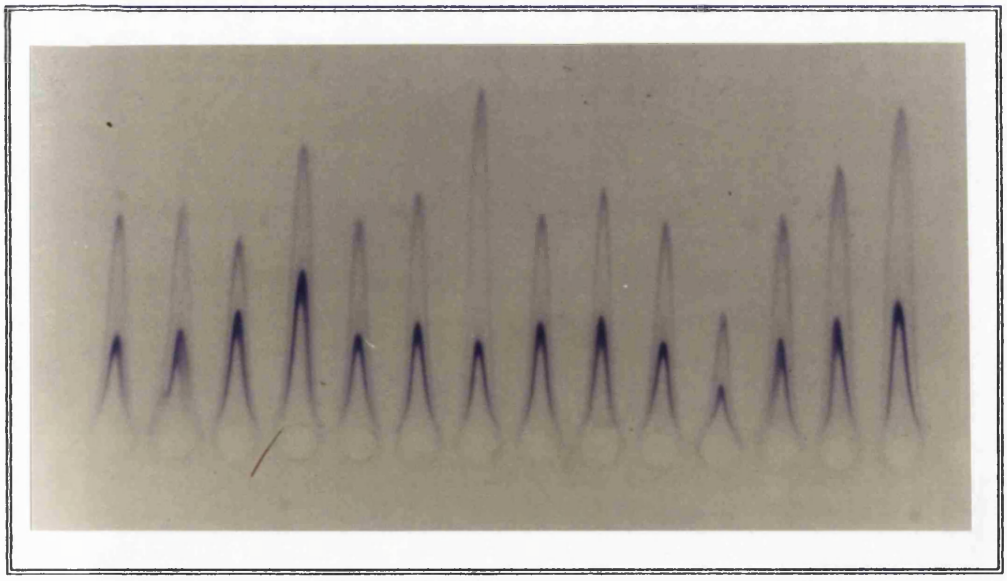
Variable	CHD	Controls
Total cholesterol	0.196	0.112
Triglycerides	-0.243	-0.070
HDL cholesterol	0.208	0.155
ApoA-I	0.110	-0.018
ApoB	0.082	-0.033
LpAI	0.013	-0.061

(c) LpAI particles in CHD and control groups

Subjects in the CHD group had significantly lower LpAI concentrations ($p<0.001$) than controls (table 20).

Figure 26. Electroimmunoassay for LpAI (higher rockets) and apoA-II (lower rockets).

Wells are from left to right: well 1 water, wells 2-11 samples, wells 12-15 standards, well 16 water.



LpAI concentrations accounted for approximately 37% in controls and 33% in the CHD group of total apoA-I.(table 20). LpAI was correlated ($p<0.05$) with HDL cholesterol and apoA-I in both CHD and controls but not with total cholesterol or apoB in either group (table 22).

Table 22. Spearman correlation coefficients of LpAI particles with other risk factors († $p<0.01$)

Variable	CHD	Controls
Total cholesterol	0.245	0.176
Triglycerides	-0.131	-0.222
HDL cholesterol	0.715†	0.596†
ApoA-I	0.682†	0.528†
ApoB	0.175	0.142
Lp(a)	0.013	-0.061

(d) Discriminant function analysis of lipids and apolipoproteins

Univariate discriminant function analysis of the combined CHD and control group showed that the proportion correctly classified, expressed as a percentage, was highest using LpAI concentrations as the discriminator (table 23). Serum triglycerides, HDL cholesterol, apoA-I and Lp(a) all had similar discriminant power. Combining LpAI with apoA-I increased the proportion correctly classified and this classification rate was further improved by including triglycerides. However adding Lp(a) to the variables LpAI, apoA-I and triglycerides did not improve the classification rate.

Table 23. Discriminant analysis of lipid risk factors

Variable	Proportion correctly classified (%)
HDL cholesterol	64.5
Triglycerides	62.8
LpAI	69.2
ApoA-I	64.0
Lp(a)	63.5
LpAI + apoA-I	75.0
LpAI + triglycerides	78.8
LpAI + Lp(a)	69.2
LpAI + apoA-I + triglycerides	80.2
LpAI + apoA-I + triglycerides + Lp(a)	78.8

3.4 The effects of moderate exercise on serum lipids and apolipoproteins in previously sedentary middle-aged men

(a) Baseline data for exercise and control groups

Baseline data for the exercise and control groups shows that the two groups were well matched for age and BMI (table 24).

Table 24. Baseline clinical characteristics (mean ± s) of exercise and control groups

Variable	Exercise	Control
Number	18	16
Age (yr)	53.3 ± 5.9	52.1 ± 6.1
BMI (kg/m^2)	25.4 ± 2.8	24.1 ± 3.3

(b) Serum lipids and apolipoproteins in the control group

There was no significant changes in body weight (or percentage of body fat, data not shown) at twelve months compared with baseline values in the control group. Although there was some seasonal variation in serum cholesterol, LDL cholesterol, HDL cholesterol and triglyceride concentrations, there was no significant changes over twelve months in the control group (table 25). There were similar variations but no significant differences in apoA-I and apoB (table 26). There were no significant differences between baseline and

twelve month LpAI and Lp(a) concentrations. LpAI and Lp(a) concentrations were not measured at three and six months (table 26). Statistical analysis was performed initially on the Lp(a) data by setting Lp(a) concentrations <5mg/dl to Lp(a)=1mg/dl. Statistical tests were then repeated after deletion of men with Lp(a) concentrations <5mg/dl (Six men in the control group had Lp(a) <5mg/dl). There was no significant difference between Lp(a) concentrations at baseline and twelve months even after removal of those individuals with Lp(a) concentrations <5mg/dl.

(c) Training diaries for the exercise group

The average time spent walking per day was 28.5 minutes (range 18 to 45 minutes) corresponding to an average distance walked of approximately 2.1 miles per day (range 1.3 to 3.3 miles). Results from one walker were removed from the calculations due to poor adherence to the training programm.

(d) Dietary intakes and changes in endurance fitness

Mean energy intakes were unchanged over 12 months in both exercise and control groups. Similarly, intakes of protein, carbohydrate, total fat and cholesterol showed no change in either group (data not shown). In walkers, the heart rate and blood lactate concentrations were lower during standardised treadmill exercise after 12 months of exercise compared with baseline (data not shown).

Table 25. Body weight and serum lipid concentrations (mean ± s, except § median and range) over twelve months in the control group

Variable	Baseline	3 months	6 months	12 months
Weight (kg)	75.6 ± 13.4	76.3 ± 13.3	76.0 ± 12.9	75.7 ± 12.9
Total cholesterol (mmol/L)	4.9 ± 0.8	4.6 ± 1.0	4.5 ± 0.9	4.6 ± 1.0
LDL cholesterol (mmol/L)	3.3 ± 0.6	3.2 ± 0.9	2.9 ± 0.7	3.0 ± 0.9
HDL cholesterol (mmol/L)	1.0 ± 0.4	1.1 ± 0.2	1.1 ± 0.3	1.0 ± 0.3
Triglyceride § (mmol/L)	1.3 (0.3-3.2)	1.2 (0.4-1.9)	1.1 (0.5-1.5)	1.1 (0.5-1.8)

Table 26. Serum apolipoproteins, LpAI and Lp(a) concentrations (mean ± s, except § median and range) over twelve months in the control group. Lp(a) data was calculated with (#) and without ($) Lp(a) values <5mg/dl. (¶ Serum Lp(a) and LpAI concentrations were not measured at 3 and 6 months)

Variable	Baseline	3 months	6 months	12 months
ApoA-I (g/L)	1.25 ± 0.22	1.27 ± 0.29	1.36 ± 0.28	1.49 ± 0.38
ApoB (g/L)	0.64 ± 0.11	0.63 ± 0.10	0.64 ± 0.10	0.68 ± 0.12
LpAI (g/L)	0.43 ± 0.10	¶	¶	0.43 ± 0.14
Lp(a) (mg/dl) § n=16 #	5 (5-26)	¶	¶	5 (5-30)
Lp(a) (mg/dl) § n=10 $	8 (5-26)	¶	¶	13 (5-30)

(e) Serum lipids and apolipoproteins in the exercise group

There was no significant changes in body weight or percentage of body fat at twelve months compared with baseline values in the exercise group. Some seasonal variation, with no significant changes over twelve months, similar to that seen in controls were observed in the exercise group for serum cholesterol, LDL cholesterol, triglyceride, apoA-I, apoB, LpAI and Lp(a) concentrations (tables 27 and 28). There was no significant difference in Lp(a) concentrations at baseline and twelve months in the exercise group even after removal of those individuals with Lp(a) concentrations less than 5mg/dl (n=2). Serum HDL cholesterol concentrations at three months were lower than baseline and just reached significance (p=0.044), although concentrations at 6 and 12 months were not significantly different from baseline values.

Furthermore no significant changes in body weight, serum lipids and apolipoproteins over twelve months were seen in a subset (n=7) of the exercise group who did the most exercise (tables 29 and 30). The average time spent walking per day in this sub-group was 35.1 minutes (range 31 to 45 minutes) corresponding to an average distance walked of 2.6 miles per day (range 2.2 to 3.3 miles), compared with an average time spent walking of 28.5 minutes (distance 2.1 miles) walked by the group overall.

Table 27. Body weights and serum lipid concentrations (mean ± s, except § median and range) over twelve months in the exercise group (significantly different compared with baseline * $p<0.05$)

Variable	Baseline	3 months	6 months	12 months
Weight (kg)	82.6 ± 11.8	82.0 ± 11.7	82.6 ± 11.7	82.8 ± 11.8
Total cholesterol (mmol/L)	5.8 ± 1.0	5.7 ± 0.9	5.6 ± 1.2	5.7 ± 1.0
LDL cholesterol (mmol/L)	3.9 ± 0.9	3.9 ± 0.9	3.8 ± 1.0	3.7 ± 1.0
HDL cholesterol (mmol/L)	1.2 ± 0.2	0.9 ± 0.2*	1.0 ± 0.2	1.1 ± 0.3
Triglyceride § (mmol/L)	1.4 (0.8-4.6)	1.5 (0.6-4.0)	1.4 (0.8-4.0)	1.6 (0.8-4.0)

Table 28. Serum apolipoproteins, LpAI and Lp(a) concentrations (mean ± s, except § median and range) over twelve months in the exercise group. Lp(a) data was calculated with (#) and without ($) Lp(a) values <5mg/dl. (¶ Serum Lp(a) and LpAI concentrations were not measured at 3 and 6 months)

Variable	Baseline	3 months	6 months	12 months
ApoA-I (g/L)	1.40 ± 0.19	1.54 ± 0.23	1.52 ± 0.17	1.50 ± 0.24
ApoB (g/L)	0.72 ± 0.13	0.69 ± 0.13	0.80 ± 0.18	0.80 ± 0.19
LpAI (g/L)	0.45 ± 0.09	¶	¶	0.47 ± 0.12
Lp(a) (mg/dl) § n=18 #	20 (5-79)	¶	¶	21 (<5-67)
Lp(a) (mg/dl) § n=16 $	20 (5-79)	¶	¶	22 (5-67)

Table 29. Body weights and serum lipid concentrations (mean ± s, except § median and range) over twelve months in a sub-set of high mileage walkers (n=7).

(significantly different compared with baseline * $p<0.05$)

Variable	Baseline	3 months	6 months	12 months
Weight (kg)	80.9 ± 7.4	80.5 ± 7.9	81.2 ± 7.9	82.0 ± 8.3
Total cholesterol (mmol/L)	5.9 ± 1.2	5.9 ± 1.3	5.9 ± 0.8	6.0 ± 1.1
LDL cholesterol (mmol/L)	3.9 ± 1.3	4.0 ± 1.3	4.1 ± 1.0	4.1 ± 1.0
HDL cholesterol (mmol/L)	1.2 ± 0.3	0.9 ± 0.3*	1.0 ± 0.3	1.1 ± 0.3
Triglyceride § (mmol/L)	1.5 (0.9-4.6)	1.5 (1.0-4.6)	1.4 (0.8-4.6)	1.7 (0.8-3.7)

Table 30. Serum apolipoproteins, LpAI and Lp(a) concentrations (mean ± s, except § median and range) over twelve months in a sub-set of high mileage walkers (n=7).

(¶ Serum Lp(a) and LpAI concentrations were not measured at 3 and 6 months)

Variable	Baseline	3 months	6 months	12 months
ApoA-I (g/L)	1.45 ± 0.20	1.46 ± 0.23	1.48 ± 0.17	1.48 ± 0.19
ApoB (g/L)	0.80 ± 0.22	0.81 ± 0.19	0.71 ± 0.10	0.73 ± 0.10
LpAI (g/L)	0.47 ± 0.07	¶	¶	0.49 ± 0.08
Lp(a) (mg/dl) §	19 (5-67)	¶	¶	17 (5-57)

3.5 Effects of lipoprotein lipase and hepatic lipase activities on HDL composition and metabolism

(a) Validation of LPL and HL assays

Time course experiments, conducted by stopping the reaction at 15, 30, 60 and 75 minutes, showed that measured LPL and HL activities were linear for up to 75 minutes incubation time (figure 27). There was essentially zero activity when plasma HL was inhibited by SDS and treated plasma was incubated with the LPL substrate in the absence of heat-treated serum. Maximum LPL activity occurred when the substrate contained 5-10% (v/v) of heat-inactivated serum (figure 28).

(b) Reference ranges for normolipaemic adults

The mean activities (and standard deviation) of LPL and HL in the 10 minute post heparin plasma from normal males and females (table 31) were similar to previously published values (Gamlen and Muller, 1980).

Table 31. Plasma post heparin LPL and HL activities (mean ± s) in normolipaemic adults

	Males	Females
Number	11	12
LPL activity (μmol/ml/hr)	3.9 ± 0.9	3.8 ± 1.0
HL activity (μmol/ml/hr)	9.3 ± 1.7	7.2 ± 1.6

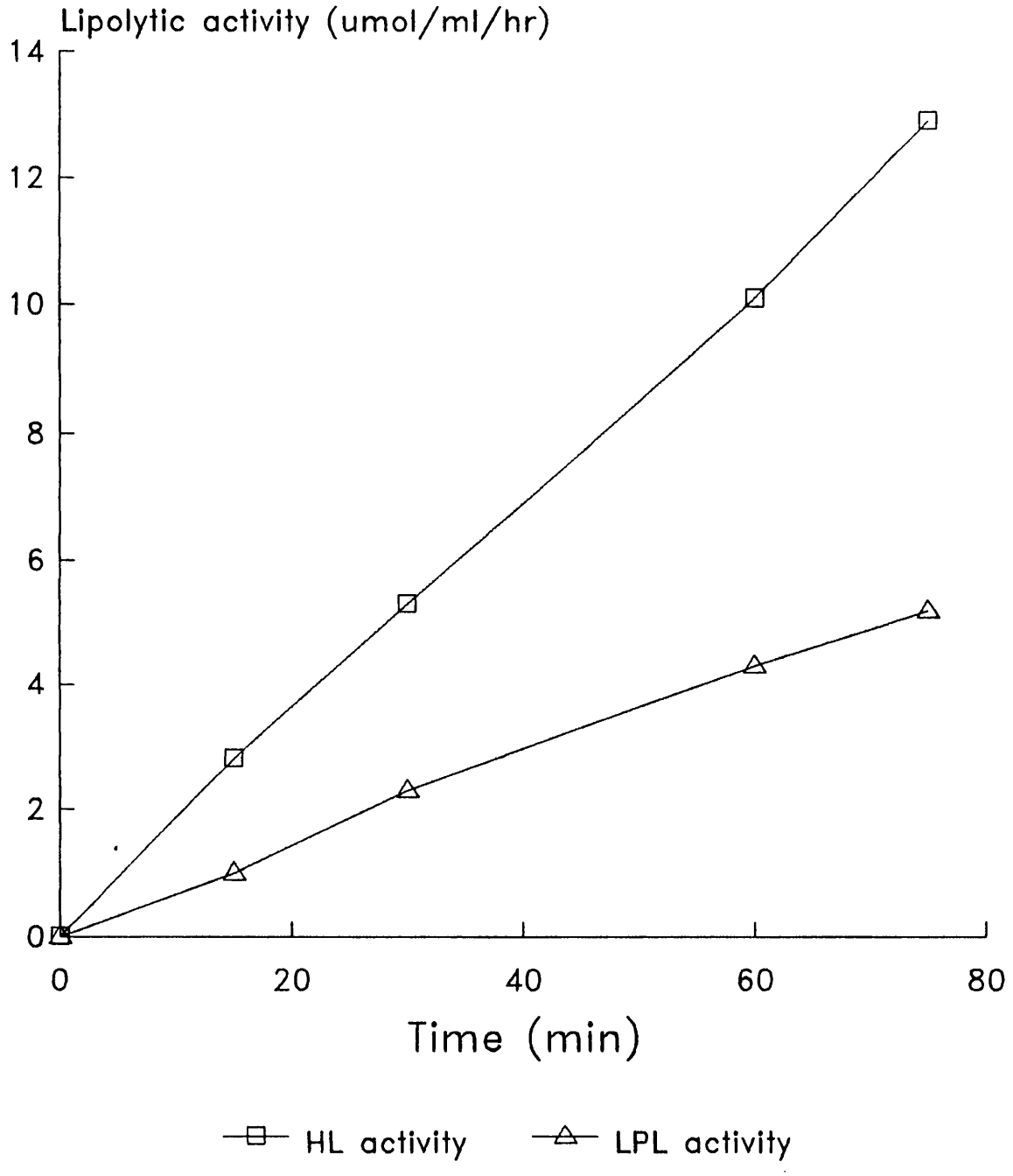


Figure 27. The relationship of post heparin LPL and HL activities to incubation time

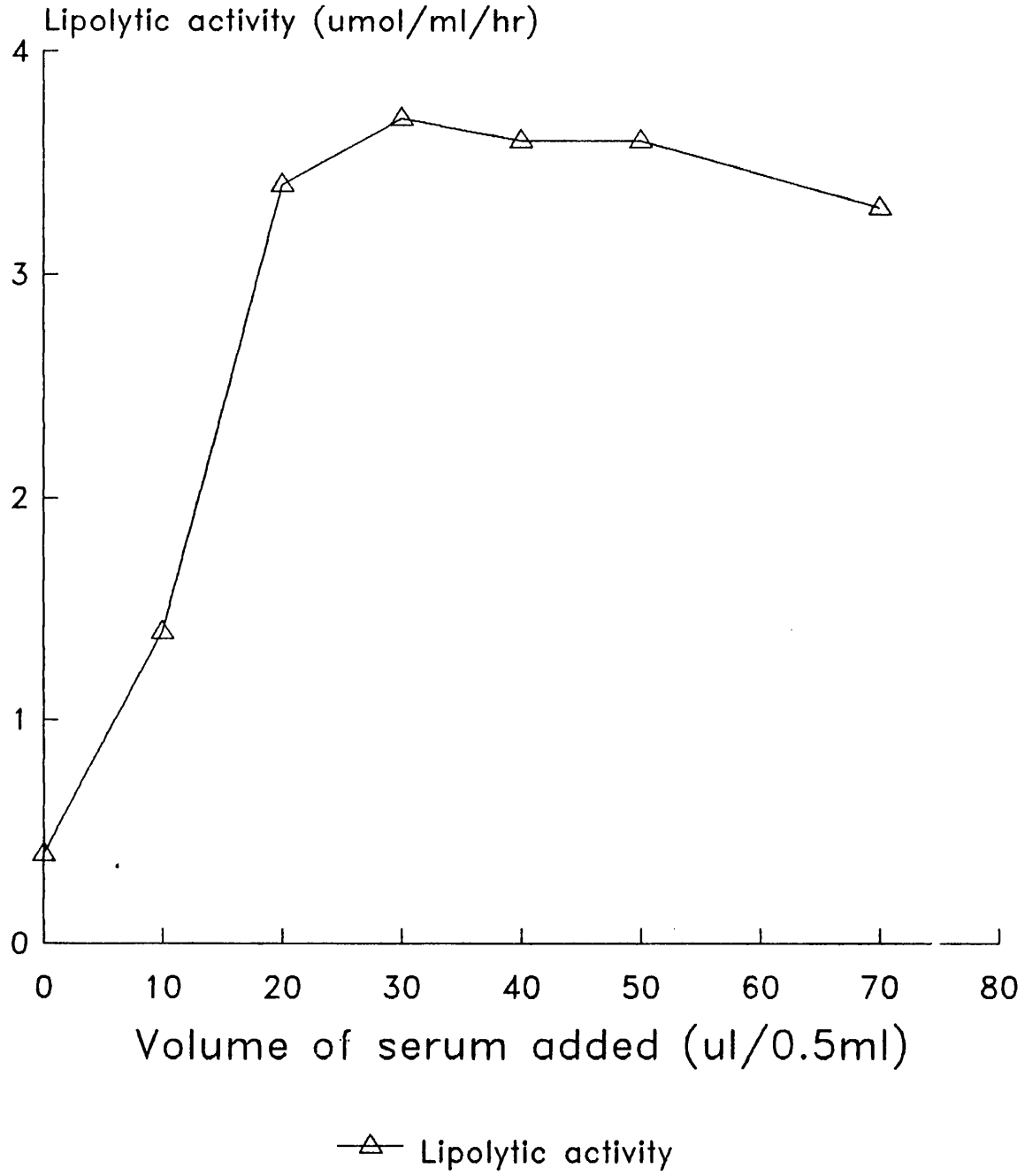


Figure 28. The effect of heat inactivated serum on SDS-treated post heparin plasma lipolytic activity

(c) Time course for post heparin lipolytic activity in normolipaemic adults

HL peak activity in normolipaemic males and females was reached within 5 minutes of intravenous injection of heparin and thereafter tended to slowly decline although there was no significant difference in HL activities at the four time points of 5, 10, 20 and 30 minutes. The maximum activity of LPL activity occurred between 20-30 minutes after heparin administration with the 5 minute LPL activity being significantly different ($p<0.01$) from the 10 minute sample. Although LPL activities continued to rise from 10 to 30 minutes there was no significant difference in the activities between the 10, 20 and 30 minute samples (males figure 29; females figure 30).

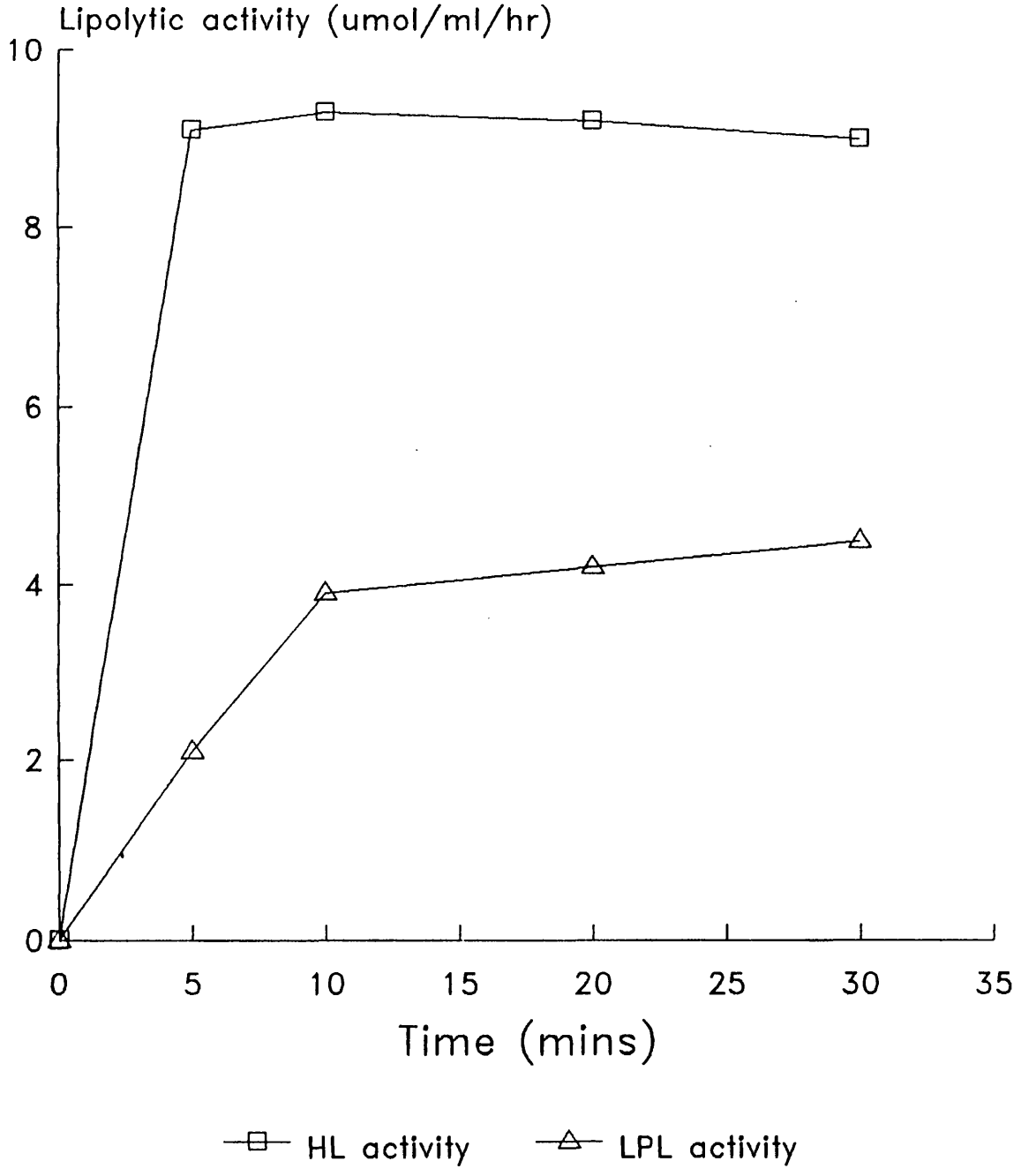


Figure 29. Time course for post heparin lipolytic activity in normolipaemic males

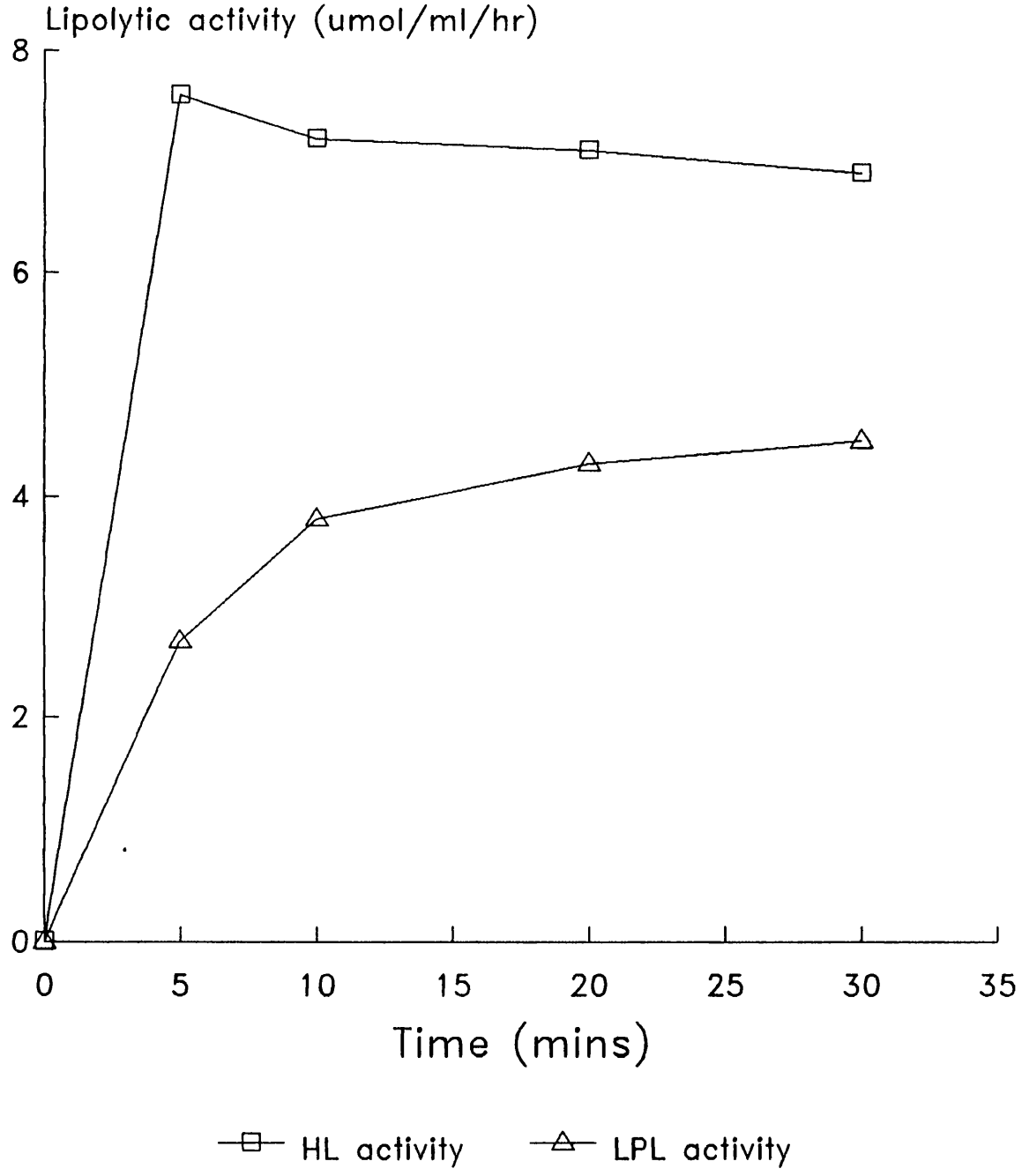


Figure 30. Time course for post heparin lipolytic activity in normolipaemic females

(d) Post heparin plasma lipolytic activity and lipoproteins in males and females with low and high HDL cholesterol

Serum lipid and apolipoprotein concentrations for adults with low HDL cholesterol (serum HDL cholesterol concentration <5th percentile) are given in table 32 and for those with high HDL cholesterol (serum HDL cholesterol concentration >50th percentile) are given in table 33.

Table 32. Serum lipid and apolipoprotein concentrations and ten minute post heparin plasma lipolytic activity in adults with low HDL cholesterol (mean ± s, except § median and range)

Variable	Males (n=13)	Females (n=13)	All (n= 26)
Cholesterol (mmol/L)	6.6 ± 0.7	5.4 ± 1.7	6.2 ± 1.3
Trig.§ (mmol/L)	4.1 (1.5-7.8)	1.7 (0.7-3.4)	3.2 (0.7-7.8)
HDL-chol (mmol/L)	0.80 ± 0.07	0.92 ± 0.13	0.84 ± 0.11
ApoA-I (g/L)	1.15 ± 0.09	1.35 ± 0.11	1.22 ± 0.14
ApoA-II (g/L)	0.36 ± 0.06	0.34 ± 0.05	0.35 ± 0.06
LpAI (g/L)	0.34 ± 0.06	0.36 ± 0.07	0.35 ± 0.07
10 min LPL (µmol/ml/hr)	3.0 ± 0.6	3.2 ± 0.8	3.1 ± 0.7
10 min HL (µmol/ml/hr)	12.8 ± 2.6	9.6 ± 1.8	11.4 ± 2.9

Table 33. Serum lipid and apolipoprotein concentrations and ten minute post heparin plasma lipolytic activity in adults with high HDL cholesterol (mean ± s, except § median and range) and significance of low versus high HDL cholesterol groups (male v. male, female v. female and all v. all;
* p<0.05, † p<0.01, ‡ p<0.001)

Variable	Males (n=12)	Females (n=13)	All (n= 25)
Chol. (mmol/L)	6.0 ± 1.0	5.2 ± 1.1	5.7 ± 1.1
Trig.§ (mmol/L)	1.9 (0.8-4.6)‡	1.2 (0.4-1.9)‡	1.7 (0.4-4.6)‡
HDL-chol (mmol/L)	1.4 ± 0.2‡	1.7 ± 0.1‡	1.5 ± 0.2‡
ApoA-I (g/L)	1.50 ± 0.15‡	1.63 ± 0.13‡	1.55 ± 0.15‡
ApoA-II (g/L)	0.46 ± 0.05†	0.37 ± 0.07	0.41 ± 0.06†
LpAI (g/L)	0.42 ± 0.08	0.53 ± 0.09†	0.47 ± 0.08
10 min LPL (µmol/ml/hr)	4.0 ± 0.8*	4.1 ± 0.6*	4.0 ± 0.8*
10 min HL (µmol/ml/hr)	9.6 ± 1.9†	7.1 ± 1.6*	8.7 ± 1.9†

Significance tests of post heparin lipolytic activity (PHLA) showed that LPL was significantly higher ($p<0.05$) in the high HDL cholesterol group compared with the low HDL group for both male and female while HL was significantly lower between the two groups in males ($p<0.01$) and in females ($p<0.05$). While apoA-I was significantly different ($p<0.001$) between the two groups for both males and females, apoA-II was significantly different in males only ($p<0.01$), but not in females. However in males, LpAI concentrations were not significantly different between the low and high HDL cholesterol groups but LpAI concentrations were significantly different between the two groups in females ($p<0.01$).

(e) Post heparin plasma lipolytic activity and lipoproteins in subjects with low and high HDL cholesterol

HDL cholesterol and apoA-I concentrations were correlated with LPL and inversely correlated with HL in both the low and high HDL cholesterol groups. The respective correlations of LPL and HL with apoA-II concentrations were much weaker and did not reach significance for either group. LpAI concentrations were inversely correlated with HL activities in both groups but not correlated with LPL activities in either group (table 34).

Table 34. Spearman correlation coefficients for ten minute post heparin plasma lipolytic activity with lipid and apolipoproteins subjects with low and high HDL cholesterol concentrations (* p <0.05, † p <0.01, ‡ p <0.001)

	Low HDL-cholesterol (n=26)		High HDL cholesterol (n=25)	
	10 minute LPL	10 minute HL	10 minute LPL	10 minute HL
HDL chol	0.645†	-0.688‡	0.436*	-0.784‡
ApoA-I	0.421*	-0.600†	0.401*	-0.452*
ApoA-II	0.104	0.144	-0.366	0.080
LpAI	0.229	-0.425*	0.150	-0.481*

Chapter 4: Discussion

4.1 Comparison of precipitation procedures used to isolate HDL

The experiments were designed to test if the differences reported between various precipitation methods were due to co-precipitation of HDL, particularly LpAI particles and to determine if the methods differed in their ability precipitate Lp(a). Warnick *et al.* (1979a) found that there was slight co-precipitation of HDL by all methods. However comparisons between evaluations by different investigators are difficult as there are often small but important differences in the protocols used. Thus Wiebe *et al.* (1985) found that dextran sulphate (MW 50,000) and sodium phosphotungstate underestimated serum HDL cholesterol compared with the heparin-manganese chloride method (initial manganese chloride concentration 1mol/L) but that there was good agreement between the heparin-manganese chloride method and PEG 6000, while Demacker *et al* (1980b) found good agreement between all methods when using serum. Inspection of the protocols shows that the final concentrations of PEG 6000 differ (Demacker *et al.* used 75g/L unbuffered PEG, Wiebe *et al.* used 100g/L PEG buffered to pH 10) and some difference in the way the sodium phosphotungstate reagent was prepared (Demacker reagent adjusted to pH 6.15, Wiebe pH unadjusted). More recent comparisons have involved a larger number of samples (82 EDTA-plasma samples) than this study but the larger study involved collecting samples over a two year period with the consequent problems of maintaining good assay precision (Warnick et al., 1985).

In the present study, HDL free cholesterol, HDL phospholipid, HDL apoA-I and LpAI concentrations were lower by the heparin-manganese chloride method 2 (initial manganese chloride concentration 1mol/L) compared with the heparin-manganese chloride method 1 (initial

manganese chloride concentration 0.5mol/L). These results are consistent with previous reports which have shown that heparin-manganese chloride method 2 precipitates some HDL from serum (Warnick and Albers, 1978b). Furthermore, results here show that LpAI particles are precipitated by the heparin-manganese chloride method 2, although the percentage of total apoA-I circulating as LpAI and the mean percentage differences for apoA-I and LpAI between the heparin-manganese chloride method 1 and heparin-manganese chloride method 2 indicate that LpAI is not selectively precipitated by the heparin-manganese chloride method 2. Both serum and plasma have been used for HDL analysis and are often used interchangeably even though the heparin-manganese chloride method 1 procedure, originally developed for serum, is not suitable for EDTA-plasma and similarly these results indicate that the heparin-manganese chloride method 2 procedure should not be used to measure HDL in serum as the higher concentration of manganese chloride may cause co-precipitation of HDL cholesterol from serum.

The agreement between heparin-manganese chloride method 1 and dextran sulphate-magnesium chloride methods was excellent with only modest differences for HDL cholesterol, apoA-I and LpAI. Others have found good agreement using dextran sulphate (MW 50,000)-magnesium chloride. This procedure has been proposed as a selected method and is reported to be less sensitive to small changes in dextran sulphate or magnesium chloride concentrations than other methods using dextran sulphate of different molecular masses (Warnick et al., 1982). HDL cholesterol concentrations using the PEG 6000 method were on average 4.1% lower than results by the heparin-manganese chloride method 1, indicating that there was slight co-precipitation of HDL.

Mean percentage differences were similar for apoA-I and LpAI measured in the HDL supernatants, indicating that although some HDL may be co-precipitated, LpAI is not selectively precipitated by the PEG 6000 method. Demacker *et al.* (1980a) found that PEG 6000 concentrations greater than 80g/L precipitated HDL while small amounts of apoB-containing lipoproteins were left in solution at PEG 6000 concentrations less than 65g/L. A final concentration of 75g/L was chosen although the results presented here indicate that even at 75g/L small amounts of HDL may be precipitated and PEG 6000 concentrations of 70g/L may be more suitable. Some of the differences observed between PEG 6000 methods and other precipitation procedures may be due to variations in the PEG 6000 preparations from different suppliers (Kostner et al., 1985), although others have reported only small differences between methods using PEG 6000 from different sources (Demacker et al., 1980a). The optimum PEG 6000 concentration for PEG 6000 from different sources could be determined using titration experiments. Another reason for the differences observed with PEG 6000 methods is the viscosity of the solutions and careful pipetting of the solutions using positive displacement pipettes is required to achieve good precision, even when using relatively low concentrations of PEG 6000.

HDL cholesterol results by the phosphotungstate method were in good agreement with those by the heparin-manganese chloride method 1. The final concentrations of phosphotungstate (5mmol/L) and magnesium (16.7mmol/L) used in this comparison were lower than those previously used and which have been found to cause co-precipitation of HDL (Warnick et al., 1979a).

Measurement of apoB in the HDL supernatants after precipitation

showed that all apoB-containing lipoproteins were precipitated by the various precipitation techniques. Similarly, Lp(a) was undetectable in the supernatants even after concentration by ultracentrifugation to approximately 20% of the original sample volume and measuring Lp(a) in lower dilutions than those used for the standard Lp(a) assay.

These results indicate that there is good agreement between different precipitation procedures, with the exception of heparin-manganese chloride method 2, when methods incorporating recent modifications to the methods are employed. Heparin-manganese chloride 2 (manganese chloride 1mol/L) method caused co-precipitation of HDL cholesterol from serum emphasising that methods cannot be applied indiscriminately to serum or plasma and that the appropriate concentrations of precipitation reagents must be used. Others have reported that activation of blood clotting factors in plasma by dextran sulphate may reduce the effective dextran sulphate concentration and cause incomplete precipitation of apoB-containing lipoproteins (Demacker et al., 1987). This interaction may also lead to the appearance of fibrin which interferes with HDL cholesterol measurement during the analytical phase causing spuriously high HDL cholesterol values (Greenberg and Warnick, 1985). These results again emphasise that different assay conditions may be required for serum and plasma.

Interlaboratory surveys have found that the between-laboratory variation was highest in hypertriglyceridaemic samples (McMillan and Warnick, 1988). Although these experiments were not designed to determine which method is most suitable for measuring HDL cholesterol in hypertriglyceridaemic samples, others have found that the PEG 6000 method yields the highest proportion of clear

supernatants and is effective at precipitating apoB-containing lipoproteins up to serum triglyceride concentrations of 18.5mmol/L (Demacker et al., 1980a). This is probably due to relatively greater co-precipitation of serum proteins which would decrease the background density of the supernatant and make sedimentation of precipitated apoB-containing lipoproteins easier.

The mean size and density of LpAI and LpAI:AII particles varies between individuals but within individuals LpAI is always larger and less dense than LpAI:AII (Kilsdonk et al., 1990). Those LpAI particles with the lowest density and greatest proportion of lipid would be the particles most likely to be precipitated if any HDL was precipitated during the isolation procedures. Results here indicate that although small differences may occur between the methods, these differences are not associated with selective precipitation of LpAI particles. Any differences reported in other studies between the different isolation procedures are unlikely to be due to contamination of the HDL supernatants with Lp(a) as all precipitation methods completely precipitated Lp(a). Furthermore, although most epidemiological data on HDL cholesterol has been gathered using the heparin-manganese chloride method 1, the small differences in HDL cholesterol, apoA-I and LpAI concentrations between this method and the dextran sulphate and phosphotungstate methods indicates that either could be routinely used to isolate HDL. Small differences were also observed using PEG 6000 although this reagent is more difficult to use due to the viscosity of the solution.

Specific recommendations for HDL cholesterol measurement have not yet been set although the Laboratory Standardisation Panel of the National Cholesterol Education Program has recommended that methods

for cholesterol measurement should have a coefficient of variation below 5% and bias below 5%, (compared with Centre for Disease Control reference values), with the aim of improving both precision and accuracy to achieve a target of 3% or less for each of these. Results here show that with careful attention to detail, all HDL cholesterol isolation techniques studied are also capable of achieving good precision, although an assessment of bias will be more difficult. No comparisons were made here with ultracentrifugation as this method itself has several drawbacks including contamination of the HDL density fraction with apoB-containing lipoproteins (Albers et al., 1972), changes in lipoprotein composition (Kunitake and Kane, 1982) and incomplete recovery of lipoproteins due to manipulative losses during fractionation. Rather, these experiments were conducted to determine the relative relationships between the precipitation methods for isolating HDL. Many factors need to be taken into account before one method can be recommended. Some have proposed dextran sulphate-magnesium chloride as a selected method (Warnick et al., 1982) while others have recommended PEG 6000 (Demacker, 1985). Additional factors which may influence the selection of a proposed method, but not studied here, include the ability to measure HDL cholesterol in hyper-triglyceridaemic sera, interference by the precipitants in enzymic cholesterol methods and whether HDL cholesterol is measured in serum or plasma. These and other factors such as storage temperature, may account for the large intralaboratory variations in HDL cholesterol measurements reported in other studies (Warnick et al., 1983; McMillain and Warnick, 1988). The present study has shown that good precision can be achieved, using carefully defined conditions, for all the methods studied here.

4.2 Dyslipoproteinaemia UK families with severe expression of coronary heart disease

The families studied were recruited through our interests in hidden factors predisposing to premature clinical coronary heart disease. The aims were to recruit affected families with no perceived risk associations and to store material for future genetic studies. However, of 15 families completely studied only one had no lipid abnormality, and overall this 26-family study showed an enhanced expression of two well-established dyslipoproteinaemic risk associations - hypercholesterolaemia with LDL excess, or reduced levels of HDL, or both, in patterns consistent with polygenic influences on lipid and apolipoprotein levels. Familial combined hyperlipidaemia may also be involved, but the underlying defects have not yet been defined and a rigorous criteria for this diagnosis have not yet been established. The was no evidence that unrecognised heterozygous familial hypercholesterolaemia (FH) contributed to the observed expression of dyslipoproteinaemia. The heterogeneity of gene defects in FH renders any direct genetic analysis impractical at present.

The changes in plasma lipids with increasing age were similar to those previously reported (Mann et al., 1988). In that and the present study, total cholesterol concentrations in women continued to increase with age and overtook levels in men around 55 years as levels in men reached a plateau and then declined. This reversal of the pattern observed in younger adults when cholesterol concentrations are usually higher in men than women is probably due to changes in lipoprotein metabolism in post-menopausal women.

HDL cholesterol did not vary with age in either males or females

although others studying North American populations have shown a progressive increase in HDL cholesterol in women and a sharp increase in HDL cholesterol in men over 55 years (Heiss et al., 1980). Triglyceride concentrations did not vary with age in females but increased with age in males, peaking at between ages 45 and 54 years and then declining. Others have found that changes in triglyceride concentrations with age are similar for both males and females in North American populations (Rifkind and Segal, 1983) although other studies of British populations have reported similar trends in HDL cholesterol and triglycerides to those found here (Mann et al., 1988).

ApoE isoform frequencies in non-probands were similar to those previously reported (Davignon et al., 1988). There was an increased frequency of apoE4/E3 and a reduced frequency of apoE3/E3 and apoE3/E2 in probands. The ϵ-4 allele is known to be associated with higher levels of total and LDL cholesterol than the ϵ-3 or ϵ-2 alleles (Davignon et al., 1988). ApoE polymorphism also influences plasma Lp(a) and triglyceride concentrations parallel to that of LDL. The ϵ-2 allele is associated with lower concentrations of Lp(a) than the ϵ-3 or ϵ-4 alleles (de Knijff et al., 1991) and a meta-analysis of 45 population studies from 17 different countries showed that individuals with the ϵ-2 allele have lower concentrations of triglycerides and higher concentrations of HDL cholesterol than those with the ϵ-4 allele (Dallongeville et al., 1992). However the number of probands studied were too few to draw any definite conclusions about apoE isoform frequencies in males with premature CHD although there was no laboratory or clinical evidence that unrecognised familial Type III dysbetalipoproteinaemia contributed to the observed

expression of dyslipoproteinaemia.

Some of the participants were taking anti-hypertensive drugs which may affect lipoprotein metabolism and have a confounding effect on the observed association between abnormal lipoprotein profiles and premature CHD. This potential contribution is now difficult to assess in detail as further follow-up is difficult owing to mobility and death of some participants. Thiazide diuretics may increase VLDL and LDL cholesterol levels but have little or no effect on HDL cholesterol, although long term studies indicate that serum lipids may return to baseline values (Ames, 1986a). Beta-blockers can increase VLDL levels, especially in individuals who are already hypertriglyceridaemic, and decrease HDL cholesterol concentrations but probably have little or no effect on LDL cholesterol levels (Durrington et al., 1985; Ames, 1986b). There are fewer reports of the effects of calcium channel blockers or angiotensin converting enzyme (ACE) inhibitors and although they may slightly decrease serum total cholesterol and triglycerides while increasing HDL cholesterol, their overall effect on serum lipid concentrations is probably minor (Ames et al., 1986b).

In the present study, 7 probands and 17 family members were taking beta-blockers and/or thiazides at the time of analysis. The most commonly prescribed drug was Atenolol, a cardioselective beta blocker, which in some studies has been shown to have little effect on total cholesterol and triglycerides when given alone (Chamshi-Pasta et al., 1988). While it is difficult to assess the precise effects of anti-hypertensive drugs in the present study, the overall effect was probably minor. The potential adverse associations of anti-hypertensive medication were not commented on by the mainly cardiac units referring the patients for this study.

Concern has been expressed about the safety and efficacy of lowering serum cholesterol concentrations (Smith and Pekkanen, 1992; Oliver, 1992) as using drugs to modify serum lipid concentrations in primary prevention trials reduced non-fatal myocardial infarction, but there was no difference in total number of deaths between individuals on treatment and those taking placebo due to the increases in non-cardiac mortality (Frick et al., 1987, World Health Authority Clofibrate Trial, 1980; Lipid Research Clinics Program, 1984). It is possible that increases in non-cardiac mortality are due to chance rather than some specific mechanism as the increase in non-cardiac mortality was spread over various unrelated causes (Jacobs et al., 1992). However many of the non-CHD related deaths in the treatment groups were due to suicide or violent deaths and it has been postulated that lowering serum cholesterol by lipid lowering drugs may alter the number of serotonin receptors on the surface of brain cells, leading to decreased intracellular serotonin and alterations in mood (Engleberg, 1992). Furthermore low plasma cholesterol concentrations in older men are associated with depression which is a major risk factor for suicides and accidents (Morgan et al., 1992) and with increased risk of mortality from injuries (Lindberg et al., 1992). Others have questioned the validity of these findings as depressed people often have poor appetites leading to weight loss and lower cholesterol concentrations (Smith and Shipley, 1993) and others have been unable to demonstrate an association between depression and low cholesterol (Strandberg et al., 1993).

However these concerns with the non-cardiovascular effects and general safety of lipid-lowering therapy should be addressed in the

knowledge that uncontrolled dyslipoproteinaemia also has severe adverse associations. Results from HMGCoA reductase inhibitor trials, drugs which are very effective in reducing LDL cholesterol, should determine if reductions in high serum cholesterol concentrations are associated with increases in non-cardiac mortality or if these increases are purely due to chance. CHD is the commonest single cause of death in the United Kingdom and abnormal serum lipoprotein profiles are a major risk association. This study has shown the extent of unrecognised dyslipoproteinaemia even in those families with severe, premature CHD.

Further genetic studies are proceeding with the DNA collected during the present study, and new insights into CHD expression may follow but it is unlikely that some major new factor will appear to explain the severe expression of CHD in the British families studied. Although the clinical expression of LDL excess may be enhanced through oxidative change to promote uptake by arterial wall, or the presence of high levels of Lp(a), LDL excess is the baseline requirement through which such other proposed mechanisms probably operate. The association of CHD with dyslipoproteinaemia and the low appreciation of this association by the cardiac referring units may at least partially explain the occurrence of CHD in these individuals.

4.3 Lipoproteins and apoA-I only containing particles in early onset coronary heart disease

LpAI particles, containing approximately 40% of total apoA-I (Koren et al., 1987) have been proposed as the anti-atherogenic fraction of HDL (Puchois et al., 1987) and might therefore be a better index of

coronary risk than either HDL or apoA-I. Results here, showing that the concentrations of LpAI particles are reduced in early onset CHD, are in agreement with other studies which have shown decreased LpAI concentrations in patients with angiographically proven coronary artery disease (Puchois et al., 1987) and in patients prior to coronary artery bypass surgery (Coste-Burel et al., 1990).

An early study measured apoA-I and apoB in male survivors of myocardial infarction and reported that apolipoproteins were as good as total cholesterol, triglycerides or HDL cholesterol below age 50 and better in the sixth to eight decades at discriminating between atherosclerotic subjects and controls (Avogaro et al., 1979). A later study of middle-aged men with and without previous myocardial infarction found that apoB was a better discriminator than total serum cholesterol, triglycerides, LDL cholesterol, HDL, HDL2 and HDL3 cholesterol. Discrimination was marginally improved by combining apoB with apoA-I (Durrington et al., 1986).

Univariate discriminant function analysis of results in the present study show that the discrimination was slightly improved by measuring LpAI particles rather than apoA-I: the percentage correctly classified was superior using LpAI particles rather than apoA-I or HDL cholesterol alone. However neither HDL, Lp(a), apoA-I or LpAI used alone resulted in a high degree of correct classification although measuring LpAI particles rather than apoA-I marginally improved discrimination between controls and those with CHD (Vallance et al., 1991). Discrimination was further improved by combining LpAI, apoA-I and triglycerides. These results differ from those of Coste-Burel *et al.* (1990) who found that the highest rate of correct classification using a single variable was obtained using

apoA-I and that measuring LpAI in addition to apoA-I did not improve discrimination between healthy and ischaemic heart disease groups. However the Coste-Burel study differed in a number of aspects from our study including the selection of patients (pre CABG versus post CABG respectively). In addition, while LpAI concentrations in the CHD groups were similar in both studies, LpAI concentrations were higher (mean 0.62 g/L) and mean age lower (mean 46 years) in the control group of Coste-Burel's study than in our study (mean LpAI 0.55 g/L, mean age 51.6 years). There is little variation in LpAI levels with age in men (Steinmetz et al., 1990) and the age differences between the two studies are unlikely to explain the differences observed between the two studies. However the percentage of total apoA-I circulating in plasma in the form of LpAI was similar to previously reported values (Koren et al., 1987). Although both apoA-I and LpAI concentrations were lower in the CHD group compared with controls, the percentage of total apoA-I circulating as LpAI was lower in the CHD group than controls, providing further evidence that LpAI is a better discriminator of CHD than total apoA-I.

LpAI:AII particles were not measured in this study, although others have shown that in normolipaemic subjects, those with angiographically defined coronary artery disease have lower serum LpAI, but not LpAI:AII, particle concentrations, when compared to those with normal coronary arteries and to the asymptomatic group (Puchois et al., 1987). Later studies showed that both LpAI and LpAI:AII were decreased in hypertriglyceridaemic subjects prior to coronary artery bypass surgery (Coste-Burel et al., 1990). The discrepancy in LpAI:AII concentrations between normolipaemic and

hypertriglyceridaemic subjects with coronary artery disease may be due to reduced formation of LpAI:AII particles during lipolysis in hypertriglyceridaemic subjects (Genest et al., 1990).

The mechanism by which HDL might exert its protective effect is unknown, the two most attractive mechanisms being reverse cholesterol transport hypothesis and the remnant particle hypothesis. High concentrations of LpAI particles could potentially benefit either mechanism. Small, discoidal apoA-I containing HDL with pre-ß mobility and LpAI particles (which include pre-ß HDL) have both been shown to be potent stimulators of cholesterol efflux from cultured fibroblasts and may therefore have important roles in the process of reverse cholesterol transport. Some have shown that LpAI but not LpAI:AII particles promote cholesterol efflux from cultured mouse adipocytes (Barbaras et al., 1987) while others have shown that both LpAI and LpAI:AII function equally well at removing cholesterol from a variety of cultured cells including human skin fibroblasts and rabbit aortic smooth muscle cells (Johnson et al., 1991). LpAI particles may be important in the remnant particle hypothesis as pre-ß HDL is generated during the lipolysis of triglyceride-rich lipoproteins (Neary et al., 1991) and those individuals who rapidly process triglyceride-rich particles would be expected to have the lowest concentrations of atherogenic remnants and the highest concentrations of LpAI and pre-ß HDL particles.

Clearly further work is required to define the precise metabolic function of the two types of apoA-I containing particles. Measurement and use of LpAI particles in cell cultures and in studying the processing of triglyceride-rich particles might be useful in identifying the precise mechanisms by which HDL exerts its

protective effects against atherosclerosis.

Neither the Coste-Burel study or the study using men with angiographically proven coronary artery disease (Puchois et al., 1987) measured Lp(a). However although Lp(a) concentrations were significantly higher in the CHD group compared with the control group, the proportion correctly classified was not improved by adding Lp(a) results to the LpAI results or by adding Lp(a) to the other three variables (LpAI, apoA-I and triglycerides). Lp(a) concentrations were not correlated with other known risk associations such as total cholesterol and apoB or with HDL cholesterol and apoA-I, again emphasising that there are some differences in the regulatory mechanisms which control the synthesis and catabolism of LDL and those which control Lp(a) even though both are apoB-100 containing lipoproteins. Cross sectional studies have reported that Lp(a) concentrations are higher in those with CHD compared with matched controls without CHD (reviewed by Morriset et al., 1987). Some cross sectional studies have reported that high serum concentrations are found in individuals with FH and CHD compared with those with FH but without CHD (Seed et al., 1990; Wiklund et al., 1990), while others have been unable to confirm this finding (Mbewu et al., 1991). Differences in the findings of these studies may arise from the methods used to assign individuals to the CHD or control groups as coronary angiography was not used to define the extent of CHD in any of the three studies nor was angiography used to assess coronary arteries in the control populations. Furthermore Lp(a) was not a discriminator of future coronary events in the Helsinki Heart study (Jauhiainen et al., 1991) although a prospective study of 50 year old Swedish men followed for 6 years showed that

those who developed clinical CHD had significantly higher Lp(a) levels (Rosengren et al., 1990), although this prospective study should be interpreted with some caution as only men, all of the same age, were studied.

The number of subjects and controls in the present study is small and case-control studies are known to be subject to many errors. All men with CHD had undergone coronary angiography and CABG at least six months before sampling. However controls did not have coronary angiography and some individuals in the control group may have had asymptomatic CHD. Inclusion of such individuals in the control group would weaken the discriminating power of the lipids and apolipoproteins measured. Additionally, the effects of diets on lipoprotein particles was not studied. Although diets commonly used to lower serum lipid concentrations do not appear to reduce Lp(a) concentrations, diets high in polyunsaturated fatty acids are known to decrease LpAI concentrations in comparison to diets high in saturated fatty acids (Fumeron et al., 1991). No specific dietary advice had been given to the men studied here although two men had adopted a Mediterranean style diet which was low in total fat with an increased proportion of mono-unsaturated fat and high in carbohydrate. Alcohol is known to increase both LpAI and LpAI:AII concentrations at least during short term moderate alcohol intakes (Valimaki et al., 1991), while chronic alcohol abuse causes an increase in LpAI:AII and a decrease in LpAI concentrations (Puchois et al., 1990). The effect of alcohol on lipoproteins was minimised by matching the two groups for alcohol consumption and by excluding those with excessive alcohol consumption.

The results obtained here, and those from larger studies, show that

LpAI concentrations are reduced in patients with early onset CHD and that LpAI concentrations may be useful in discriminating between those who will develop premature CHD and those who will not. However the discriminating power was only marginally better using LpAI rather than using apoA-I concentrations and a prospective study would be required to determine if measurement LpAI concentrations (and similarly Lp(a) concentrations) would be a better discriminator of CHD risk rather than the serum lipid and apolipoproteins traditionally measured. Measurement of LpAI particles may therefore be useful in research to identify the precise mechanisms by which HDL may exerts its protective effects against atherosclerosis but LpAI measurement cannot be recommended as a useful addition to the lipid and apolipoprotein tests currently available for assessing the risk of CHD in patients.

4.4 The effects of moderate exercise on serum lipids and apolipoproteins in previously sedentary middle-aged men

Samples generated during the study were batched up and measured together at the end of the study after storage at -70°C. Preliminary work had shown that there was no deterioration in lipid measurements compared with day 1 (data not shown), a finding confirmed by others for HDL cholesterol (Nanjee and Miller, 1990) and for apoA-I and apoB (Albers and Marcovina, 1989).

Low intensity, moderate exercise over a one year period in previously sedentary middle-aged non-smoking men did not produce

any significant changes in serum lipids or apolipoproteins. Total cholesterol concentrations were not influenced by exercise in the present study, a finding reported by other longitudinal studies (Table 35). Similarly no changes occurred in serum triglyceride concentrations. Others have suggested that exercise training only lowers serum triglycerides when pre-training values are greater than 1.7 mmol/L (Haskell, 1986) and in the present study median triglyceride levels were below 1.7mmol/L for both the control and exercise group. No changes in Lp(a) concentrations were observed with the present study, possibly because Lp(a) concentrations are influenced more strongly by genetic rather than environmental factors. Dietary changes and drugs which may be effective in modifying other serum lipid and apolipoprotein concentrations appear to have little or no effect on Lp(a) concentrations (Lawn, 1992), although diet-induced weight loss reduced Lp(a) concentrations in obese patients (Sonnichsen et al., 1990).

The men participating in the trial had relatively low HDL cholesterol concentrations (mean HDL cholesterol in both walkers and controls was <50th percentile). It would be reasonable to expect such a group would be more likely to show any changes rather than men who already had high HDL cholesterol concentrations. There were also no significant changes in body weight in either the control or exercise group during the study. Men in the exercise group (BMI 25.4, mean weight 82.6kg) were heavier than the control group (BMI 24.1, mean weight 75.6kg) and again it would be reasonable to expect any exercise induced weight loss to be greater in this group than in a group of leaner men. Furthermore no changes in serum lipid and apolipoprotein concentrations were observed in those walkers who

showed a decrease in body fatness as assessed by hydrostatic weighing.

Although cross-sectional studies have shown that physically active people have higher HDL cholesterol concentrations than sedentary controls, conflicting reports have appeared in longitudinal studies concerning the effects of exercise on serum lipids and apolipoproteins. Most longitudinal studies have shown that regular exercise is associated with favourable changes in serum lipoprotein profiles (Huttunen et al., 1979; Sopko et al., 1985; Baker et al., 1986; Schwartz, 1987; Thompson et al., 1988; Marti et al., 1990) while other studies have failed to demonstrate any beneficial effect of exercise on serum lipoproteins (Gaesser and Rich, 1984; Hagen et al., 1986; Raz et al., 1988; Despres et al., 1988). These apparently contradictory results between different studies may be due partly to differences in design and partly due to the lack of understanding of the precise mechanisms by which exercise can influence serum HDL cholesterol concentrations. Most studies have been relatively short and many studies lack statistical power either because of small numbers of participants (Nye et al., 1981; Stubbe et al., 1983; Sopko et al., 1985; Thompson et al., 1988) or because a control group was not properly assigned (Stubbe et al., 1983; Schwartz, 1987; Despres et al., 1988). Some studies have shown that exercise-induced increases in serum HDL cholesterol concentrations occur only when there is weight loss (Williams et al., 1983), although other studies have demonstrated significant increases in serum HDL cholesterol concentrations without weight loss (Sopko et al., 1985; Thompson et al., 1988) or no change in serum HDL cholesterol concentrations with significant weight loss (Gaesser and Rich 1984; Findlay et al., 1987; Despres et al., 1988).

Others have reported that exercise and weight loss induced by dieting could both produce similar increases in serum HDL cholesterol concentrations (Wood et al., 1988). Furthermore a meta-analysis of 95 published reports (Tran and Weltman, 1985) and a study of the effects of exercise and weight loss on plasma lipoproteins (Sopko et al., 1985), concluded that exercise and weight loss independently increase HDL cholesterol concentrations and that the effects are additive when both weight loss and exercise occur together. These findings imply that increases in HDL cholesterol can occur in the early phases of training programs and that further increases in HDL cholesterol concentrations may occur if weight loss subsequently occurs (Krauss, 1989). Indeed a study of the effects of prolonged exercise training in men found that HDL cholesterol concentrations and apoA-I concentrations were significantly higher after 14 weeks of the exercise program but although HDL cholesterol concentrations remained significantly increased, apoA-I concentrations were not significantly different from baseline levels after 32 weeks of the training program (Thompson et al., 1988). The authors concluded that these results and studies on the fractional catabolic rate of apoA-I during the training program indicate that the metabolic changes that occur early in training are different from those that occur with continued endurance exercise.

The effects of exercise on body weight, lipids and apolipoproteins for some of the more important longitudinal studies are summarised in table 35.

Table 35. Summary of some longitudinal studies on the effects of exercise on body weight, serum lipids and apolipoproteins. (NS not significant, S significant, § not measured)

Authors	Date (yr)	Exercise	No.	Duration (weeks)	Change in value from baseline					No. of controls
					Weight	T. chol	Trig	HDL-C	ApoAI	
Huttunen et al	1979	Jogging	50	16	NS	NS	S	S	NS	50
Nye et al	1981	Aerobics	17	10	NS	NS	NS	NS	§	0
Wood et al	1983	Jogging	48	52	S	NS	NS	NS	NS	33
Williams et al	1983	Jogging	36	52	S	NS	NS	S	§	28
Sopko et al	1985	Walking	5	12	NS	NS	S	S	§	5
Baker et al	1986	Walking	20	20	S	S	S	S	NS	14
Schwartz et al	1987	Aerobics	14	12	NS	NS	NS	S	S	0
Findlay et al	1987	Jogging	51	30	S	S	NS	NS	§	0
Despres et al	1988	Cycling	12	3	S	NS	S	NS	NS	0
Thompson et al	1988	Cycling	8	48	NS	NS	S	S	NS	0
Wood et al	1988	Jogging	47	52	S	NS	S	S	§	42
Raz et al	1988	Jogging	28	9	NS	NS	S	NS	§	27
Weintraub et al	1989	Jogging	6	7	NS	NS	S	NS	§	0
Marti et al	1990	Jogging	39	16	NS	NS	NS	S	NS	22

Exercise might produce favourable changes in serum lipoprotein profiles as a result of improved processing of triglyceride-rich particles. Some longitudinal studies have shown that exercise can increase post heparin plasma LPL activity (Thompson et al., 1988; Weintraub et al., 1989), while others have been unable to detect any change in LPL activity (Stubbe et al., 1983). Cross sectional studies have produced conflicting reports with one study being unable to demonstrate any difference in post heparin plasma LPL activity between endurance athletes and sedentary men (Sady et al., 1988) while another study found that plasma LPL activities were higher in long distance runners than in sedentary men (Nikkila et al., 1978). However even when there was no difference in LPL or HL activities between athletes and sedentary controls, the fat clearance rate following an intravenous infusion of a standard triglyceride emulsion in the athletes was almost twice that of sedentary controls (Sady et al., 1988) and postprandial lipaemia was reduced in athletes due to shorter chylomicron-triglyceride half-life in comparison with sedentary controls (Cohen et al., 1989). This indicates that athletes can process triglyceride-rich particles quicker than sedentary controls even when there was no apparent difference in post heparin LPL and HL activities in plasma. Therefore the high serum HDL cholesterol concentrations observed in endurance athletes and the increases in serum HDL cholesterol concentrations associated with exercise programs may partly be due to increased catabolism of triglyceride-rich lipoproteins, the surface components of these particles being picked up by nascent HDL (and HDL3) leading to an increase in HDL mass. This is supported by a study which found that peripheral HDL3 cholesterol production in the human forearm was

increased in exercising muscle but that there was no measurable production of HDL3 in patients with lipoprotein lipase deficiency (Ruys et al., 1989). There are two possible mechanisms by which exercise might increase muscle LPL activity. Fatty acids, derived from intramuscular triglyceride stores, are the primary energy source for exercising muscles and depletion of these intramuscular triglyceride stores may stimulate LPL synthesis and secretion in muscle capillaries (Hurley et al., 1986). Furthermore, training enhances the ability of muscle to oxidise fatty acids which would reduce the degree of end product inhibition of LPL activity (Kiens et al., 1989). Secondly, increased LPL activity during prolonged exercise training may also be due to increased capillarisation of muscles (Kiens et al., 1980). The increased activity of muscle LPL leads to a reduction in serum triglyceride concentration as lipid is hydrolysed from VLDL and surface components transferred to HDL leading to an acute increase in serum HDL cholesterol concentration without a corresponding change in serum apoA-I (Sady et al., 1986).

However the controversy about whether weight loss is required to produce changes in serum lipoprotein concentration and composition is still unresolved. In non-obese people, fasting is known to decrease adipose tissue LPL but muscle LPL either increases or remains the same (Eckel, 1989) and any effect on serum HDL composition may depend on the relative contribution to total lipolytic activity from these two tissues. In addition, changes in dietary habits can produce changes in HDL cholesterol concentrations with high carbohydrate diets tending to decrease HDL cholesterol and increase triglyceride concentrations. Finally plasma volume is known to expand with training (Thompson et al., 1988) and reduce with weight loss (Reisin

et al., 1983). HDL cholesterol concentrations uncorrected for changes in plasma volume may be underestimated during exercise and overestimated during weight loss. Any changes in HDL cholesterol concentrations are likely to be strongly influenced by the design of the study and while weight loss may be important to provoke changes, it is more likely that changes in LPL activity exert a stronger influence on HDL cholesterol.

In the present study, there were no significant changes in serum triglyceride or HDL cholesterol concentrations nor were there any exercise-induced changes in HDL subclass distribution towards a potentially anti-atherogenic pattern as there were no changes in total apoA-I or LpAI concentrations. The failure to demonstrate any beneficial changes in the serum lipoproteins in the present study may have been due to a failure to exceed an exercise threshold limit which could result in changes in LPL activity and/or weight loss leading to alterations in serum lipid concentrations and lipoprotein composition. Similar low intensity exercise in women was able to demonstrate a 27% increase in serum HDL cholesterol after one year without any significant changes in weight or body fat (Hardman et al., 1989). However even men who walked for longer than the average for the exercise group failed to show any changes in serum lipids and apolipoproteins or lose weight over the twelve months, Also although 12/17 men walked for two or more miles per day, only 2/17 men achieved the original set target of 3 or more miles per day, despite encouragement and psychological back-up from the exercise supervisors.

One of the major potentially useful ways of modifying serum lipids and apolipoprotein concentrations is by moderate exercise. However

the exercise undertaken by the participants of this study might not have been of sufficient intensity, even in the sub-group of high mileage walkers, to cause a large enough change in muscle blood flow to produce significant changes in the processing of triglyceride-rich lipoproteins or to cause an increase in the synthesis and secretion of LPL and a consequent increase in HDL.

Although other studies (Paffenbarger et al., 1986; Shaper and Wannamethee, 1991) have shown that low intensity exercise such as brisk walking may reduce the risk of CHD, the present findings provide further evidence that the reduction in risk may not be related to changes in serum lipid and apolipoprotein concentrations, at least in normolipaemic men. Low intensity, moderate exercise may have other important effects such as improvements to psychological well-being, improved cardiac function and changes to haemostatic factors. There was clear evidence of training-induced adaptations in the walkers. Both heart rate and blood lactate concentrations were reduced during standardised treadmill exercise tests in walkers and the decrease in heart rate was related to the amount of walking done but despite the improvements in indices of endurance fitness in walkers, brisk walking did not influence serum lipid and apolipoprotein concentrations.

Lifestyle changes more comprehensive than simply increasing the amount of exercise taken may prevent the progression, and even cause regression, of coronary atherosclerosis. Patients with angiographically documented coronary artery disease were assigned to a regime consisting of low fat vegetarian diet, stress management training, cessation of smoking and moderate exercise of similar intensity to that used in the present study (Ornish et al., 1990).

After one year, over 80% of the patient group showed evidence of regression of coronary artery lesions as assessed by quantitative angiography. Serum total and LDL cholesterol concentrations and apoB concentrations fell significantly in the experimental group while HDL cholesterol and apoA-I concentrations were not significantly different from baseline after 12 months. Body weight was significantly lower after one year compared with baseline. Although no attempt was made to determine the relative contribution of each lifestyle change to changes in coronary atherosclerosis, these results suggest that regular, moderate exercise combined with other lifestyle changes may reverse, or at least halt progression of, coronary atherosclerosis. However from this present study, it appears that the potential for exercise-related changes to lipoprotein profiles using low intensity exercise is modest in sedentary middle-aged men. Further work could involve a critical evaluation of the level and intensity of exercise and other lifestyle changes which may be required to produce favourable changes in serum lipid and apolipoprotein concentrations, lipoprotein particle composition and subclass distribution and changes to LPL and HL activities.

4.5 Post heparin plasma lipoprotein lipase and hepatic lipase activities and HDL composition

The modifications, first described by Baginsky and Brown (1979), and adapted here for use in the method described by Nilsson-Ehle and Ekman (1977) allow direct measurement of post heparin plasma LPL and HL activities. LPL activity has traditionally been calculated by measuring total lipolytic activity and then measuring residual

lipolytic activity (equivalent to HL activity) after inhibition of LPL using 1mol/L sodium chloride solution, the difference between the two results being LPL activity. This indirect measurement of LPL activity is clearly an unsatisfactory approach as the choice of buffer pH for measurement of total lipolytic activity is a compromise because the pH optima for the two enzymes differs (Baginsky and Brown, 1979) and the imprecision of the calculated LPL activity is affected by the imprecision of both the total and residual lipolytic activity assays. LPL can be directly measured by using a specific antiserum to HL (Huttunen et al., 1975) or by using SDS to inhibit HL. However SDS has some advantages over HL antiserum as SDS is widely available and inexpensive. This approach to measuring plasma lipolytic activity has been validated here by showing that the assays were linear up to 75 minutes, that HL was inactivated by pre-incubation with SDS as negligible lipase activity was measured using the HL lipase substrate with SDS-treated post heparin plasma, that SDS-resistant lipolytic activity (which should be equivalent to LPL activity) would only hydrolyse the substrate in the presence of serum activator and that reference values for normolipaemic adults were similar to previously published values.

Experiments designed to follow the release of LPL and HL into the systemic circulation following intravenous administration of heparin showed that the time to reach maximum activity for LPL and HL differs. Others have shown that lipolytic activity was dependant on the dose of heparin used. With high doses (100U heparin per kg), there was a more prolonged response for both LPL and HL activities and a higher maximum for LPL activity than with lower doses (20U heparin per kg) although the maximum HL activity is similar for both

doses (Nilsson-Ehle and Ekman, 1977). After injection of heparin, the initial response (from 2 to 10 minutes) for both LPL and HL activity when high doses of heparin were used was almost identical to that when low doses were used (Nilsson-Ehle and Ekman, 1977). A balance has to be struck between administering a large enough dose of heparin to ensure release of LPL and HL from endothelial binding sites and administering a heparin dose that would cause excessive risk to the patient. In this study 100U heparin per kg body weight was used. HL peak activity in normolipaemic males and females was reached within 5 minutes of intravenous injection of heparin and thereafter slowly declined while LPL activities continued to rise from 10 to 30 minutes. There was no significant difference in LPL activities between the 10, 20 and 30 minute samples, although the 5 minute LPL activity was significantly different from the 10 minute sample (Vallance et al., 1993). These results indicate that although the sampling time for optimal activity of both enzymes differs, for convenience 10 minute samples can be used. Reference ranges for normolipaemic adults are similar to previously published values (Gamlen and Muller, 1980), with similar LPL activity in both males and females and higher HL activity in men than women. This gender-related difference in HL but not LPL activity has been described by others (Wang et al., 1981; Kuusi et al., 1989), and may be due to the influence of sex hormones on HL production and secretion, with androgens tending to increase HL production and oestrogens tending to decrease it (Applebaum-Bowden et al., 1985). Normolipaemic controls used to construct the reference ranges for ten minute post heparin LPL and HL activities were not stratified by age but others have found no changes in activity with increasing age (Wang et al., 1981).

Similarly although alcohol consumption and cigarette smoking were not taken into account, others have found that LPL and HL activities are not related to alcohol consumption or to cigarette smoking in either men or women (Applebaum-Bowden et al., 1985).

Measurement of LPL and HL activities in adults with low and high HDL cholesterol showed that LPL activity was higher and HL activity lower (although difference in HL activity did not quite reach significance in women) in the group with high HDL cholesterol compared with the low HDL cholesterol. ApoA-I was also significantly higher in the high HDL cholesterol group for both males and females, although apoA-II was significantly higher in males only, but not in females with high HDL cholesterol. This indicates that elevations in HDL cholesterol are associated with increases in both apoA-I and apoA-II in males but that in females with high HDL cholesterol, apoA-I is predominantly increased without a proportionate increase in apoA-II. Further evidence for this hypothesis is provided by LpAI results. In males, LpAI concentrations were not significantly different between the low and high HDL cholesterol groups but LpAI concentrations were significantly different between the two groups in females (Vallance et al., 1993). LpAI concentrations are known to be higher in women than men (Steinmetz et al., 1990) and although the numbers in each group studied here are small, the elevation of HDL cholesterol and apoA-I in the female high, compared with low, HDL cholesterol groups is associated with a predominant increase in LpAI particles while in men the elevation is probably due to an increase in LpAI:AII particles, with a much smaller increase in LpAI particles. Variations in the concentration of the larger less dense HDL2 subfraction accounts for the most of the variation in total HDL

cholesterol while the smaller denser HDL3 particles vary only within narrow limits. Similarly the difference in LpAI concentrations would account for the some of the difference seen in HDL cholesterol concentrations between men and women. Studies of the fractional catabolic rate (FCR) of HDL apolipoproteins have shown that FCR of apoA-I is reduced in females with high HDL cholesterol and apoA-I while serum apoA-II concentrations are correlated with the apoA-II synthetic rate rather than the FCR (Brinton et al., 1989).

In the combined male and female groups, LpAI concentrations were inversely correlated with HL activities in both low and high HDL cholesterol groups but not correlated with LPL activities in either group. Therefore HL activity appears to influence serum LpAI concentrations more than LPL activity does and those individuals with the highest HL activities are likely to have the lowest concentrations of LpAI particles. Similar results to those described here were found in a study which examined the relationship between plasma HDL cholesterol and post heparin LPL and HL activities and which reported that LPL activity was elevated and HL reduced in those individuals with high HDL cholesterol (mean HDL cholesterol = 2.25 mmol/L) compared with those with low HDL cholesterol (mean HDL cholesterol = 0.88 mmol/L), the difference in HDL cholesterol between the two groups being almost entirely due to a four fold difference in HDL2 cholesterol (Kuusi et al., 1989). In that study the influence of LPL and HL on LpAI particles was not examined although the apoA-I:A-II ratio was significantly correlated with HL but not LPL activity in men.

The risk of CHD within and between populations is inversely related to serum HDL cholesterol concentrations. Any factors which influence

HDL composition and metabolism would be a very important determinant of HDL cholesterol concentrations and therefore CHD risk. Higher HDL2 seen in athletes compared with sedentary people may be due to the differences in LPL and HL activities observed between the respective groups (Nikkila et al., 1987) and one of the mechanisms through which exercise may lead to a less atherogenic lipoprotein profile is by enhanced processing of triglyceride-rich particles, possibly through changes in LPL and HL activities. Furthermore, the relative activities of LPL and HL will strongly influence HDL composition and studies in rats have shown that HDL phospholipids and HDL cholesterol are both increased when HL is inactivated by antibodies to HL (Kuusi et al., 1979; Grosser et al., 1981) and a study using monkeys has reported that inhibition of LPL using monoclonal antibodies caused a fall in HDL protein, HDL cholesterol and total apoA-I (Goldberg et al., 1990b). Other studies have reported that the LPL:HL ratio is lower in individuals with low HDL cholesterol and that this ratio determines HDL composition which is a major determinant of apoA-I FCR and which in turn determines HDL cholesterol levels (Brinton et al., 1988).

Plasma HDL cholesterol concentrations are determined by both the secretion of HDL precursors and intravascular processing of triglyceride-rich lipoproteins by the action of lipolytic enzymes. Rapid lipolysis of triglyceride-rich lipoproteins by LPL releases surface remnants which are taken up by HDL, leading to the formation of HDL2 and thereby increasing the cholesterol carrying capacity of HDL. Impaired lipolysis increases the possibility of neutral lipid exchange, mediated by CETP and other lipid transfer proteins, in which triglycerides are transferred from triglyceride-

rich lipoproteins to HDL in exchange for cholesterol ester. Triglycerides transferred to HDL (and LDL) are hydrolysed by HL while cholesterol ester transferred to triglyceride-rich lipoproteins makes these particles more resistant to lipolysis. The overall effect of this process is to produce the "atherogenic lipoprotein phenotype" consisting of HDL3, small dense LDL and cholesterol-enriched remnant particles (Austin et al., 1990).

The influence of lipolytic enzymes on HDL2 concentrations is consistent with both the reverse cholesterol transport hypothesis and the remnant particle hypothesis. The transfer by CETP of cholesterol from HDL into triglyceride-rich particles could be considered beneficial in the reverse cholesterol transport hypothesis as cholesterol is transferred to particles which will be eventually taken up by the liver but potentially harmful in the remnant particle hypothesis as cholesterol would be transferred from anti-atherogenic particles to atherogenic particles. However the transfer of cholesterol between particles is driven by lipolysis of triglyceride-rich particles. Rapid clearance of triglyceride-rich lipoproteins, leading to HDL2 production and low concentrations of triglyceride-rich particles, would prevent excessive transfer of cholesterol into triglyceride-rich lipoproteins, but impaired clearance and accumulation of triglyceride-rich lipoproteins would lead to low levels of HDL2 and transfer of cholesterol into lipoprotein particles associated with high risk of CHD. Those individuals with genetic deficiency of CETP have high levels of HDL cholesterol and do not develop premature CHD, possibly because there is little transfer of cholesterol from HDL into atherogenic particles and also because circulating levels of lipid-enriched atherogenic LDL are also reduced (Koizumi et al., 1985),

while hyperalphalipoproteinaemia due to HL deficiency is associated with premature atherosclerosis, possibly due impaired metabolism of chylomicron and VLDL remnants and cholesterol enrichment of VLDL from HDL2 by the action of CETP. The three enzymes LPL, HL and CETP are, therefore, all important in the intravascular processing of HDL and may influence the development of atherosclerosis.

Results here imply that LPL and HL have different roles in the metabolism of LpAI and LpAI:AII particles and that the lower concentrations of LpAI seen in men compared with women are due in part to the higher male HL activities. If the protective mechanism mediated by HDL is dependent on interconversion of HDL subfractions and uptake of lipids and apolipoproteins by HDL3 to produce HDL2, then increases in HDL2 would reflect the anti-atherogenic activity of HDL and similarly any shift in the distribution of apoA-I containing particles towards a less atherogenic pattern would be a major potential benefit in reducing atherosclerosis. Furthermore any intervention which might cause an increase in LPL activity and/or a decrease in HL activity such as exercise or treatment of dyslipoproteinaemic patients with fibric acid derivatives would be expected to lower serum triglyceride concentrations and increase HDL cholesterol and apoA-I concentrations.

The factors which regulate HDL composition and metabolism are of fundamental importance to the understanding of lipoprotein metabolism and to the mechanisms through which HDL may protect against CHD. The serum concentrations of LpAI particles are determined by the rate of apoA-I synthesis, the fractional catabolic rate of apoA-I and the distribution of apoA-I within different HDL particles and between other serum lipoproteins. The present results

show that serum LPL and HL activities influence serum HDL cholesterol concentrations and distribution of apoA-I within the spectrum of apoA-I containing particles. However as LpAI concentrations were correlated with HL activity but not LPL activity, it is unlikely that moderate exercise, even if of sufficient intensity to provoke changes in LPL activity, would cause a substantial alteration in LpAI concentrations. This finding has recently been confirmed by a cross-sectional study which examined the distribution of apoA-I in HDL subfractions and found that LpAI particle concentrations did not differ between sedentary and endurance trained normolipaemic men (Frey et al., 1992).

The studies reported here have shown that LpAI particles are not selectively precipitated by HDL isolation procedures but that LpAI is only marginally better than apoA-I at discriminating between those individuals with CHD and those without. Furthermore, moderate exercise did not alter the distribution of apoA-I in the HDL particle spectrum. Observational data reported here on the discriminating power of LpAI particles, the failure of exercise to influence LpAI particle concentrations and the factors which may influence LpAI concentrations in plasma, indicate that further work will be needed, possibly using autologous ^{125}I-labelled particles, to clarify the roles of LpAI particles and LpAI:AII particles and the role of LPL and HL in the metabolism of HDL particles with different apolipoprotein composition. The mechanisms through which HDL might protect against atherosclerosis remain controversial and more studies will be required before LpAI can truly be described as the anti-atherogenic fraction of HDL.

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Appendix A: Fish-eye Disease

Appendix A: Fish-eye disease

Introduction

Fish-eye disease (FED) is a familial syndrome with corneal opacification and ocular appearances resembling boiled fish. Plasma HDL cholesterol ester and apoA-I concentrations are markedly reduced but cholesterol ester concentrations in apoB-containing lipoproteins are normal probably because LCAT is able to esterify cholesterol in apoB-containing lipoproteins but is ineffective when HDL is the substrate. This may be due to changes in LCAT structure and modified substrate preference, as a fish-eye-like syndrome also arises in some individuals with normal LCAT but with a variant apoA-I structure (Funke et al., 1991a). Seven cases of FED have now described, with six cases from three FED families having a homozygous base change at codon 123 of the LCAT gene (Funke et al., 1991b). FED may involve structural defects affecting LCAT at non-catalytic sites, thus modifying substrate specificity (Funke et al., 1991b), as recombinant enzyme with this defect also shows modified substrate specificity (Karmin et al., 1993). Currently, the essential diagnostic procedure is demonstration of negligible esterification of cholesterol associated with HDL or apoA-I proteoliposomes, but normal esterification of cholesterol in VLDL and LDL.

Clinical presentation of the proband

The male patient, born in 1916, had a probable first myocardial infarction (MI) in 1966 age 49 years, having smoked 30 cigarettes per day for 30 years. In 1990 age 73 years he was admitted with chest pain and ECG evidence of infarction but without characteristic enzyme changes. Mixed lipaemia with very low HDL, and cloudy

corneas, were then noted. A diagnosis of Tangier disease was considered. Tonsils had been removed in early adult life.

On detailed review three months later he was generally well, overweight but on a diet and on diuretics (Burinex K). Xanthelasmata and cutaneous or tendon xanthomata were not present, although corneas were hazy. Neck, throat and axillae were normal and liver and spleen were not palpable.

Family history

The parents were British, from different areas of the UK and not known to be related. The father died age 76 years (MI) and the mother, reported to have had very large tonsils in her 60's, and cloudy corneas later, died age 77 (stroke). Neither had any previous clinical history of ischaemic heart disease. The proband's brother died at age 76 and sister at age 81 years both from unspecified cardiac causes and with unremarkable tonsils. The brother had cloudy corneas but the corneal status of the sister is not recorded.

The patient is married to an unrelated spouse and has one son age 43 years and one daughter age 37 years, both without obvious corneal abnormality. Both are resistant to venipuncture.

Laboratory investigations

Routine haematological investigations and renal, electrolyte, liver and thyroid profiles were within reference ranges with the exception of a mildly raised serum urea (7.1-8.0 mmol/L). Serum cholesterol and triglyceride concentrations were moderately increased with markedly reduced levels of HDL cholesterol (measured by a heparin/manganese chloride procedure) and of apoA-I (table 36).

Date	Cholesterol (mmol/L)	Trigs (mmol/L)	HDL-cholesterol (mmol/L)	ApoA-I (g/L)
19.04.90	8.0	4.3	<0.1	*
18.06.90†	5.9	3.5	<0.1	*
15.07.90†	4.7	2.1	<0.1	*
14.09.90†	4.3	2.2	<0.1	*
13.11.90†	4.6	1.9	<0.1	*
23.01.91†	5.4	1.8	0.1	*
21.01.92	7.1	2.1	0.1	*
07.07.92	6.0	2.0	<0.1	<0.16

Table 36. Lipid profiles on patient with fish-eye disease, at diagnosis, on treatment with Simvastatin (†), and later off active therapy except for diet advice (* not measured).

No bands with α-mobility were seen on serum lipid electrophoresis (figure 31).

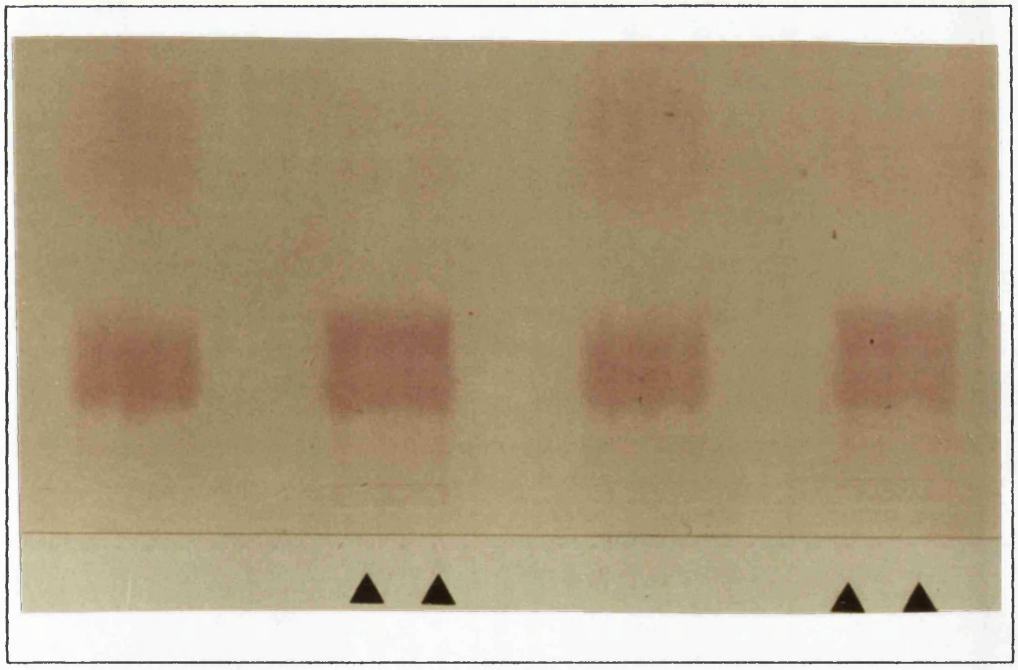

Figure 31. Electrophoresis of serum from proband stained for lipid. Note absence of band with α-mobility in lanes marked by arrows.

Ophthalmological examination

There were no ocular or visual symptoms and ophthalmological examination showed bilateral coarse punctate corneal haze (figures 32 and 33).

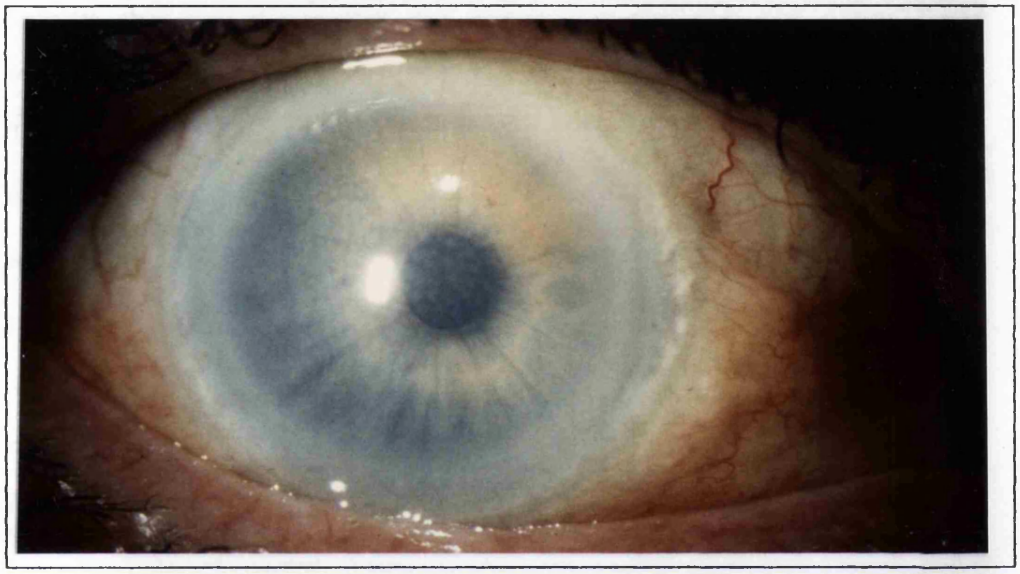

Figure 32. The eye of proband showing hazy cornea.

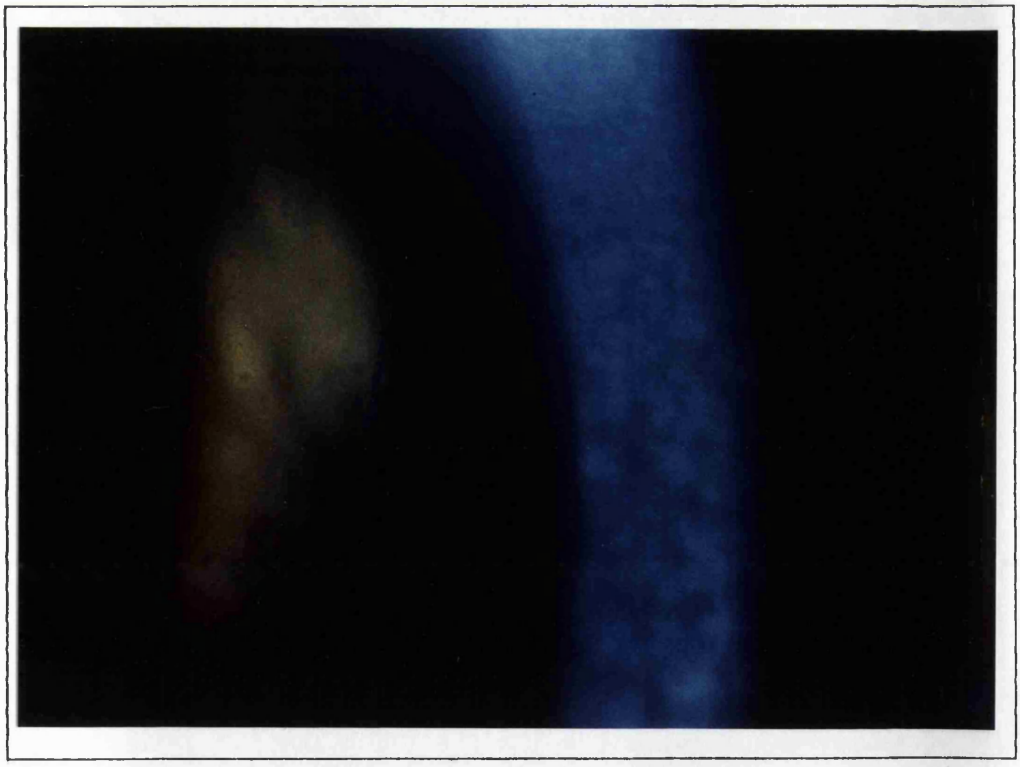

Figure 33. Slit lamp illumination of eye of proband

Analysis of serum total cholesterol and cholesterol ester by enzymatic CHOD-PAP assay (Boehringer assay kit), and also after extraction with chloroform:methanol 2:1 (v:v), followed by thin layer chromatography in hexane:diethyl ether:acetic acid 80:20:1 (v:v:v) and scanning densitometry, indicated that 67-72% of total cholesterol was present in ester form.

Table 37 shows that plasma from the proband has very little cholesterol esterification activity using apoA-I proteoliposomes as substrate (Chen and Albers, 1982) but (in contrast to the result in classic LCAT deficiency) reasonable activity when whole plasma was used (Stokke and Norum, 1971), presumably because esterification can occur with the d<1.063 lipoproteins (LDL,VLDL) as substrates.

	Proteoliposomes*	Whole plasma†	
Subject	% esterified per 30 min	% esterified per hour	nmol/ml/h
FED	0.18	1.71	31.5
No. 1	4.93	5.41	71.5
No. 2	5.04	4.94	79.2
No. 3	5.52	6.62	52.9
No. 4	5.25	7.47	53.9

Table 37. Cholesterol esterification rate using proteoliposomes and whole plasma as substrates in FED and normals (numbers 1-4).

* Chen and Albers method † Stokke and Norum method

These definitive findings of FED were confirmed by using specific lipoprotein fractions (HDL-3 and the d<1.063 fraction) as substrates (Stokke-Norum method modified by Gillett and Owen, 1992) and which showed impaired esterification of HDL-cholesterol but normal activity when VLDL and LDL were used as substrates.

Plasma LCAT mass, measured after isolation using affinity column chromatography and SDS-PAGE electrophoresis, showed that patient LCAT was of normal mobility but reduced mass in comparison with normal reference samples, suggesting that the patient's LCAT molecular structure was grossly normal. [All plasma LCAT activities and mass measurements were undertaken by Dr J Owen, Dept of Medicine, Royal Free Hospital School of Medicine, London.]

Preliminary analysis (Frohlich and Pritchard, UBC Vancouver) of the LCAT gene suggests that the proband is heterozygous for a C to T base change at codon 123 of the LCAT gene as reported for other families, and resulting in a threonine-isoleucine substitution with presumably a second, as yet undetermined, further defect. No gross defects in the apoA-I gene coding sequence were found.

Discussion

The term fish-eye disease is probably a misnomer because underlying cataract affecting visual performance and appearance was found at corneal surgery in an early case (Philipson, 1982). Subsequent cases including the proband reported here have had corneal clouding but no cataract and adequate vision. Special features of this first reported case of FED in a British family include an expression of premature coronary disease but good longevity, an absence of haematological abnormalities and a gene defect associated with a base change at codon 123 with a presumed second defect at another site. The close relationship between LCAT deficiency and FED was apparent from biochemical analysis of corneal buttons from a patient with FED (Philipson, 1982) which was essentially identical to that reported for LCAT deficiency (Winder et al., 1985).

Investigation of patients with defects in lipoprotein metabolism may provide information on the general relationship and associated mechanisms linking HDL deficiency and premature cardiovascular disease and the processes of reverse cholesterol transport. Premature disease is reported not to be a gross feature of Tangier disease, some apoA-I structural variants, familial LCAT deficiency, and fish-eye disease (Schaefer, 1984). In the family now reported the proband with definite fish-eye disease had a first cardiovascular event, probably an infarct, age 50 years, but was then a heavy cigarette smoker. Other family members, including a brother (who from the clinical description probably also had fish-eye disease), enjoyed longevity without premature cardiovascular disease. From the proband and the small number of cases described, it appears that not all individuals with FED and HDL deficiency are free from premature cardiovascular disease. Any association with cardiovascular events could also be confounded by variation in plasma VLDL and LDL composition and concentrations (Assmann et al., 1991). Results here also confirm that cholesterol transport and metabolism does proceed through the plasma lipoprotein pathway, but without significant involvement of HDL.

The fish-eye syndrome, described in association with an apoA-I structural variant and the codon 123 defect in LCAT, is consistent with the proposal that through conformational change of enzyme, enzyme-substrate complex or substrate, the resulting modification of fit alters substrate specificity, impairing interaction with HDL fractions but not with other plasma lipoproteins. Further in vitro studies of expressed LCAT mutations have identified other variant sites distant from the active centre, potentially also associated with

reduced α-LCAT activity (Klein et al., 1992) A spectrum of structural changes and associated changes in catalytic activity or enzyme specificity have now been reported (Pritchard et al., 1992). In the present family, the mother was reported to have had corneal clouding and enlarged tonsils in later life, again raising the prospect that the codon 123 or more likely the presumed second defect in this family can be sufficiently disruptive to have dominant expression in late life. From the gel analytical data for the proband, neither defect has caused substantial disruption of the LCAT structure. Further analysis of the LCAT and apoA-I genes are now being undertaken.

Appendix B : Tangier Disease

Appendix B: Tangier disease

Introduction

Tangier disease is a rare autosomal recessive disorder associated with markedly reduced serum concentrations of HDL cholesterol and apoA-I. LDL and total cholesterol are low but with a normal proportion of cholesterol in ester form, and triglyceride-rich chylomicrons and VLDL may be in moderate excess (Assmann et al., 1989). The basic defect is unknown but cell culture studies indicate that low levels of serum HDL may be due to rapid catabolism of HDL associated with defects in intracellular lipid and lipoprotein trafficking (Schmitz et al., 1990; Robenek and Schmitz, 1991). Tissues such as spleen, liver, lymphatic and reticulo-endothelial systems, tonsils and cornea accumulate phospholipid and cholesterol ester. The disorder is relatively benign, with many patients surviving beyond 50 years, although the degree of disability may then be severe. Heterozygotes are clinically unaffected, although abnormal storage of lipid may be detected by rectal biopsy and plasma levels of HDL cholesterol (and apoA-I) are often reduced. Lipid accumulates in the cornea in other disorders of HDL metabolism including familial LCAT deficiency (Winder et al., 1985) and Fish-eye disease (Philipson, 1982; Winder and Borysiewicz, 1982). The first structural and biochemical analysis of the cornea in Tangier disease is reported here.

Clinical details of the proband

The Canadian male patient of British ancestry and from a consanguineous first cousin marriage, died age 62 years having undergone a splenectomy at age 38 years and tonsillectomy in

childhood. Fasting plasma lipids were total cholesterol 1.9mmol/L with 62% as esterified cholesterol, triglycerides 3.4mmol/L, HDL-cholesterol <0.1 mmol/L, apoA-I <0.1g/L and apoA-II <0.1g/L. Corneas were removed post mortem and dissected into central buttons and outer corneoscleral rings prior to analysis.

Histology

Corneal tissue was severely disrupted with massive vacuoles. Small amounts of cholesterol, cholesterol ester and phospholipids were detected using histological stains for lipid. Transmission electron microscopy showed many membranous lamellar bodies in the corneal stroma (figure 33B).

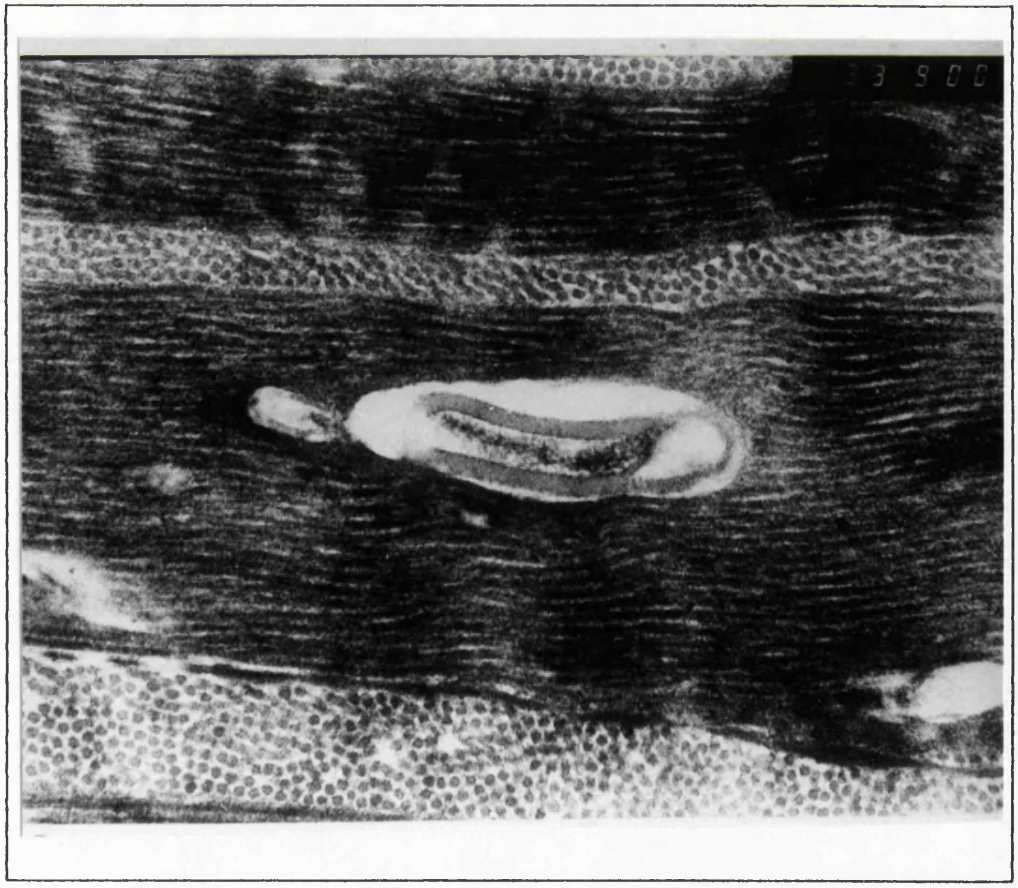

Figure 33B. Electron microscopy of Tangier cornea

Biochemistry

Lipids were extracted from the corneal button by homogenising slices of cornea with chloroform:methanol 2:1 (v:v). Extracts were evaporated to dryness, re-dissolved in chloroform and lipids separated by thin layer chromatography in hexane:diethyl ether:acetic acid 80:20:1 (v:v:v) followed by quantitative scanning densitometry. Lipid composition of the corneal extract is given in table 37B. Cholesterol esters accounted for 65% of the total cholesterol extracted from the cornea.

Table 37A. Lipid composition of corneal extract in Tangier disease.

Component	% lipid extracted
Total phospholipids	27
Free cholesterol	26
Esterified cholesterol	47

Differential scanning calorimetry

Corneas were scanned using a Perkin Elmer 7 Series Thermal Analysis system by Dr D. Johnston [Department of Protein and Molecular Biology, Royal Free Hospital School of Medicine, London]. Normal cornea without evident arcus showed one major transition at 29°C (figure 24B). Cornea with a heavy arcus showed several distinct transitions over the range studied (figure 25B). Tangier central cornea showed a bimodal peak with transitions in the range 25-33°C. The cooling profiles were not consistent on repeat, suggested that interaction of lipid with structural elements of cornea was disturbed by temperature cycling.

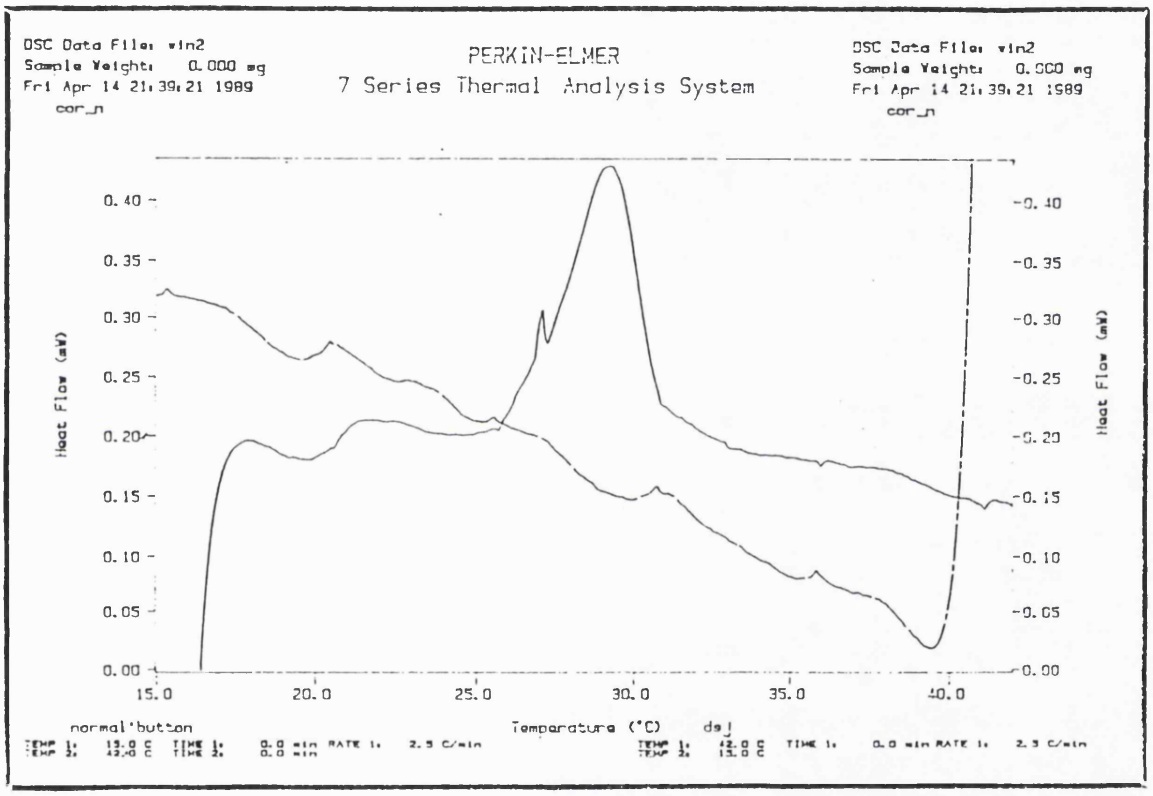

Figure 35. Differential scanning calorimetry of normal cornea

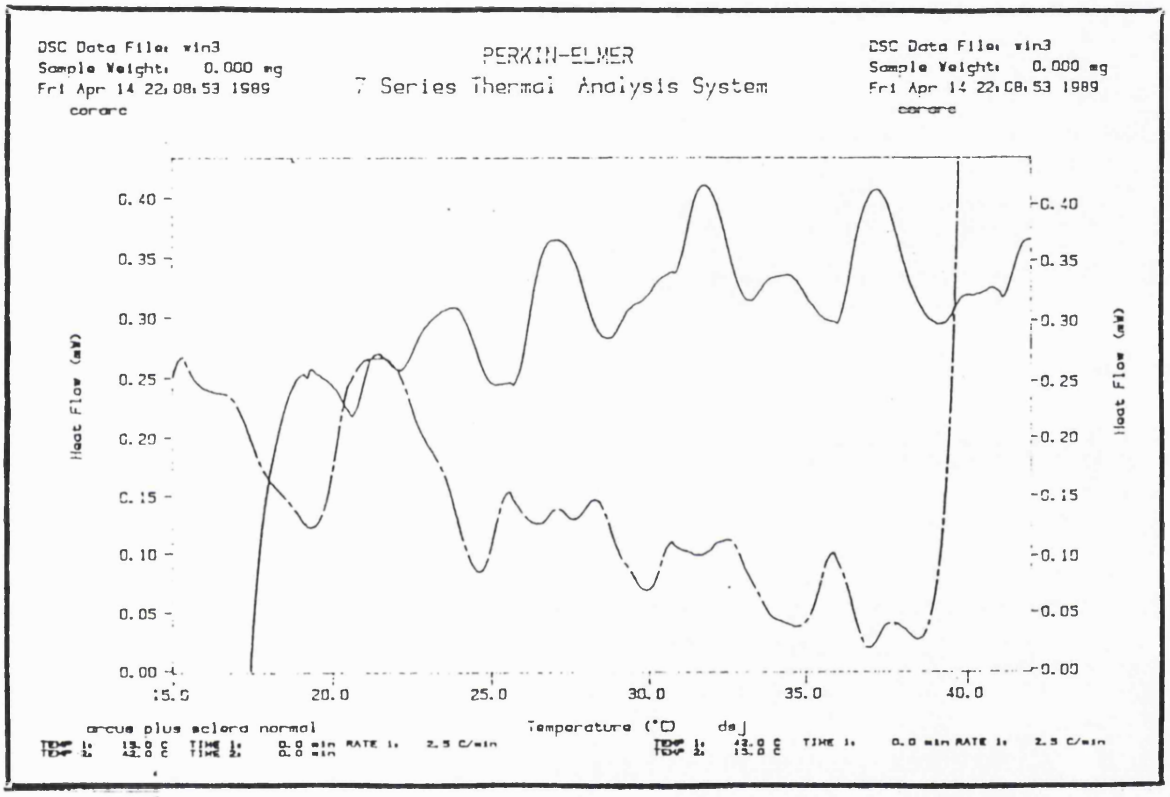

Figure 36. Differential scanning calorimetry of normal cornea with heavy corneal arcus

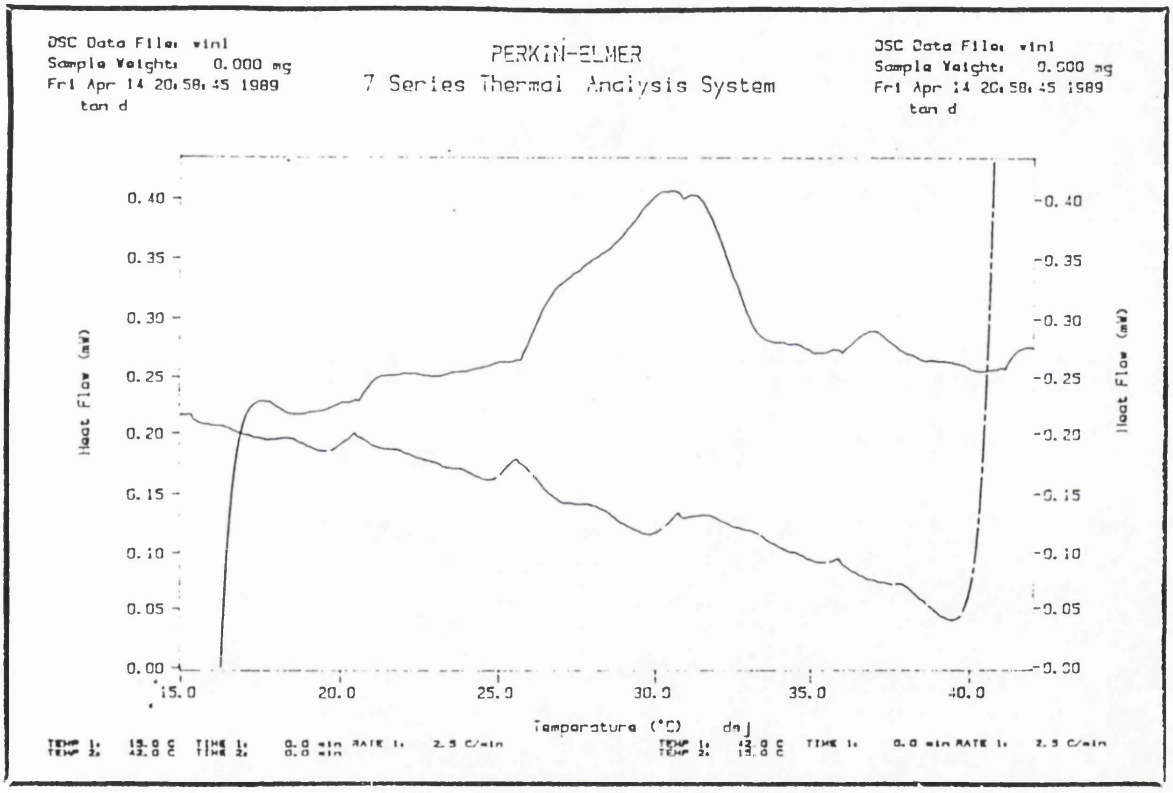

Figure 37. Differential scanning calorimetry of Tangier cornea

Discussion

Direct biochemical and histological analysis has confirmed that corneal opacification in Tangier disease is associated with accumulation of lipid as has been previously described for other sites. Cholesterol was mostly esterified in comparison to familial LCAT deficiency when cholesterol is mostly unesterified. By analogy with lipid accumulation in tonsils exposed to lower ambient airway temperatures, the accumulation of lipid, initially in the 3 and 9 o'clock positions, has been attributed to the reduced temperature in the medial and lateral cornea (Fielder et al., 1981). Lipid, particularly longer chain saturated cholesterol esters, may accumulate at sites were the temperature is below the transition temperature for that lipid (Davis et al., 1970).

However the extent to which ingestion of modified lipoprotein, or alternatively local synthesis, contributes to the accumulation of lipid is not known, but abnormal circulating particles are more obvious after splenectomy and decrease in number after diet (Herbert et al., 1978). Uptake by phagocytosis into macrophages is therefore likely as deposits in most tissues are intracellular and in foam cells. The findings in this present study are consistent with an influence of local corneal temperature on the unusual pattern of accumulation described in some cases of this disorder.