The Use of Stable Isotopes to Study ApoB Metabolism in Dyslipidaemia.

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Abstract.

The primary aim of this thesis was to characterise the metabolism of apoB containing lipoproteins in various dyslipidaemic states, using stable isotope methodology and multicompartmental modelling techniques. Human apoB kinetics have been extensively studied in normolipidaemic subjects, both using radio-isotope methodology and, more recently, using stable isotope techniques. Radio-isotopes have also been widely used to study various dyslipidaemias, but the use of stable isotopes in such conditions is more limited.

To provide a basis on which to understand lipid and lipoprotein metabolism in dyslipidaemias, a non-kinetic study of 95 normolipidaemic individuals was performed. Eighty of these subjects were healthy (41 males and 39 females) with the remaining 15 being sufferers of coronary heart disease (CHD). The relationships between the subfractions of VLDL, LDL and HDL and indices of obesity, insulin resistance and lipase activity were established. In particular, differences between the sexes and between the healthy group and the CHD sufferers were noted. The main observation of this study was that even within the normal range of plasma lipid levels (cholesterol<6.0mmol/l, triglyceride<2.0mmol/l) several subgroups of differing lipid patterns appear to exist. Plasma triglyceride appeared to play a strong role in determining measured lipoprotein profiles and hence risk for CHD. Those subjects with marginally higher triglyceride levels tended to have lower levels of HDL cholesterol and higher levels of the small, dense species of LDL (primarily LDLIII). Thus they could be described as having the Atherogenic Lipoprotein Phenotype despite having plasma triglyceride levels within the normal range. The likely causes of the marginal increase in triglycerides were highlighted by further comparing the subgroups. Relatively obese individuals, as determined by increased body mass indices and waist:hip ratios, and those with a tendency towards insulin resistance, as determined by increased fasting glucose and insulin levels, had higher triglyceride levels and a trend towards developing the atherogenic lipoprotein phenotype. Those individuals most strongly exhibiting these features were the 15 subjects already suffering from CHD. The influence of sex on the lipoproteins appears to be mediated through the lipolytic enzymes, in particular hepatic lipase which has a higher activity in men than women. From this study a metabolic model was drawn, with particular reference to the factors that may influence lipoprotein secretion and their further metabolism. This in turn directed the kinetic studies to look in particular at the nature of the apoB containing lipoprotein being predominantly secreted, i.e. triglyceride-rich $VLDL_1$ or relatively cholesterol-rich $VLDL_2$.

Kinetic studies of the metabolism of apoB containing lipoproteins were then performed in certain dyslipidaemic states. Subjects with heterozygous familial hypercholesterolaemia (FH) were compared to subjects heterozygous for familial defective apoB-100 (FDB), as in both conditions there is a disruption of receptor-mediated LDL catabolism. Both exhibited the expected reduced catabolic rate of LDL, but in addition there was reduced transfer of the lipoproteins down the delipidation chain, possibly as a result of the nature of the lipoproteins being produced by the liver or due to a disruption of receptor-lipase interaction. In FH there was a

reduced catabolism of LDL precursors that was not seen in FDB, probably reflecting normal apoE-mediated clearance in FDB.

Two rare and very different subjects were then studied. A hypobetalipoproteinaemic subject, with very low levels of plasma cholesterol and apoB, showed greatly reduced hepatic lipoprotein production, in particular VLDL production, possibly as a consequence of a genetic defect in apoB. In contrast, in a unique study of an analbuminaemic subject, massive overproduction of $VLDL_2$ was shown to be the cause of the grossly elevated plasma cholesterol and apoB levels. This secondary hyperlipidaemia reflects a compensatory response to negligible plasma albumin levels, identifying a further factor that is influential in apoB metabolism.

The kinetics of apoB in mixed hyperlipidaemia were studied in seven individuals with plasma cholesterol levels of 6.0-9.0mmol/l and triglyceride of 2.0-4.0mmol/l. These subjects, with only moderate elevations in their plasma lipids, showed an increase in total apoB production, specifically $VLDL_1$ production, a decrease in the transfer of apoB down the delipidation chain, and decreases in the catabolism of $VLDL_1$ and LDL. Further analysis suggested that it is the production of $VLDL_1$ as the predominant lipoprotein (manifest as elevated plasma triglycerides) that influences the further metabolism of the apoB.

Finally, the mechanisms of action of two HMG-CoA reductase inhibitors, simvastatin and atorvastatin, were compared in the mixed hyperlipidaemic group. This type of dyslipidaemia is more usually treated with one of the fibrate class of drug but the triglyceride-lowering action of the HMG-CoA reductase inhibitors was particularly under study here. In addition to the expected increase in LDL catabolism, these drugs (notably atorvastatin) had surprising effects on $VLDL_1$ metabolism. Treatment caused an increase in both $VLDL_1$ production and catabolism, seemingly resulting in 'futile cycling' of triglyceride. In addition, atorvastatin caused a shift in the LDL subfraction profiles from those of mainly small, dense LDL to a predominance of the larger, less dense species. This is presumably due to the triglyceride-lowering action of atorvastatin, an effect that has previously only been reported for the fibrates.

The results of the above studies were drawn together in a scheme describing the regulation of apoB metabolism. It was suggested that obesity and insulin resistance cause the liver to become relatively triglyceride-rich, whilst analbuminaemia may increase the cholesterol ester content. The intrahepatic lipid content in turn determines the amount and type of lipoprotein secreted - triglyceride-rich $VLDL_1$ or relatively cholesterol-rich $VLDL_2$. The VLDL particles have different metabolic properties with $VLDL_1$ being relatively slowly metabolised to form predominantly small, dense LDL, whilst $VLDL_2$ is more rapidly metabolised to the larger species of LDL. Hepatic lipase also has an effect on the LDL subfractions by catalyzing the interconversion from LDLI to LDLIII. The different LDL species are then catabolised at different rates with LDLI being cleared more avidly by the receptor than LDLIII.

In conclusion, this work characterises the metabolism of apoB containing lipoproteins in a number of dyslipidaemic states, and in so doing highlights points of regulation of apoB kinetics namely, intrahepatic lipid availability, the nature of the particle

secreted, and the subsequent metabolism of the lipoproteins. It is clear that stable isotope methodology and multicompartmental modelling techniques have tremendous potential in the further elucidation of the causes of dyslipidaemia and the mechanism of action of lipid-lowering therapy.

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Author's Declaration.

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

Dr. Lorne F. Forster,
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Dedication.

This thesis is dedicated to my husband, Michael, for all his love and support over the past few years, and for his healthy disinterest in lipoprotein metabolism. It is also dedicated to our unborn child whose imminent arrival we are so eagerly awaiting.

Chapter 1 Introduction.

1.1 Hyperlipidaemia, Atherosclerosis and Coronary Heart Disease.

Coronary heart disease (CHD) is currently responsible for the majority of morbidity and mortality in most developed countries, with mortality rates from CHD in the United Kingdom, in particular Scotland, being amongst the highest in the world.

Over one hundred years ago Marchand coined the term *atheroma* or *atherosclerosis* to describe the pathological feature now familiar to us as the major cause of CHD (Aschoff 1924). He took the term from the Greek, *athere* meaning gruel, *oma* meaning mass and *skleros* meaning hard. In 1847 his contemporary, Vogel, described the principal histological characteristic of these lesions, that of lipid accumulation, predominantly cholesterol, in the arterial wall.

Keele (1952) credits Leonardo da Vinci (1452-1519) as giving us the first theory on the pathogenesis of atherosclerosis when, whilst illustrating an autopsy of an elderly man, he suggested it may be caused by 'excessive nourishment' from the blood. This euphemistic term for hyperlipidaemia serves to remind us that lipids are not purely pathological but do have a physiological function in the 'nourishment' of cells. It is, however, ironic that Marchand's terminology for this excessive nourishment could be equally well applied to the traditional diet of many Scots, namely porridge: a mass of hard gruel.

1.1.1 Epidemiology and Risk Factors.

Research throughout this century has further elaborated on the link between lipids and atherosclerosis formation, confirming the direct relationship between elevated plasma cholesterol levels and CHD development. Large epidemiological studies have done much to identify risk factors for CHD. Perhaps one of the most famous of these is the Framingham Study - a prospective, observational study, set up in 1948, and now studying the off-spring of the original subjects (Wilson and Castelli 1989). This study was responsible for the identification of many of the now accepted risk factors for CHD, such as hypercholesterolaemia, hypertension, cigarette smoking and a low level of high density lipoprotein (HDL) cholesterol. It also highlighted the fact that these factors are not additive but multiplicative in increasing the risk for CHD development. Male sex and increasing age were observed to have higher associated incidences of CHD, with the incidence for women catching up with that for men post-menopausally.

From this study came interesting observations on the various cholesterol levels at which CHD was more common. Approximately 50% of CHD events occurred at cholesterol levels of 5.28-6.80mmol/l - a sobering observation given the average cholesterol level in Scotland of 6.4mmol/l. Only 20% of events in men and 45% of events in women occurred at levels above 6.80mmol/l. CHD events below 5.28mmol/l were 25% and 10% in men and women respectively. From this and other studies has emerged the threshold cholesterol value of 5.20mmol/l. An individual cholesterol level below this value is said to be ideal for minimising the risk of CHD development.

However, the Multiple Risk Factor Intervention Trial, MRFIT, demonstrated that there is not a critical level of serum cholesterol above which CHD will develop, but that the risk is 'continuous and graded' (Stamler et al 1986).

The MRFIT also observed racial differences in predisposition to CHD and cerebrovascular disease, studying diastolic blood pressure (DBP) in black and white males (Neaton et al 1984). An increase in DBP was associated with an increased incidence of CHD in whites that was greater than that in blacks, whereas for cerebrovascular disease the converse was true. An earlier study, the Seven Countries Study (Keys 1970), illustrated the large variations in CHD incidence between populations. However, despite this variation the all cause death rate for each country reflected the CHD death rate. Population differences in CHD incidence open the way for 'nature versus nurture' debates of the relative importance of endogenous and exogenous risk factors. Extensive research by Goldstein and co-workers in the 1970's has done much to characterise the familial aggregation of lipid phenotypes and CHD (Hazzard et al 1973). A family history of premature CHD is now considered an independent risk factor.

Further studies have identified a range of other risk factors including obesity, inactivity, glucose intolerance and hypertriglyceridaemia. The more recently identified risk factors include certain lipoprotein and apolipoprotein characteristics, and most recently elevations in coagulation factors such as fibrinogen.

1.1.2 Treatment Strategies - populations versus the high risk individual.

Strategies for the primary and secondary prevention of CHD by correction of hyperlipidaemia include both lifestyle modification and pharmacological intervention. Because of the multiplicative nature of risk factors it is essential that all modifiable risks are addressed. Guidelines published by the British Hyperlipidaemia Association (BHA) in 1993 stress the need for lifestyle modification prior to the introduction of lipid-lowering agents (Betteridge et al 1993). In some individuals lifestyle modification is all that is required to correct their lipid profile, thus avoiding the need for lifelong drug therapy with the inevitable potential for side effects. Lifestyle modifications include weight loss if over-weight, cessation of smoking, moderation of alcohol intake and adherence to a cholesterol-lowering diet, as follows -

Table 1.1 The Cholesterol-lowering Diet (BHA 1993).

	% total calories
Total fat	<30
Saturated fat	<10
Monounsaturated fat	<10
Polyunsaturated fat	<10
Carbohydrate	50-60
Protein	10-20
Fibre	35g/day
Cholesterol	<300mg/day

These modifications should be followed for three to six months before pharmacological intervention is considered, and continued on the introduction of therapy.

Such modifications are readily applicable to whole populations without the need for consideration of cost effectiveness. (Some would argue that we all should be adhering to the healthy lifestyle irrespective of our lipid profile). Commencement of lipid-lowering therapy, however, cannot be applied to whole populations but must be tailored to the individual. For each individual all risk factors must be taken into account in order to calculate his overall risk of developing CHD. For a high risk individual, for example an overweight middle-aged male smoker with hypertension and a family history of premature CHD, the question of lipid-lowering agents is much more pertinent than for a low risk individual with the same cholesterol level. In addition, the nature of the lipid abnormality must be taken into account.

1.1.3 Classification of Hyperlipidaemia.

There are several different approaches to the classification of hyperlipidaemia, for example - i. primary/secondary,
ii the Fredrickson classification,
iii. family phenotyping.
Each has shortfalls and they are not mutually exclusive.

1. Primary/secondary. A secondary hyperlipidaemia is one that results from such underlying causes as metabolic disease, for example diabetes mellitus or hypothyroidism, hormonal disturbance such as pregnancy, renal disease or drugs such as certain anti-hypertensive agents. In primary hyperlipidaemia no underlying cause can be found save, in certain cases, the primary defect e.g. an enzyme or receptor deficiency. In all patients presenting with hyperlipidaemia it is important to screen for secondary causes as often their treatment will lead to the correction or improvement of the lipid abnormality.

2. The Fredrickson classification. In 1967 Fredrickson and colleagues devised their classification of hyperlipidaemia based on the electrophoretic pattern of the lipoproteins. This was subsequently modified by the World Health Organisation (Beaumont et al 1970) to subdivide type II into two groups. This classification makes no allowance for the primary or secondary nature of the hyperlipidaemia nor of its familiarity but it is widely used as a convenient shorthand in the clinical setting.

3. Family phenotyping. In the 1970s work by Goldstein and colleagues (Hazzard et al 1973) delineated the familial aggregation of lipoprotein phenotypes using survivors of myocardial infarction as probands. They identified six classes of hyperlipidaemia -

1. familial hypercholesterolaemia,
2. familial hypertriglyceridaemia,
3. familial combined hyperlipidaemia,
4. polygenic hyperlipidaemia,
5. sporadic hypertriglyceridaemia,
6. type III hyperlipidaemia.

While this classification gives a more accurate impression of the heritability of the hyperlipidaemia, it necessitates screening of relatives which may not always be possible in a clinical situation.

In practice, a combination of all three approaches is generally used. The primary hyperlipidaemias are summarised below, based on the Fredrickson classification.

Table 1.2 The Classification of Primary Hyperlipidaemia.

Phenotype	Elevated Lipoprotein	Electro-phoretic Pattern	Elevated Lipid	Associated Genetic Disorders
I	chylomicrons	origin	triglyceride	LPL deficiency, ApoCII deficiency.
IIa	LDL	β	cholesterol	Familial hypercholesterolaemia, Polygenic hypercholesterolaemia, Familial defective apoB-100.
IIb	VLDL & LDL	pre-β & β	cholesterol & triglyceride	Familial combined hyperlipidaemia Familial hyperlipidaemia.
III	β-VLDL	broad β	triglyceride = cholesterol	ApoE2 homozygosity.
IV	VLDL	pre-β	triglyceride	Familial hypertriglyceridaemia, Familial combined hyperlipidaemia
V	chylomicrons & VLDL	origin & pre-β	triglyceride	ApoCII deficiency, Familial hypertriglyceridaemia, Familial combined hyperlipidaemia

1.1.4 Lipid-lowering Agents.

Intervention trials of lipid-lowering agents have shown that these drugs significantly decrease the incidence of major coronary events.

1. Resins. These are non-absorbable basic anion-exchange resins that bind bile acids in the intestine leading to their excretion in the faeces, thus interrupting the enterohepatic circulation. As a result of the decreased return of bile acids to the liver, there is an increase in their hepatic synthesis so reducing the intrahepatic cholesterol pool. This results in up-regulation of the low density lipoprotein (LDL) receptors and an increase in cholesterol uptake from the circulation. Resins can achieve a 20-30% reduction in LDL cholesterol but have the adverse effect of a moderate increase in triglycerides due to stimulation of hepatic very low density lipoprotein (VLDL) production. Their efficacy is dependent on functioning LDL receptors and they are therefore of questionable benefit in homozygous familial hypercholesterolaemia.

Because they are not absorbed, the resins do not have major systemic side effects and are thus of use in children and women of child-bearing age. However, compliance is often poor due to the gastro-intestinal side effects of bloating, flatulence and constipation. In addition, they can interfere with the absorption of other drugs such as digoxin, warfarin and thyroxine (this can be minimised by taking the drug one hour before or four hours after the resin).

The Lipid Research Clinics Coronary Primary Prevention Trial (1984) studied the effect of the resin cholestyramine on CHD incidence in approximately 4,000 men (35-59 years of age) with moderate primary hypercholesterolaemia who were free of CHD at entry into the study, and compared them to similar controls on placebo. Treatment with cholestyramine was associated with a decrease in the incidence of CHD in line with a reduction in the levels of total cholesterol and LDL cholesterol.

2. Fibrates. This class of fibric acid derivatives increases the clearance of triglyceride-rich lipoproteins by activating lipoprotein lipase (one of the lipolytic enzymes). Their primary effect is the 50% reduction in triglycerides and thus their target recipients are those with elevations in this lipid. They have an additional beneficial effect of elevating the HDL cholesterol by 15-20% and some of the newer fibrates can also lower LDL cholesterol by 10-25%. The side effect profile is good, but with two important, but rare and reversible, adverse effects of myositis and elevated liver transaminases.

The first major intervention study of this class of drug was that performed by the World Health Organisation, Committee of Principal Investigators, (1980) of the parent compound, clofibrate. Unfortunately, due to the increased lithogenic index of the bile in subjects taking the drug there was an increase in mortality in the treatment group of 25% due to gall bladder disease. However, this increased lithogenicity does not appear to be a problem with the newer compounds. The Helsinki Heart Study (Frick et al 1987), a primary prevention trial, observed significant reductions in the incidence of CHD in those subjects treated with gemfibrozil. The effect was first noted after two years of treatment.

3. HMG-CoA reductase inhibitors (statins). These relatively new drugs were developed from fungal metabolites and act by the competitive inhibition of the rate-limiting enzyme in cholesterol synthesis, 3-hydroxy-3-methylglutaryl Coenzyme A reductase. The intracellular cholesterol production and pool size are reduced resulting in an up-regulation of the LDL receptors and hence an increased clearance of circulating cholesterol. There is also a reduction in LDL production and an increase in VLDL clearance. They can achieve 30-40% reductions in LDL cholesterol, with a 10-20% reduction in triglycerides and a small elevation in HDL cholesterol. The effects are dose-dependent, reaching a maximum within four weeks. As with the resins, they reportedly have little efficacy in homozygous familial hypercholesterolaemia. Side effects are uncommon but again myositis and elevated liver transaminases are rare possibilities.

Within the past two years two major intervention trials of these drugs have been published. The first of these, the Scandinavian Simvastatin Survival Study (4S), a secondary prevention trial, studied the effect of cholesterol lowering with simvastatin on mortality and morbidity in patients with pre-existing CHD. 4444 subjects were randomised to treatment or placebo groups and followed for an average of 5.4 years. The cholesterol range was 5.5-8.0mmol/l. Simvastatin produced highly significant reductions in CHD mortality and morbidity, with this effect being first observed at about one year. This finding extended to women and individuals over the age of 60 years, two groups previously unstudied. There was also noted a beneficial effect on

cerebrovascular disease. More importantly, there was no increase in non-CHD mortality.

The second large intervention trial was the West of Scotland Coronary Prevention Study (WOSCOPS). This primary prevention trial studied the effect of pravastatin on the subsequent development of CHD in men with primary moderate hypercholesterolaemia (mean 7.0mmol/l) but no past history of myocardial infarction. 6595 men were randomised to treatment or control and followed for an average of 4.9 years. The impact of pravastatin was seen within six months, with significant reductions in the incidence of CHD. Again there was no increased incidence of non-CHD death.

Therefore, this class of drug has two recent large intervention trials, one primary and one secondary, in its favour. One of the many important outcomes is the finding that there is no increase in non-CHD death with treatment, hopefully finally laying to rest the concerns over possible serious adverse effects of lipid-lowering.

4. Other lipid-lowering agents. Less commonly used drugs include nicotinic acid and its derivatives, probucol and omega-3 fatty acids.

Nicotinic acid is a water-soluble B vitamin that inhibits the breakdown of triglyceride in the adipose tissue, lowering the fatty acid supply to the liver and hence reducing VLDL and LDL with an increase in HDL. It is, however, poorly tolerated due to the side effects of severe flushing, gastro-intestinal disturbance and pigmentation. These can be ameliorated to a certain extent by the concomitant use of aspirin (prostaglandin inhibition). It also exacerbates gout and glucose intolerance. Synthetic analogues, such as acipimox, have been developed but although these are better tolerated they are less efficacious.

Probucol, a lipophilic bis-phenol, has a limited effect on lowering cholesterol and also adversely lowers HDL. Its benefit lies in its ability to increase LDL clearance by receptor-independent mechanisms and to block the oxidation of LDL. It mobilises cholesterol from the skin and xanthomata. Due to its lipophilicity, it is stored in the adipose tissue and is present for up to six months after cessation of treatment.

Omega-3 fatty acids (fish oils) reduce VLDL synthesis by the liver and thus primarily lower triglyceride. There is some concern that they may increase the level of LDL but they may also have a beneficial effect on certain coagulation factors.

1.2 Properties of Lipids and Lipoproteins.

1.2.1 Lipids.

Lipids are a heterogeneous class of naturally occurring substances, grouped together by common solubility properties. They are all insoluble in water but highly soluble in certain organic solvents such as ether, chloroform, benzene and acetone, the 'lipid-solvents' (Brown 1981). In the human body, the three principal lipids are cholesterol, triglycerides and phospholipids.

Cholesterol is a white, water-insoluble sterol found in practically all living organisms except bacteria. Its primary functions are i). as a component of all cell membranes, where it is present in the unesterified form, and ii). as a precursor for the synthesis of steroid hormones in the adrenal glands and gonads, and vitamin D and bile acids in the liver. Despite most tissues being capable of de-novo cholesterol synthesis it is carried out for the most part by the liver with some contribution from the distal small intestine. 3-Hydroxy-3-methylglutaryl Coenzyme A reductase forms mevalonic acid from acetate. This rate limiting enzyme is subject to feedback inhibition from cholesterol which is formed from acetate via a series of some 20 precursors. Hepatic cholesterol synthesis is regulated by absorption of dietary cholesterol and re-absorption of biliary cholesterol, in the form of bile acids, via the enterohepatic circulation. Excretion is by way of bile acids into the faeces.

Triglycerides are the most abundant naturally occurring lipids and are familiar to us in the form of fats and oils. Exogenous triglycerides enter the circulation following absorption by the small intestine, whilst endogenous triglycerides are produced mainly by the liver. They are hydrolysed in the plasma to one glycerol molecule and three fatty acid molecules per triglyceride molecule, and taken up into the tissues where they are available for energy production, or reconstituted into triglyceride for storage. The majority of the triglyceride in the body is found in adipose tissue. Hydrolysis of this stored triglyceride by hormone sensitive lipase, the rate limiting enzyme found within the adipocytes, releases fatty acids into the plasma where they circulate as free fatty acids bound to albumin (Coppack et al 1994). The relatively small amount of triglyceride stored in muscle and liver turns over more rapidly than that in adipose tissue, providing a ready supply of fatty acids until those from adipose tissue are available. Fatty acids provide oxidative fuel for the liver, kidneys, skeletal muscle and myocardium. They differ in chain length and saturation, thus determining differences in physical properties such as melting point. Triglycerides differ from one another by variation in their fatty acid component. In addition, the majority of cholesterol in plasma is present as esters of long-chain fatty acids, mainly linoleic acid and oleic acid.

Phospholipids, the second most abundant lipid, are amphipathic molecules containing glycerol, fatty acids, phosphoric acid and a low-molecular weight alcohol such as choline or ethanolamine. They are an essential component of biological membranes, which typically consist of 40-50% phospholipid and 50-60% protein. As with cholesterol, all tissues are able to synthesise phospholipid but the majority is produced by the liver.

In the routine management of hyperlipidaemic patients, phospholipid and fatty acid levels are not usually measured. Instead, treatment strategies are based on plasma levels of cholesterol, triglycerides and the lipoproteins.

1.2.2 Lipoproteins.

Plasma is largely water. Therefore, in order that lipids may circulate in the plasma, they associate with protein to form spherical macromolecules called lipoproteins. These lipoproteins consist of a hydrophobic core of cholesterol esters and triglceride surrounded by a hydrophilic monolayer of phospholipid, free cholesterol and protein, known as apolipoprotein (apo). The phospholipids are aligned such that their polar heads interact with the aqueous environment of plasma whilst their fatty acyl chains abut onto the non-polar neutral lipid core.

The lipoproteins can be classified into five major groups, namely chylomicrons, very low density lipoproteins (VLDL), intermediate density lipoproteins (IDL), low density lipoproteins (LDL) and high density lipoproteins (HDL). They can be differentiated according to size, density and composition (Alaupovic 1971). These differences, summarised in the tables below, enable their separation by methods such as electrophoresis and centrifugation. They each have specific but complimentary roles in the transport of lipids in the circulation. With the exception of chylomicrons, the particles within each lipoprotein class have been shown to be heterogeneous and so can be further sub-classified (Krauss 1987).

Table 1.3 Physical Properties of Plasma Lipoproteins (Thompson 1994)

	Density (g/ml)	Flotation Rate* Sf 1.063	Sf 1.20	Mean Diameter (nm)	Electrophoretic Mobility
Chylomicrons	<0.95	>400		100-1000	origin
VLDL	<1.006	20-400		43	preβ
IDL	1.006-1.019	12-20		27	β
LDL	1.019-1.063	0-12		22	β
HDL_2	1.063-1.125		3.5-9.0	9.5	α
HDL_3	1.125-1.21		0-3.5	6.5	α

**Flotation rate (Sf), in Svedberg units, is the rate at which lipoproteins float in the analytical ultra-centrifuge.*

Table 1.4 Mean Percentage Composition of Plasma Lipoproteins (Warwick 1991).

	Free Cholesterol	Cholesterol Ester	Triglyceride	Phospholipid	Protein
Chylomicrons	5	2	84	7	2
VLDL	12	7	55	18	8
IDL	23	8	32	21	16
LDL	38	10	9	22	21
HDL_2	16	6	4	30	44
HDL_3	12	3	4	26	55

Chylomicrons are triglyceride-rich lipoproteins synthesised in the small intestinal epithelium following the absorption of dietary fat. They are not normally found in the plasma in the fasted state. Following a meal, dietary cholesterol, triglycerides and phospholipid are hydrolysed in the small intestine by intestinal lipases and absorbed as bile salt micelles. Within the enterocytes they are reconstituted and packaged with apolipoproteins B-48, AI and AIV to form chylomicrons. They are then released into the plasma via the lymphatics whereupon they gain more protein, in the form of apolipoproteins CI, CII, CIII and E, transferred from HDL. The chylomicron concentrations in the plasma start to rise at approximately one hour following a meal, peak at two to four and normally return to fasting levels within eight.

Once in the circulation chylomicron triglycerides undergo hydrolysis by lipoprotein lipase, a lipolytic enzyme present on the vessel endothelium. HDL transfers cholesterol esters to chylomicrons while in return it accepts the surface components, including the apoC's. With loss of the core lipid, chylomicrons become smaller, remnant particles which are removed from the circulation by apoE-facilitated hepatic uptake. The outcome of this process is delivery of dietary triglceride, in the form of fatty acids, to the adipose tissue and skeletal muscle for use in storage and energy production, and of dietary cholesterol esters to the liver as chylomicron remnants for storage or synthesis of bile acids and VLDL.

VLDL are similar to chylomicrons in that they are triglyceride-rich lipoproteins but they have two important differences. Firstly, the triglycerides transported are of endogenous origin, and secondly, the major apolipoprotein is apoB-100 rather than apoB-48. VLDL are secreted primarily by the liver with a minor contribution from the small intestine (mainly for the reabsorption of endogenous cholesterol and fatty acids of biliary origin). Hepatic lipids are added to the apoB-100 to form VLDL.

Following hepatic synthesis, the VLDL are released into the space of Disse and here, in a manner similar to that of chylomicrons, they acquire apolipoproteins C from HDL before undergoing stepwise delipidation by lipoprotein lipase (LPL) in the systemic circulation. Again HDL provides cholesterol esters and apoE. As the core triglyceride is hydrolysed, the particle becomes smaller so forming a remnant particle or IDL. At any point during delipidation the particle may be removed from the plasma by hepatic uptake mechanisms.

VLDL can be further classified into two major subfractions, distinct in their metabolic properties and separable by their hydrated density - $VLDL_1$ (Sf 60-400), larger, triglyceride-rich particles and $VLDL_2$ (Sf 20-60), a smaller, relatively cholesterol-enriched species. Both can be synthesised directly by the liver and, in addition, $VLDL_2$ are formed by the delipidation of $VLDL_1$. $VLDL_1$ are the preferred substrate of cholesterol ester transfer protein (CETP) and readily undergo neutral lipid exchange whilst $VLDL_2$ are overproduced in common hypercholesterolaemias (Shepherd and Packard 1987, Gaw et al 1995).

IDL provide an intermediate step between VLDL and LDL. Their concentration in plasma is only one tenth of that of LDL and they are not routinely measured in the assessment of hyperlipidaemia. However, they can accumulate in the plasma in certain

pathological states. IDL are smaller, denser and more cholesterol-enriched than VLDL, with the main protein component being apoB, the remainder having been lost with the surface components. They are rapidly delipidated by hepatic lipase to form LDL, or alternatively cleared from the circulation by hepatic receptor-mediated uptake, binding to the receptor by virtue of their apoB and apoE moieties. As with VLDL, IDL can be further divided into several subfractions (Musliner et al 1986).

LDL transport the bulk of cholesterol in the plasma. They are the most cholesterol-rich of all the lipoproteins and are formed by the delipidation of IDL. Their only protein is apoB-100, present as one protein molecule per lipoprotein particle.

LDL are cleared from the plasma by receptor-mediated uptake. This receptor, the LDL or B/E receptor, recognising apoB-100 on the lipoproteins, binds and internalises them. Some LDL are cleared by receptor-independent mechanisms, such as scavenging by macrophages, which become more relevant in cases of cholesterol excess or receptor deficiency (Goldstein and Brown 1978, Slater et al 1982).

LDL comprise several subpopulations of heterogeneous particles (the exact number depending on the method of separation used). In the early 1980s, Krauss and Burke separated LDL into four subclasses using density gradient ultracentrifugation (DGUC) and gradient gel electrophoresis. The DGUC technique was later refined by Griffin et al (1990) to separate LDL into three subfractions - LDLI (d=1.025-1.034), LDLII (d=1.034-1.044), LDLIII (d=1.044-1.060). LDLI is a large lipid-rich species whereas LDLIII is small, dense and rich in protein and phospholipid. The **LDL subfraction profile** can be visualised by passing the LDL that has been separated within the density gradient through an optical unit so giving a curve with peaks corresponding to the relative amounts of each subfraction present in the total LDL. From this, the concentration of each subfraction can be determined. These profiles provide a visual impression of the LDL pattern in any one individual. This study and others (Krauss 1991, Griffin et al 1994) have shown associations between LDL subfraction profiles and criteria such as sex and CHD. The majority of healthy individuals have a profile comprising mainly LDLII. Premenopausal females have a predominance of LDLI whereas sufferers of CHD often have a predominance of LDLIII.

Metabolic studies have shown that LDL is kinetically as well as structurally heterogeneous. When plasma and urine data are considered together in the same model it is possible to define two metabolically distinct pools (Caslake et al 1992). Pool A is a rapidly catabolised pool whilst pool B is only slowly cleared from the plasma. This heterogeneity in LDL metabolism and structure is clinically important as different lipoprotein subfractions are believed to have different atherogenic potential. Small, dense LDL is particularly atherogenic. Its small size enables it to filter through the arterial wall into the sub-endothelial space where the cholesterol then accumulates. It is reported to bind more readily to arterial wall proteoglycans (Tribble et al 1992) so prolonging its residence time in the wall. It is susceptible to oxidation, enhancing macrophage uptake and hence foam cell formation (Packard 1994). The accumulation of cholesterol within the arterial wall plus a local connective tissue reaction characterise atherogenesis.

HDL are the most abundant lipoproteins in plasma but also have a significant extravascular presence. They are synthesised by both the liver and the small intestine, as discoid apoA containing particles and are present in the plasma in two major forms, HDL_2 and HDL_3 (Schaeffer 1990). These can be further subclassified (Skinner 1994), for example by immunoaffinity methods depending on their apoA content (Cheung and Albers 1984). Some particles contain both apoAI and apoAII, Lp(AI-AII), and these are thought to be of hepatic origin. Other particles contain apoAI only, Lp(AI) and appear to originate in the intestine (Schaeffer 1990).

HDL function as mediators of reverse cholesterol transport, the process responsible for the return of cholesterol from the peripheral tissues to the liver (Barter 1993). Free cholesterol from cells and the surface layer of triglyceride-rich lipoproteins transfers to the nascent HDL. This is esterified by lecithin:cholesterol acyl transferase (LCAT) and these cholesterol esters then move from the surface to the core of the HDL particle, changing its shape from that of a flattened disc into that of a more spherical particle. In so doing, a gradient is established allowing more free cholesterol to transfer to HDL. The cholesterol ester is then transferred to triglyceride-rich lipoproteins, preferentially $VLDL_1$ (Eisenberg 1985), in exchange for triglyceride, catalysed by cholesterol ester transfer protein (CETP). The apoB containing lipoproteins are cleared by the liver, thus completing the reverse cholesterol transport. The larger, triglyceride-rich HDL (HDL_2) are then hydrolysed by hepatic lipase (HL), forming smaller HDL_3 which are then available for free cholesterol acquisition.

An additonal role for HDL is that of a repository for the small, exchangeable apolipoproteins (apoC and apoE) donating them to chylomicrons and VLDL prior to lipoplysis. The apolipoproteins are later shed back into the HDL density range. HDL catabolism is performed by the liver via apoE-facilitated receptor uptake.

Lp(a), lipoprotein (a), is a more recently discovered lipoprotein whose physiological function is as yet unclear. It is positively associated with CHD being both thrombogenic and atherogenic. Plasma levels vary from nil to 200mg/dl and have strong heritabilty. It can simplistically be described as LDL with an additional protein, apo(a), attached by a disulphide bond to the apoB. Apo(a) is synthesised by the liver and has a high degree of sequence homology with plasminogen. It is a highly glycosylated, hydrophilic protein with low affinity for lipid. Despite the similarity with LDL, Lp(a) binds less avidly to the LDL receptor (Marcovina and Morrisett 1995).

1.3 Apolipoproteins.

Alaupovic, in his 1971 editorial, described apolipoproteins as "the most probable determinants of the compositional and structural stability of lipoproteins". He was also instrumental in their classification, introducing the alphabetical nomenclature that is now widely used. Apolipoproteins are responsible for the regulation of lipoprotein inter-relationships and the maintenance of lipoprotein homeostasis. They act as ligands for receptor binding and as cofactors for lipolytic enzymes (Mahley et al 1984). Characteristics of the principal apolipoproteins are summarised in Table 1.5.

Table 1.5 Composition, Locations and Function of Human Apolipoproteins (Jordan-Starck et al 1992).

Apolipoprotein	Molecular Weight (kD)	Lipoprotein Association	Other Function	Chromosomal Location	Plasma Concentration (mg/dl)
AI	28	HDL & chylomicrons	LCAT activator.	11	100-150
AII	17	HDL	Hepatic lipase regulation.	1	30-40
AIV	46	HDL & chylomicrons	? LCAT activator.	11	~15
B-100	512	VLDL, IDL & LDL	LDL receptor ligand.	2	80-100
B-48	241	Chylomicrons	Triglyceride secretion.		
CI	6.6	HDL, VLDL & chylomicrons	LCAT activator.	19	~6
CII	8.8	HDL, VLDL & chylomicrons	Lipoprotein lipase activator	19	~4
CIII	8.7	HDL, VLDL & chylomicrons	Modulation of chylomicron and VLDL clearance.	11	~12
E	34	VLDL & HDL	Ligand for chylomicron and VLDL clearance.	19	3-7

The A apolipoproteins consist of three main apoA's - apoAI, apoAII and apoAIV. ApoAI is the main protein component of HDL accounting for 70% of its protein. It is also present to a lesser extent on chylomicrons but is transferred to HDL on hydrolysis of the former. It is synthesised by both the liver and small intestine and has been shown to have several isoforms. Its main role is as the structural apolipoprotein for HDL and as a cofactor for LCAT. ApoAII, synthesised by the liver, is the second most abundant protein in HDL. It activates LCAT and appears to enhance the lipid binding properties of apoAI. ApoAIV is present on newly secreted chylomicrons but it is readily displaced and the majority of it is found free in the plasma. Its role is as yet uncertain but it possibly acts as an LCAT activator.

Apolipoprotein B in the delipidated state is insoluble in aqueous solutions and must therefore be denatured with, for example, sodium dodecyl sulphate or urea in order to be characterised. Two types of apoB are found in normal plasma - apoB-100 and apoB-48.

ApoB-100 is the main protein moiety of VLDL, IDL and LDL. It is synthesised primarily by the liver and is an obligatory component of these lipoproteins, being present as one mole per particle. It does not exchange between lipoproteins but remains with the particle from secretion to catabolism. It acts as a ligand for receptor recognition and uptake of the particle. However, conformational changes of the apoB during lipolysis of VLDL regulate the lipoprotein uptake so that it is only IDL and LDL that are cleared by this mechanism (Chatterton et al 1995). VLDL appear to be cleared by an apoE-mediated mechanism.

ApoB-48 is synthesised by the small intestine and secreted on chylomicrons. It is identical to the amino terminal 48% of apoB-100 (hence the nomenclature), being formed by post-translational modification of the apoB-100 mRNA (Scott et al 1988). ApoB-48 does not bind to the B/E (LDL) receptor but, as with apoB-100 and VLDL, it is a prerequisite for chylomicron secretion.

The C apolipoproteins are three - apoCI, apoCII and apoCIII. They are present on HDL, chylomicrons and VLDL, the first supplying the last two with this protein on their entry into the circulation. ApoCI is the smallest of the three and acts as an activator of LCAT. ApoCII is an essential cofactor of lipoprotein lipase. ApoCIII appears to have an inhibitory effect on the hydrolysis and clearance of chylomicrons and VLDL (Thompson 1994). It exists in three forms depending on sialylation - $CIII_0$, $CIII_1$ and $CIII_2$, the subscript reflecting the number of sialic acid residues present.

Apolipoprotein E, present on chylomicron remnants, VLDL and large HDL particles, is synthesised by the liver and functions as a ligand for the hepatic uptake of chylomicron remnants and VLDL. Iso-electric focusing reveals three common apoE isoforms - E3, E2 and E4. These have arisen from point mutations of the wild type, E3. The differing isoforms do have physiological effect, in particular on the metabolism of LDL precursors.

1.4 Enzymes and Receptors Involved in Lipoprotein Metabolism.

In addition to the apolipoproteins, there are various enzymes and receptors intimately involved in the regulation of lipoprotein metabolism.

1.4.1 Enzymes of Cholesterol Metabolism.

3-Hydroxy-3-methylglutaryl Coenzyme A reductase (HMG-CoA reductase) catalyses mevalonic acid formation from acetate. This is the rate limiting step in cholesterol synthesis. Cholesterol is then formed from mevalonate via a series of some 20 steps and exerts feedback inhibition on HMG-CoA reductase thus regulating its own production. **Lecithin:cholesterol acyltransferase** (LCAT) and **cholesterol ester transfer protein** (CETP) are involved in reverse cholesterol transport. LCAT esterifies cholesterol with fatty acids, preferably linoleic acid, taken from lecithin. CETP catalyses the neutral lipid exchange of cholesterol esters from HDL and LDL to the triglyceride-rich lipoproteins in exchange for triglyceride.

1.4.2 Enzymes of Triglyceride Metabolism.

Lipoprotein lipase (LPL) hydrolyses triglyceride in chylomicrons and VLDL and requires apoCII as a cofactor. It is found mainly in adipose tissue and skeletal muscle where it is bound to the capillary endothelium. In women LPL activity is greater in the gluteal adipose tissue than in the abdominal tissue, whereas in men the converse is true (Arner et al 1991). Overall, the amounts of LPL in adipose tissue are higher in women than in men. Regular alcohol consumption increases the amount of LPL in the adipose tissue, whereas exercise increases that in the skeletal muscle.

Hepatic lipase (HL) hydrolyses triglyceride in HDL_2 and by so doing mediates the conversion of HDL_2 to HDL_3. Additionally it delipidates IDL to form LDL and is thought to be responsible for the interconversion between LDL subclasses. It is found on hepatic endothelial cells and concentrations in women are lower than those in men.

The triglyceride lipases are structurally related to pancreatic lipase. In addition to their role as enzymes, both have been shown to act as ligands for the binding of triglyceride-rich lipoproteins to cell membranes. Although each lipase is capable of hydrolysing each lipoprotein, they are selective - LPL preferentially acts on triglyceride-rich lipoproteins, and HL on HDL and LDL (Decklebaum 1987).

Hormone sensitive lipase (HSL) is the rate limiting enzyme in the intracellular hydrolysis of triglyceride in adipose tissue, releasing fatty acids into the circulation. It is regulated reciprocally to LPL (Coppack et al 1994).

1.4.3 Receptors.

The **LDL (or B/E) receptor** was first identified by Goldstein and Brown (1977) winning them a Nobel prize for their discovery. The receptors are found predominantly on hepatocytes but a significant proportion are present in the adrenals and gonads. They are responsible for the clearance of IDL and LDL from the plasma,

with the apoB-100 on these lipoproteins acting as the ligand for receptor binding. Their expression on cell surfaces is regulated by the intracellular cholesterol concentration such that when cholesterol is plentiful within the cell the LDL receptors are down-regulated and vice versa, thus maintaining a balanced intracellular cholesterol content.

The **LDL receptor-related protein (LRP)** has been described as a receptor for chylomicron and VLDL remnants (Beisiegel 1995). In this case the ligand is said to be apoE. It is present on numerous cell types, including macrophages and smooth muscle cells, suggesting a possible involvement in atherogenesis.

Other receptors that have more recently been described include extrahepatic VLDL receptors, scavenger receptors on macrophages and HDL receptors (Beisegel 1995, Thompson 1994).

1.5 An Overview of Lipoprotein Metabolism.

In health the apolipoproteins, receptors and enzymes interact intimately with the lipoproteins to maintain lipid homeostasis. This system can be described as having three main components. Firstly, the backbone of the system is the delipidation cascade for the sequential delipidation of apoB-100 containing lipoproteins and the dispersion of lipids to tissues in the fasted state. Secondly, the intermittent presence of chylomicrons serves to provide the system with exogenous lipids, with minimal disruption to endogenous turnover. Thirdly, the HDL removes excess cholesterol from the periphery and remodels apoB containing lipoproteins. Thus the homeostasis is maintained despite dramatic swings in plasma lipid levels (particularly triglyceride) between the fasted and fed states.

In the post-prandial state the plasma is flooded with chylomicrons. They are delipidated by the action of lipoprotein lipase and their remnants are cleared by the liver. These chylomicrons compete with VLDL for lipoprotein lipase and appear to be preferentially delipidated. Thus post-prandially there is an increase in plasma VLDL, mainly $VLDL_1$, although the majority of the triglyceride increase is due to chylomicrons (Karpe et al 1993). HDL mediates neutral lipid exchange, depleting the chylomicrons of triglyceride. The longer chylomicrons reside in the plasma the more cholesterol-enriched they become (and the more triglyceride-rich the HDL become). These cholesterol-rich chylomicrons are believed to be highly atherogenic (Packard and Shepherd 1990).

VLDL are secreted into the plasma by the liver in response to hormonal control of hepatic triglyceride synthesis, entering at the top of the delipidation cascade. They are sequentially delipidated firstly to IDL by lipoprotein lipase, mediated by apoCII, and then to LDL by hepatic lipase. The LDL are then cleared from the plasma by the LDL receptor, following binding mediated by apoB.

The liver can directly secrete any class of lipoprotein and so apoB can enter into any point of the delipidation cascade. In addition, any lipoprotein can be directly cleared from the plasma, thus removing the apoB from the cascade. The concept of direct LDL synthesis is controversial, with some workers suggesting that LDL are not secreted directly, but in fact as a small pool of VLDL which are very rapidly metabolised to LDL. The topic is reviewed by Shames and Havel (1991) who summarise the results from many radio-isotope metabolic studies. The authors conclude that the issue is far from solved, but they argue that the apparent direct LDL synthesis can be explained by VLDL heterogeneity. A recent study (Gaw et al 1995) noted that the degree of direct LDL synthesis appeared to be inversely related to plasma triglyceride level. This fits with the concept that it is the availability of lipid within the hepatic pool that determines the class of lipoprotein secreted - in states of low triglyceride the triglyceride-poor lipoproteins are secreted.

1.5.1 Metabolic Channelling of ApoB-100 Containing Lipoproteins.

The idea of metabolic channelling was first introduced by Fisher in the 1980s (Fisher 1982). He described the concept whereby "a chemical substance may be metabolised

along alternate metabolic pathways which do not necessarily intersect". Dual tracer radio-isotope studies have shown that this concept can be applied to the delipidation cascade (Packard 1995, Griffin and Packard 1994). VLDL entering the cascade at $VLDL_1$ appears to be delipidated slowly through $VLDL_2$ and IDL to LDL, predominantly forming LDLIII following neutral lipid exchange by CETP and subsequent hydrolysis by hepatic lipase. On the other hand, VLDL entering directly as $VLDL_2$ is channelled rapidly down the cascade to LDLI. The LDL are then cleared from the plasma at different rates, with LDLI being rapidly cleared by receptor uptake, while LDLIII is less attractive to the receptor and is cleared slowly, mostly by receptor-independent mechanisms (Caslake et al 1992). The synthesis of the two VLDL subfractions also appears to respond to different stimuli. $VLDL_1$ synthesis is under hormonal control e.g. increasing in the presence of insulin resistance and oestrogen, whereas $VLDL_2$ synthesis is increased in states of hypercholesterolaemia.

1.5.2 Regulation of ApoB Metabolism.

The metabolism of lipoproteins is regulated by local factors such as lipid concentrations, but also responds to more distant hormonal influences such as the sex steroids and, more importantly, insulin.

Insulin is the major anti-lipolytic hormone (Coppack et al 1994), functioning, primarily in the post-prandial state, by the following three mechanisms (Frayn 1993) - i). inhibition of hormone sensitive lipase thereby suppressing fatty acid release from adipocytes, ii). decreasing hepatic VLDL production, and iii). increasing LPL activity in adipose tissue and so clearing triglyceride from the circulation.

In states of insulin resistance, such as obesity, non-insulin dependent diabetes mellitus (NIDDM) and hypertriglyceridaemia, this anti-lipoplytic effect is lost. Excess lipolysis leads to worsening of glucose tolerance and hypertriglyceridaemia, and is seen at its most unrestrained in diabetic ketoacidosis (Coppack et al 1994). Excess fatty acid is present in the plasma and hepatic VLDL synthesis is increased. Reduced LPL activity prolongs the residence time of the triglyceride-rich VLDL in the plasma so exposing them to prolonged CETP and HL action with the resultant formation of small, dense LDL and a lowered HDL cholesterol level. This pattern of hypertriglyceridaemia, low HDL cholesterol and small, dense LDL has been characterised by Austin (1990) as the Atherogenic Lipoprotein Phenotype. The dyslipidaemia may predate the development of NIDDM and persists despite glycaemic control, resulting in an increased incidence of CHD. In obesity (which often co-exists with NIDDM) LPL loses its sensitivity to insulin (Frayn 1993).

The Insulin Resistance Syndrome was first described by Reaven in the Banting lecture in 1988. This polymetabolic syndrome describes the clustering of insulin resistance and the characteristic dyslipidaemia with central obesity and hypertension. There appears to be a genetic component to its development and it is associated with an increased risk of CHD.

1.6 The Triglyceride Issue - a deeper look at the 'at risk' lipoprotein profile.

Whilst for some time the relationship between plasma cholesterol and CHD has been accepted as 'continuous and graded' (Stamler et al 1986) the role of triglyceride is less clear cut. Triglyceride is not found to any appreciable degree in atheromatous plaques and for many years was ignored as a risk factor.

Austin, in her 1991 review, summarised the problems in assessing the relationship between triglyceride and CHD. Epidemiological studies have shown a univariate relationship but in many studies this relationship is lost with multivariate analysis, especially those that introduce LDL and HDL as variables. Additional complications include the large intra-individual variation in triglyceride and its relatively imprecise measurement as compared, for example, to that of HDL. Intervention studies thus far have only addressed the triglyceride issue to a limited degree. Furthermore, genetics appear to play a role in determining triglyceride levels. However, what has emerged from these studies is the positive correlation between triglyceride and small, dense LDL, and the negative correlation with HDL cholesterol.

In moderate hypertriglyceridaemia (2.3-6.0mmol/l) the primary metabolic defect is increased VLDL production. However, in more severe elevations (>6.0mmol/l) the problem appears to be one of defective catabolism (Stalenhoef et al 1986). Overall, triglyceride levels greater than 1.5mmol/l show an increasing association with CHD up to a level of 5.0mmol/l. It then appears to tail off with the main risk of severe hypertriglyceridaemia being pancreatitis, a condition with a mortality rate of 5-60%. Therefore, although the relationship between excess triglyceride and CHD is less clear than that of cholesterol, it has specific and detrimental effects on lipoprotein metabolism that can result in the atherogenic lipoprotein phenotype. This in turn, has been shown to be associated with increased risk of CHD.

We thus have three main categories of lipoprotein profile which are associated with an increased risk of CHD, i). a simple elevation of LDL, as epitomised by familial hypercholesterolaemia, ii). a low level of HDL, the hypoalphalipoproteinaemias, and iii). the atherogenic lipoprotein phenotype, encompassing elevated triglycerides, small, dense LDL and low HDL cholesterol. The aetiology of each is different and each requires a different approach in its management, but the clinical manifestation of each if left untreated is the same.

1.7 Studying and Modelling Lipoprotein Kinetics.

The detailed study of lipoprotein kinetics requires both an appropriate tracer and an applicable model.

1.7.1 The Choice of Tracer.

Over the years several tracers have been tried and tested. A tracer is merely a label for the system, or tracee, under study, in this case lipoproteins or apolipoproteins. In their recent review, Barrett and Foster (1996) described the ideal tracer as having the following characteristics - "i). it is detectable, in experimental samples, by an observer, ii). its introduction into the system does not perturb the system, i.e. steady state is maintained, and iii). it is indistinguishable with respect to the properties of the system being studied, that is the kinetics of the tracer are identical to those of the tracee." These last two points are largely assumed although in some instances may not be strictly correct.

Much of the earlier kinetic work utilised radio-isotope tracers but more recently stable isotopes have come to the fore. The use of stables isotopes in metabolic studies actually predates that of radio-isotopes, being first used in the 1930's.

The use of each has advantages and disadvantages. Radio-isotopes are generally less expensive and more easily processed. Their use is limited by the safety aspects of handling radioactivity, their preclusion for use in children and pregnant or lactating women, and their restricted repeatability in any one individual. Stable isotopes, at doses used in metabolic studies, have a good safety profile (Jones and Leatherdale 1991) and can be widely and repeatedly used. However, they are costly and require labour-intensive methods of analysis. The latter is being overcome to a certain extent by the development of sophisticated mass spectrometry techniques.

With few exceptions, radio-isotopes are attached to a lipoprotein or apolipoprotein ex vivo whilst stable isotopes are administered as small molecular weight precursors to lipid (e.g. ^{13}C-acetate) or protein (e.g. d_3-leucine). Each method has advantages and disadvantages. Exogenous tracers allow specific labelling of the lipoprotein or lipoproteins under study. (This potential for separate labelling of individual lipoproteins has been utilised in several kinetic studies, see below). Of concern, however, is that the isolation, labelling and re-introduction of the lipoprotein may alter its kinetics such that it is no longer a true representation of the system (see the third point of Barrett and Foster).

With endogenous labelling a naturally occurring substance is administered and becomes incorporated into the protein or lipoprotein as it is synthesised. However, this incorporation is non-specific, for example, when using an amino acid tracer all body protein becomes labelled rather than solely the apolipoprotein of interest. In addition, as these proteins are catabolised the tracer is recycled, necessitating complex modelling methods to accomodate this 'tail' that is seen in the enrichment curves. In general, endogenous tracers give a better representation of synthetic rates but are less accurate for interconversions and catabolic events (Packard 1995).

'The notion of kinetics of a lipoprotein particle as a whole becomes vague when all of its components can undergo transitions independently, unless it contains at least one marker with which it can be totally identified'. (Mones Berman 1982).

When studying the kinetics of VLDL, IDL and LDL, apoB-100 is that marker. It can be identified with amino acids labelled with stable isotopes. Various amino acids have been compared - leucine, valine and lysine (Lichtenstein et al 1990), leucine and glycine (Parhofer et al 1991) - with the conclusion that all produce similar results. For this thesis apoB-100 was labelled with tri-deuterated (d_3) leucine. Leucine is one of the better amino acids for this purpose as it is an essential amino acid, is ubiquitous in body protein, plentiful within apoB and is minimally recycled due to its catabolism to ketoisocaproate.

The natural abundance of deuterium (^2H) is 0.015%, substantially lower than ^{13}C at 1.11% and ^{15}N at 0.37%. Additionally, the relative mass difference from the natural element is greater for deuterium than the others. The use of multiple labelling increases the sensitivity of measurement by minimising the background of natural isotope (Schaefer et al 1992).

Parhofer et al (1991) compared two methods of administration of stable isotopes - primed constant infusion and bolus dose - and found that the method used had no significant effect on the metabolic parameters provided that both were analysed using multicompartmental modelling. Both methods have been used in this thesis.

1.7.2 Multicompartmental Modelling and Model Design.

As research progresses the lipoprotein system as we understand it becomes increasingly complex and thus requires detailed modelling for its interpretation. The three commonly used methods of analysis are linear regression, monoexponential functions and multicompartmental modelling. Parhofer et al (1991) compared the three and found that, while in a group of subjects the fractional catabolic rates did not differ significantly between methods, in any one individual there was no correlation between values obtained by each method. Of the three, multicompartmental modelling is the gold standard. Linear regression analysis is lacking in physiological basis and monoexponential functions are not applicable to a heterogeneous population of particles such as lipoproteins.

A model is simply a mathematical representation of a physiological system, from which we can calculate the rates of production, interconversion and catabolism of the particles under study. Model design has increased in complexity over the past two decades. In 1975 Phair et al proposed a 'delipidation chain' to account for the shoulder in the VLDL data. This delipidation chain or cascade is still prominent in more recent models and represents the decreasing size of the lipoprotein as lipid is removed. Berman et al (1978) expanded on Phair's VLDL model by adding a pathway for the slow catabolism of VLDL. The consensus model of 1980 included the above two features plus a delay component to reflect the intrahepatic assembly of lipoproteins.

LDL was modelled by a single compartment within the plasma but allowed extravascular exchange. This single compartment is appropriate if the LDL is monodisperse but an additional compartment is required if it is polydisperse, as in the case of hypertriglyceridaemia (Fisher et al 1980).

Beltz et al (1985) modelled with all of the preceeding but included a compartment of IDL sequestered outwith the plasma. Allowance was made for irreversible loss of apoB from the system at the level of VLDL and IDL. In addition, a 'direct' input into LDL was allowed, representing a very rapidly metabolized VLDL compartment.

Parhofer's model of 1991 is illustrated in Figure 1.1. Compartment 1 represents the precursor amino acid in the plasma and is modelled with a forcing function. Compartment 2 is the delay component. All apoB enters the plasma as VLDL (compartment 11) and from here can proceed to a slowly metabolised VLDL pool (compartment 12) or directly to IDL (21) or LDL (31). Compartments 11 and 12 represent a minimum delipidation chain and allow for VLDL heterogeneity. ApoB is irreversibly lost from the plasma at the level of VLDL or IDL. This model was designed as the simplest structure based on previous publications and the authors own experimental data. Its expansion is necessary in hyperlipidaemic subjects.

Dual tracer metabolic studies enable the concept of metabolic channelling to be taken into account. Using ^{131}I-labelled $VLDL_1$ and ^{125}I-labelled $VLDL_2$ Packard et al (1995) developed a model with parallel delipidation pathways to explain the different kinetics of $VLDL_1$ and $VLDL_2$ (Figure 1.2). This would not be possible using endogenous stable isotope labelling techniques.

As models become increasingly complex so the number of parameters increases. Thus the concept of identifiability must be introduced. This is reviewed by Cobelli and DiStefano (1980) as the possibility of obtaining 'unique solutions for unknown parameters of interest in a mathematical model, from data collected in well defined stimulus-response experiments performed on a dynamic system represented by the model'. A model can be made uniquely identifiable by the introduction of parameter constraints or dependencies to reduce the number of unknowns. Ideally, these constraints should have a physiological basis.

One of the most recent models to be published is that of Demant et al (1996). It has many similarities to that of Packard et al (1995). Six normolipaemic individuals underwent turnover studies using tri-deuterated leucine with extension of the length of the turnover to 14 days to include the more slowly turning over LDL. (Details of the turnover procedures are given in the following chapter). Isotopic enrichments in the lipoprotein fractions $VLDL_1$, $VLDL_2$, IDL and LDL were measured by sensitive gas chromatography-mass spectrometry techniques and a multicompartmental model developed from the enrichment curves. The model was made uniquely identifiable by the application of certain physiologically plausible constraints and is described fully in the following chapter.

1.8 Aims and Objectives.

The primary aim of this thesis was to characterise the kinetics of apoB containing lipoproteins in certain dyslipidaemic states. In order to achieve this, stable isotope methodology was used that has already been established for use in normolipidaemic states (Demant et al 1996). Thus a secondary aim was to determine whether this methodology is appropriate for use in these abnormal states. In particular, the multicompartmental model, designed for modelling normal apoB kinetics, was applied to the pathological situations and its ability to explain abnormal apoB kinetics tested. Studies of apoB kinetics have been performed in the past but the majority used radio-isotope techniques and different methods of data analysis. Stable isotope studies combined with multicompartmental modelling are perhaps more ideal for this sort of work.

To meet the above aims three main objectives were set. First, a non-kinetic study was performed on a group of eighty normolipidaemic individuals to characterise the inter-relationships between lipoprotein subfractions and indices of obesity, insulin resistance and lipase activity. The same parameters were determined in a smaller group of normolipidaemic CHD sufferers and the two groups compared. In particular, the differences between males and females and between non-CHD and CHD sufferers were noted despite all the individuals having lipid levels within the same range. This provided an insight into the normal regulation of lipids and lipoproteins on which to interpret the findings from the kinetic studies.

Secondly, stable isotope studies of apoB metabolism were performed on individuals with selected pathological conditions. Familial hypercholesterolaemia and familial defective apoB-100, two conditions with a similar clinical presentation and both due to defective LDL receptor-mediated catabolism but each with a different underlying aetiology, were studied and compared both to each other and to the results obtained in a study of normolipidaemic individuals performed using identical methodology (Packard, unpublished). Two unusual individuals, one with hypobetalipoproteinaemia and one with analbuminaemia, with plasma apoB levels at opposite ends of the range were studied and their apoB metabolism characterised. Although these conditions are rare and their apoB metabolism extreme, they can add understanding to apoB metabolism in more common states. Lastly, a group of subjects with a more common form of dyslipidaemia, mixed hyperlipidaemia, was studied. This type of individual is probably the most common to attend Lipoprotein Clinics and so it is important to determine the abnormalities in the apoB metabolism.

Finally, using the mixed hyperlipidaemic group the mechanisms of action of two HMG-CoA reductase inhibitors, simvastatin and atorvastatin (a new HMG-CoA reductase inhibitor), were determined by observing the effect of treatment on apoB metabolism. The HMG-CoA reductase inhibitors have come to the fore in recent years as drugs of choice for the treatment of various forms of dyslipidaemia and so an understanding of the effect that they have on apoB metabolism is important.

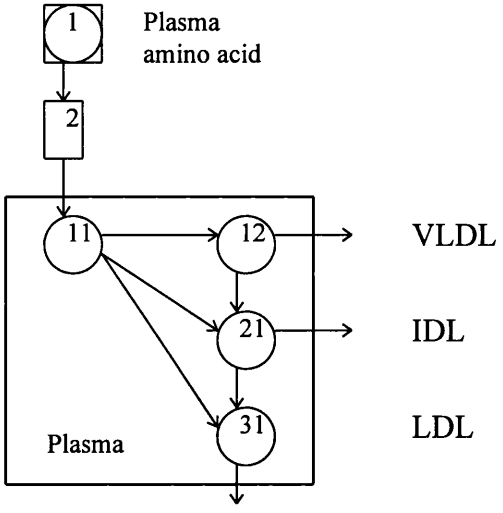


Figure 1.1 Parhofer's 1991 Model.

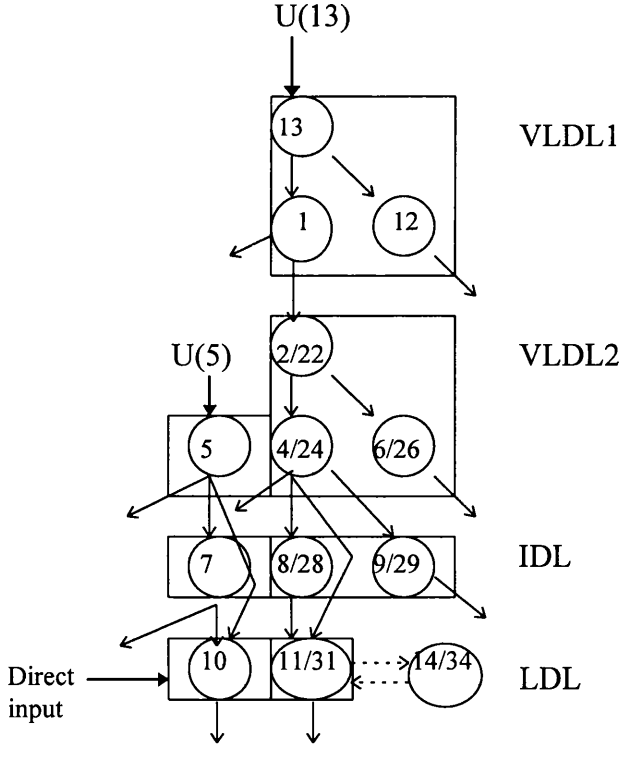


Figure 1.2 Packard's 1995 Model.

Chapter 2 General Methods.

2.1 Materials.

Names and addresses of manufacturers and suppliers of reagents, hardware and software used for this thesis are given in Appendix 1.

2.2 Subject Selection and Clinical Measurements.

2.2.1 Hyperlipidaemic Subjects.

For each study a computer printout (courtesy of Ms Jennie Johnson, Department of Biochemistry) was obtained for all those patients attending Glasgow Royal Infirmary within a specified time period whose lipid levels fell within the required range. The patients' case notes were screened to exclude those who fell out with selection criteria. Suitable subjects were contacted by letter with a pre-paid reply slip enclosed to indicate their interest or otherwise in participating. Interested subjects were then contacted by telephone and asked to attend for a screening visit. The majority of subjects were patients attending the Lipoprotein and Cardiology Clinics. Where more than a single blood sample was required all gave signed informed consent.

2.2.2 Normolipidaemic Controls.

Normolipidaemic subjects were recruited from laboratory staff, friends and colleagues from elsewhere in the hospital. In the case of any previously unrecorded abnormality being found on physical examination or blood sampling the subject's general practioner (GP) was informed. In all cases where drugs were administered the GP was informed.

For all studies, a medical history, including family and social histories, was taken and examinations performed as specified by the relevant protocol. Blood pressure was taken with the subjects seated for at least five minutes using an Accoson mercury sphygmomanometer. Diastolic pressure was recorded as Korotkov phase five. Height and weight were measured with shoes and outdoor clothing removed. Anthropometric indices were measured in the standing position as follows - waist = the smallest circumference between the rib cage and iliac crest, hip = the largest circumference between waist and thigh. Where specified by protocol, a standard full physical examination was performed.

Venous blood samples were taken via a 22G needle from the ante-cubital fossa with subjects seated and the tourniquet applied for the minimum time necessary. All lipid and lipoprotein measurements, glucose and insulin levels were taken following an overnight fast. Appropriate haematological and endocrine samples were taken to exclude a secondary cause for the hyperlipidaemia. These were analysed by the haematology and biochemistry laboratories in this hospital. Plasma was either separated immediately or samples stored temporarily at $4^{o}C$.

2.2.3 Protocol for Post-heparin Lipase Sampling.

Prior to the administration of heparin the subjects completed a questionnaire to exclude any containdications to its use, such as bleeding disorders, hypersensitivity to heparin, recent ingestion of aspirin or non-steroidal ant-inflammatory drugs, and history of cerebrovascular accident, peptic ulcer disease or rheumatic fever. In addition, a recent normal haematology report was required.

Following an overnight fast subjects received an intravenous bolus of heparin at a dose of 70iU/kg body weight. Twelve minutes later a 10ml venous sample was taken into lithium heparin and placed immediately on ice. Plasma was separated as soon as possible.

2.2.4 Protocol for Stable Isotope Administration and Sampling.

Subjects were admitted at 8am after an overnight fast and a 22G cannula placed in one forearm. Samples were taken through this during the day, the cannula being kept patent by flushing with normal (0.9%) saline. Sterile tri-deuterated leucine (Isotec Inc), made up in normal saline, courtesy of the hospital pharmacy, was administered into the contralateral arm either as a bolus dose (7mg/kg body weight) or as a primed constant infusion using an Imed volumetric infusion pump (0.7mg/kg prime, 0.7mg/kg/hour infusion for 10 hours). The subjects remained fasted during the day but were allowed unlimited low calorie fluids. At 6pm they were given a standardised low fat meal before the final samples were taken and they were allowed home. Over the following two weeks a community nurse came daily to their home or work to take a further fasting sample. In total, less than one pint of blood was taken per turnover and haemoglobin levels were monitored throughout. The bleeding schedule (with division of plasma for analysis) is given in Appendix 2.

2.3 Ethical Approval.

All studies were approved by the Ethical Committee of Glasgow Royal Infirmary.

2.4 Laboratory Methods.

2.4.1 Beta Quantification of Lipids and Lipoproteins.

The method used is a modification of the standard Lipid Research Clinics Protocol (1982) involving the selective precipitation of lipoproteins by the addition of sulphated polysaccharides and divalent cations to plasma. This routine analysis was kindly performed by the Lipid Section of the Biochemistry Department.

One aliquot (100µl) of plasma is set aside for measurement of total cholesterol and triglyceride (see below). 5ml plasma in an ultracentrifuge tube is overlayered with normal saline, density 1.006, and centrifuged overnight at 35,000rpm, 4°C. The tubes are then 'sliced' at a constant height to separate the top fraction containing VLDL from the bottom fraction containing HDL and LDL. HDL is then separated from LDL by precipitation of the latter with heparin and manganous chloride leaving the HDL in solution. Cholesterol is measured in the bottom fraction, top (VLDL) fraction and HDL. Calculations are performed as follows:-

bottom fraction chol. - HDL chol. = calculated LDL chol.
total chol. - bottom fraction chol. = calculated VLDL chol.

The measured top (VLDL) cholesterol should equal the calculated VLDL cholesterol to within 0.35mmol/l.

The analysis of lipoprotein composition, apoAI and B, apoE phenotyping, Lp(a), postheparin lipases and HDL subfractions, and the preparation of tubes for ultacentrifugation were kindly performed on a semi-routine basis by the following members of the Lipid Research team - Mr Michael McConnell, Mrs Liz Murray, Ms Julie MacKenzie and Mrs May Stewart.

2.4.2 Compositional Analysis.

1. Cholesterol -
This was measured by automated analysis on a Hitachi 717 analyser of an enzymatic calorimetric test (Boehringer Mannheim). The test principle is as follows:-

$$\text{chol. esters} + H_2O \xrightarrow{\text{chol. esterase}} \text{chol.} + \text{RCOOH}$$
$$\text{chol.} + O_2 \xrightarrow{\text{chol. oxidase}} {}^4\text{cholestenone} + H_2O_2$$
$$2H_2O_2 + \text{4-aminophenazone} + \text{phenol} \xrightarrow{\text{POD}}$$
$$\text{4-(p-benzoquinone-monoimino)-phenazone} + H_2O$$

Sample volume - 3µl, 2 point calibration, read at wavelength 505nm.

2. Triglycerides -
These were measured by automated analysis on a Hitachi 717 analyser of an enzymatic calorimetric test (Boehringer Mannheim). The test principle is as follows:-

$$\text{triglycerides} + 3H_2O \xrightarrow{\text{lipase}} \text{glycerol} + 3RCOOH$$
$$\text{glycerol} + ATP \xrightarrow{GK} \text{glycerol-3-phosphate} + ADP$$
$$\text{glycerol-3-phosphate} + O_2 \xrightarrow{GPO} \text{dihydroxyacetone phosphate} + H_2O_2$$
$$H_2O_2 + \text{4-aminophenazone} + \text{4-chlorophenol} \xrightarrow{\text{peroxidase}} \text{4(p-benzoquinone-monoimino)-phenazone} + 2H_2O + Hcl$$

Sample volume - 3µl, 2 point calibration, read at wavelength 505nm.

3. Phospholipids -
These were measured by an enzymatic colorimetric test kit (Boehringer Mannheim Cat. No. 691844), measured at 500nm, using a Centrifichem Encore analyser (Baker Instruments).

Quality controls — Precinorm (Boehringer Mannheim Cat. No. 781827)
Precipath (Boehringer Mannheim Cat. No. 1285874).

4. Free Cholesterol -
This was measured using an enzymatic calorimetric test kit (Boehringer Mannheim Cat. No. 310328) by the CHOP-PAP method of total cholesterol but omitting the enzyme cholesterol esterase. It was analysed using a Centrifichem Encore analyser (Baker Instruments) and read at wavelength 500nm

Quality controls — as for phospholipids.

5. Esterified Cholesterol -
This was calculated from the total and free cholesterol as follows:-

1.68 x (total chol. - free chol.)

The correction factor of 1.68 is to account for the molecular weight of the different cholesterol esters.

Conversion Factors -
The factors used to convert from mmol/l to mg/dl are as follows:-

cholesterol in mg/dl = 38.7 x value in mmol/l
triglycerides in mg/dl = 88.7 x value in mmol/l

2.4.3 Measurement of ApoAI and ApoB.

These were measured using liquid-phase immunoprecipitation test kits (Orion Diagnostica Cat. Nos. 67249 & 67265), read at 340nm, in a Centrifichem Encore analyser (Baker Instruments).

2.4.4 ApoE Phenotyping.

ApoE phenotyping was carried out using the method of Havekes et al (1987) which is based on isoelectric focusing of delipidated plasma followed by electroblotting with an anti-apoE antibody. Plasma is incubated with neuraminidase in sodium acetate buffer. Samples are delipidated and dried using ethanol:ether, 3:1, and ether and dissolved in solubilising buffer with 2-mercaptoethanol. Iso-electric focusing is performed overnight on acrylamide gels followed by Western (electro) blotting for 3 hours. Blots are incubated overnight with the first antibody, an anti-apoE monoclonal antibody produced within the laboratory. Following washing, the second antibody is incubated with the blots for a minimum of 2 hours. This is a horseradish peroxidase anti-mouse IgG antibody (SAPU Prod. No. SO81-201). Plasma from a subject known to have the apoE 2/2 phenotype is used as quality control. Blots are developed and read.

2.4.5 Measurement of Lp(a).

Lp(a) was quantitatively determined using an enzyme linked immunoassay (ELISA) in the form of an Innotest Lp(a) kit (Innogenetics NV), (Dagen et al 1991). All solutions, standards, controls and antibodies (mouse monoclonal Lp(a), polyclonal sheep anti-apoB labelled with horseradish peroxidase) are supplied. Polystyrene microplate strips are pre-coated with the 1st antibody. Lp(a) binds to this and unbound substances are removed by washing. The 2nd antibody binds to the 1st antibody/Lp(a) complex due to the apoB moiety of Lp(a). A blue colour forms on incubation which turns to yellow when the reaction is stopped with sulphuric acid. The colour, proportional to the Lp(a) concentration, is read on a spectrophotometric plate reader at 450nm.

2.4.6 Post-heparin Lipase Assay.

Post-heparin plasma (PHP) is incubated with ^{14}C-labelled triglyceride/gum arabic emulsion. Free fatty acids, released by the action of lipase, are captured by albumin and extracted into a solvent. The radioactivity present in this solvent allows calculation of the lipase activity. LPL activity is measured by inactivating HL with sodium dodecyl sulphate and adding apoCII (Baginsky and Brown 1979). HL activity is measured at 0.1M NaCl to inactivate LPL. Activity is measured in a Packard scintillation counter and results calculated as follows:-

Lipase activity in μmoles free fatty acid (FFA) released/ml/hour

$$= \frac{(\text{CPM samples} - \text{CPM blank}) \times 755.1}{\text{CPM total} - \text{background}}$$

2.4.7 Isolation of High Density Lipoprotein Subfractions from Plasma.

Two methods have been used for this isolation - analytical ultracentrifugation and precipitation with dextran sulphate.

1. Analytical Ultracentrifugation of HDL -
HDL subfraction masses were estimated in a Beckman Model L8 ultracentrifuge equipped with an ultraviolet scanning attachment, (Beckman Instruments) using an AnF rotor with double sector centrepiece cells. Plasma density is adjusted by the addition of d = 1.31g/ml solution and centrifuged at 40,000rpm, 18°C for 30 hours. The top 1ml is gently aspirated and diluted 1:8 with d = 1.20g/ml solution to adjust the optical density at 280nm to between 0.8 and 0.9. The double-sectored cell is filled with solvent and sample and centrifuged at 42K, 26°C for 2 hours, 11 minutes. The cell is then scanned and the HDL_2 and HDL_3 masses calculated (Shepherd et al 1984).

2. HDL_2 and HDL_3 Measurement by Dextran Sulphate Precipitation -
ApoB containing lipoproteins were precipitated with 10g/l dextran sulphate (Genzyme Dextralip 50), 0.5M $MgCl_2$. The concentration of the supernatant was raised to 1.5M which precipitates specifically HDL_2. The cholesterol contents of the supernatants were measured and HDL_3 cholesterol calculated by subtraction (Warnick et al 1982).

2.4.8 Surface Modification of Beckman Ultraclear Centrifuge Tubes.

This modification of the tubes with polyvinyl alcohol allows solutions to gravity-feed down their sides (Holmquist 1982).

2.4.9 Protein Assay.

The measurement of protein was performed using a modification of the Lowry et al (1951) method involving measurement of proteins with the Folin phenol reagent after alkaline copper treatment.

Reagents -

1) Stock Reagents	solution A - 2% Na_2CO_3 in 0.1N NaOH
	solution B - 2% Na K Tartrate in deionized water
	solution C - 1% Cu SO_4 in deionized water
	Folin Ciocalteu reagent (BDH, Prod 19058 3Q)
2) Working Reagents	Biuret reagent =100ml solution A + 1ml B + 1ml C
	- if the sample to be analysed is turbid, e.g. VLDL, add sodium dodecyl sulphate (1mg/ml) to the Biuret reagent
	Folin Ciocalteu diluted 1:1 with deionized water

Standards -

1) Stock Standard	human serum albumin (Sigma A-8763),
	- 1mg/ml, stored at -70°C
2) Working Standard	a standard curve in the range 0-50µg is made by taking 0, 15, 25, 35, 50µl of stock standard and adjusting to a final volume of 400µl with deionized water

Quality Control -

Stock Solutions	bovine serum albumin (Sigma A-4503) - 0.15mg/ml and 0.30mg/ml, stored at -70°C, 100µl adjusted to 400µl with deionized water

Sample Preparation -

Samples were made up to 400µl with deionized water as necessary. Volumes used are as follows -

Total subfractions	$VLDL_1$	200µl
	$VLDL_2$	100µl
	IDL	50µl
	LDL	25µl
Isopropanol subfractions		400µl

Method -

i) to 400µl of standard, control and sample add 2ml Biuret reagent
ii) vortex and stand for 10 minutes
iii) add 200µl of working Folin Ciocalteu reagent
iv) vortex and stand for 30 minutes
v) read optical density (OD) at 750nm
vi) plot OD_{750} against concentrations of standards and read the unknowns from the standard curve.

2.4.10 Isolation of Low Density Lipoprotein Subfractions from Plasma.

This was performed as part of collaborative work with Dr Muriel Caslake. The method used is that of Griffin et al (1990) involving a density gradient centrifugation procedure for the rapid separation of discrete LDL fractions directly from plasma.

Density solutions -

d = 1.006g/ml	11.4g NaCl + 0.1g Na_2EDTA + 1ml NNaOH in 1litre + 3ml H_2O, NaCl $conc^n$ = 0.195M			
d = 1.182g/ml	24.98g NaBr + 100ml of d = 1.006g/ml solution NaCl $conc^n$ = 0.195M, NaBr $conc^n$ = 2.44M			
Remainder	g/ml	ml 1.006g/ml		ml 1.182g/ml
	1.019	100	+	8.5
	1.024	100	+	13.6
	1.034	100	+	18.6
	1.045	100	+	27.8
	1.056	100	+	42.9
	1.060	100	+	49.3

All densities are checked with a digital densitometer.

Method -

The density of 3ml fresh (not frozen) plasma is adjusted to 1.09g/ml by the addition of 0.25g solid KBr. The sample and a discontinous six step salt gradient of the above densities are introduced into a coated Beckman Ultraclear tube by peristaltic pump, as follows:-

	g/ml	ml
top	1.019	1
	1.024	2
	1.034	2
	1.045	1
	1.056	1
	1.060	1
	sample	3
bottom	1.182	0.5

This is then centrifuged at 40K, 23°C, 24 hours and stopped without braking. The tubes are placed in a purpose-built tube piercing apparatus (Beckman) and the separated LDL fractions are upwardly displaced by the introduction of a dense, inert, hydrophobic material, Maxidens 1.9g/ml (Nycomed Pharma AS). The eluate is passed through a UV detector, with computer link (Beckman Data Graphics), and continuously monitored at 280nm to provide an LDL subfraction density profile. In most cases it is possible to detect three distinct subfractions, LDLI (1.025-1.034g/ml), LDLII (1.034-1.044g/ml) and LDLIII (1.044-1.060g/ml). The LDL concentration at 280nm is corrected to lipoprotein mass equivalence by applying specific extinction coefficients - LDLI 1 optical density unit (1 OD) = 2.63mg lipoprotein/ml, LDLII 1 OD = 2.94mg lipoprotein/ml, LDLIII 1 OD = 1.92mg lipoprotein/ml.

2.4.11 Sequential Isolation of $VLDL_1$, $VLDL_2$, IDL and LDL.

The four subfractions were prepared from plasma by a modification of the method of Lindgren et al (1972).

Density solutions -
d = 1.006g/ml and d = 1.182g/ml were prepared as for LDL subfraction isolation. The remainder were prepared as follows:-

g/ml	ml 1.006g/ml		ml 1.182g/ml
1.0988	50	+	57.78
1.0860	50	+	41.66
1.0790	75	+	53.16
1.0722	75	+	46.50
1.0641	75	+	36.93
1.0588	100	+	42.92

All densities were checked with a digital densitometer.

Method -
The density of 2ml plasma is adjusted to 1.118g/ml by the addition of 0.341g NaCl. A six step salt gradient is set up in a coated Beckman Ultraclear tube as follows:-

	g/ml	ml
top	1.0588	2
	1.0641	2
	1.0722	2
	1.0790	2
	1.0860	1
	1.0988	1
	sample	2
bottom	1.182	0.5

The tube is centrifuged at 39,000rpm, 23°C for 1 hour 38 minutes. 1ml, containing $VLDL_1$, is carefully removed from the top by aspiration and replaced with 1ml d = 1.0588 solution. $VLDL_2$ is removed in 0.5ml following centrifugation at 18,500rpm for 15 hours 41 minutes. Further volumes are not replaced. IDL is removed in 0.5ml following centrifugation at 39,000rpm for 2h 35min and LDL is removd in 1ml following 30,000rpm for 21h 10min. The subfractions are stored at 4°C until analysis.

2.4.12 Preparation of Lipoproteins for Gas Chromatography-Mass Spectrometry.

ApoB is precipitated by the addition of an equal volume of isopropanol (Egusa et al 1983) and allowed to stand overnight at 4°C. The samples are centrifuged at 3,000rpm, 4°C for 30 minutes and the supernatant removed by aspiration. The supernatant is saved for protein measurement by the method of Lowry. 3ml ethanol:ether, 3:1, is added to each sample (6ml to IDL and LDL) and allowed to stand overnight at 4°C. Following centrifugation at 3,000rpm, 4°C for 30 minutes the supernatant is removed. This step is repeated for IDL and LDL to ensure complete delipidation (the apoB pellet should now be white in colour). The samples are dried by the addition of 3ml ether which is allowed to stand for a minimum of 1 hour. This is then removed following centrifugation as above and the apoB pellets dried at 37°C overnight. The pellets are hydrolysed by addition of 2ml 6N 'Aristar' HCl (BDH) and heated at 110°C for 24 hours. The amino acid hydrolysate is concentrated in a vacuum concentrator centrifuge (Howe) and transferred to Chromacol vials before being dried completely.

2.4.13 Calculation of Lipoprotein ApoB Plasma Pools.

The apoB content of $VLDL_1$, $VLDL_2$, IDL and LDL was calculated as the difference between the total protein and the isopropanol soluble protein for each lipoprotein fraction, both measured by the Lowry method. Plasma volume (dl) was estimated as 40% body weight (kg). ApoB plasma pool sizes (mg) were calculated as the apoB mass (mg/dl) multiplied by the plasma volume. The leucine content of the apoB pools was then calculated from the apoB amino acid composition.

2.4.14 Correction for Centrifugal Losses.

Compositional analysis of $VLDL_1$, $VLDL_2$, IDL and LDL was performed as described above. The recovery of $VLDL_1$ + $VLDL_2$ + IDL + LDL cholesterol was compared to the 'non-HDL' cholesterol in plasma. In addition, the cholesterol content of Lp(a) was taken into account (taken as 25%) and the final pool sizes corrected accordingly:-

$$\frac{VLDL_1 + VLDL_2 + IDL + LDL + Lp(a)\ chol.}{\text{'non-HDL' chol.}} \times 100 = \%\ \text{recovery}$$

Analysis of samples by gas chromatography-mass spectrometry was kindly performed by Mrs Dorothy Bedford and Mr Philip Stewart. Preparation of the plasma free amino acids was carried out by Mrs Dorothy Bedford and Mrs Grace Stewart.

2.4.15 Preparation of Plasma Free Amino Acids for Gas Chromatography-Mass Spectrometry.

1ml 10% trichloroacetic acid (TCA) is added to 1ml plasma to precipitate protein. Following centrifugation at 3,000rpm, 4°C for 30 minutes the supernatant is poured down a cation exchange column pre-treated with 1N HCl and filled with Dowex AG-50W-X8 resin (H^+ form, 50-100 mesh, Bio-Rad). The columns are washed with deionized water to remove remaining TCA and the amino acids desorbed by twice washing with 3ml fresh 4M NH_4OH. Samples are then partially dried by centrifugal evaporation (Howe) using high temperature before transferring the reduced volume to Chromacol vials and taking to dryness.

2.4.16 Sample Analysis by Gas Chromatography-Mass Spectrometry (GC-MS).

Amino acids prepared from plasma and apoB are first derivitised to tert-butyl-dimethyl-silyl- (TBDMS-) derivatives before GC-MS analysis. The specific enrichment of tracer amino acid in the plasma and apoB derivatives, (the atom percent excess or APE), is then measured. The Fisons Trio 1000 quadrupole system (Fisons Instuments) was used. The gas chromatograph performs the volatilisation and separation of the particles of interest with helium as the carrier gas. The particles are then ionised in the mass spectrometer and deflected in an electric field. The resultant spectrum shows the relative abundance of each ionised particle. Selected ion recording increases the accuracy of measurement by focusing on only a few specific masses. Sensitivity is increased by multiple labelling of the tracer amino acid and by measuring the m/z 277:m/z 276 (m+3:m+2) ion ratio (Demant et al 1994). The ability to measure these higher mass fragments against a low background allowed a more precise estimate of enrichment to be made than is possible with direct assay of the m/z 277:m/z 274 ratio. The m/z 277:m/z 276 ratio is transformed into values for specific isotopic enrichment (E) by multiplication by the m/z 276:m/z 274 value which is constant. E is calculated as follows:-

$$E = (R-R_0)/[(1+R)+(1+R_0)]$$

where R and R_0 are the m/z 277:m/z 274 ratios of the sample and naturally occurring leucine respectively. The tracer:tracee ratio (Z) is then calculated as follows:-

$$Z = E/(E_t-E)$$

where E_t is the isotopic abundance of the infused [$^2H_3$]leucine over that which is naturally occurring. This method can reliably distinguish 0.1% APE from 0% APE.

2.5 Data Analysis.

2.5.1 Statistical Analysis.

This was performed using Minitab Release 10 for windows (Minitab Inc).Variables were analysed either as normal distributions or transformed where necessary. Specifications of the statistics used are given where results are reported.

2.5.2 Multicompartmental Modelling.

This was performed using the Simulation Analysis and Modelling programme II (SAAM II) version 1.0.2 for Windows (SAAM Institute, 1994). This is an interactive programme for the creation of models, design and simulation of experiments, and analysis of data. Using the Compartmental Module a model is designed to provide a visual representation of the system under study. SAAM II automatically creates systems of ordinary differential equations from the model structure. The experiment is then specified and added to the differential equations. Samples are associated with data. SAAM II then solves and fits the model to the data using complex mathematical and statistical techniques.

The model used throughout this thesis is that of Demant et al (1996) and is illustrated in Figure 2.1. The plasma leucine is modelled by four compartments. Compartment 3 is the plasma itself with the input representing the intravenous administration of the tracer and the output representing the irreversible loss of leucine from the plasma. Compartment 2 is a rapidly turning over pool of body protein, such as immunoglobulins, whilst compartment 1 is a more slowly turning over pool, for example skeletal muscle. Compartment 4 is the intracellular leucine pool. Exchange of leucine is allowed between these compartments. A delay component (15) represents the intrahepatic synthesis of apoB. The backbone of the model is the delipidation chain representing the stepwise delipidation of VLDL through to LDL. There are pathways for the slow removal of remnants from $VLDL_1$ ($k_{7,5}$ & $k_{0,7}$), $VLDL_2$ ($k_{10,8}$ & $k_{0,10}$) and IDL ($k_{12,9}$ & $k_{0,12}$). Allowance is made for direct input into each of the four fractions and there is irreversible loss of apoB from the system at every level. Finally, LDL is allowed to exchange with an extravascular compartment (14). The model was made uniquely identifiable by the application of certain physiologically plausible constraints. This model is in keeping with current knowledge of the lipoprotein system. However, it is perhaps limited by the lack of parallel delipidation pathways

for VLDL (difficult to accommodate with stable isotope tracers) and by the single compartment for LDL (not applicable for polydisperse LDL).

The constraints applied to the model are as follows:-

$k_{(3,4)} = k_{(4,3)}$	plasma leucine
$k_{(7,5)} = k_{(10,8)}$	remnant formation
$k_{(0,7)} = k_{(0,10)}$	remnant removal
$k_{(6,5)} = k_{(9,8)}$	$VLDL_1$ & $VLDL_2$ peak widths vary together
$k_{(0,9)} = k_{(0,11)}$	$VLDL_2$ & IDL catabolism
$k_{(0,12)} = k_{(12,9)}$	IDL remnant formation and removal
$k_{(13,14)} = 2.5 \times k_{(14,13)}$	established from a previous model of LDL kinetics (Packard 1995)

A systematic approach to the modelling of each experiment was followed. The data sets were created and the data weighted accordingly. A model of the plasma only was drawn and fitted. This usually took 5-8 iterations. The plasma parameters were then fixed. $VLDL_1$ then $VLDL_2$ were added and fitted, first unconstrained and then with all constraints included. In some instances a constraint was omitted if it worsened the fit. The model remained uniquely identifiable provided no more than 21 parameters were undefined. Only when these fits were acceptable were IDL and LDL similarly added to the model and fitted. A fit was deemed acceptable when the calculated curves closely approximated the observed curves, the fractional standard deviations were less than 20% and the calculated pool sizes came to within 10% of those observed. In all instances good fits were obtained.

Figure 2.2 shows a typical fit for the plasma data with an illustration of the plasma model below. Table 2.1 gives the associated parameter and fractional standard deviation (FSD) values. Figure 2.3 shows the fits for $VLDL_1$, $VLDL_2$, IDL and LDL from the same individual.

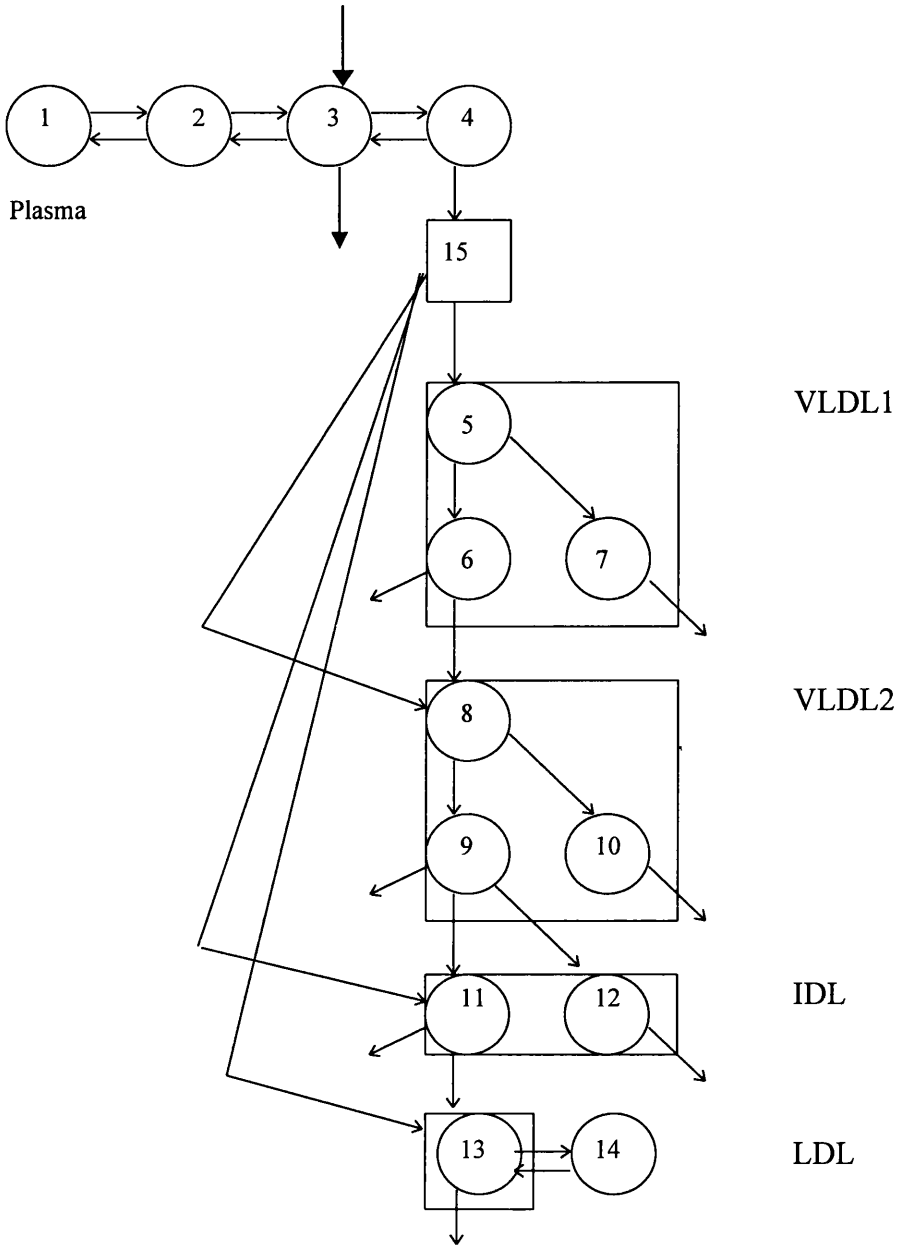


Figure 2.1 The Complete Model (Demant 1996).

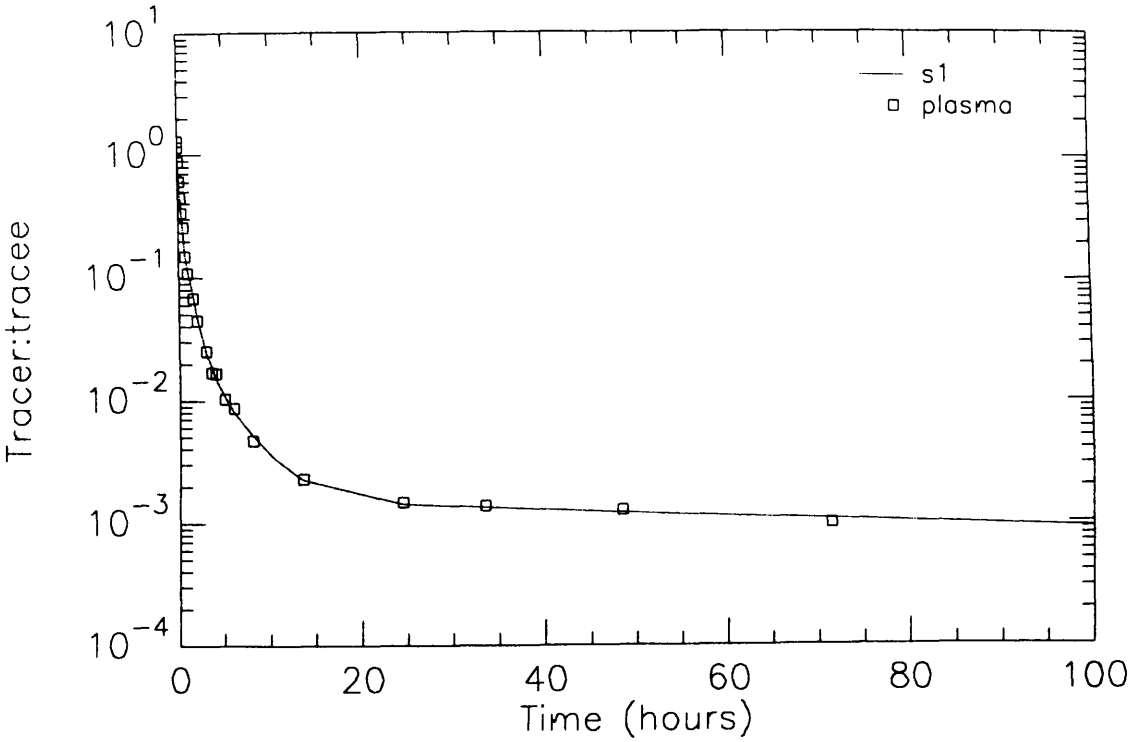


Figure 2.2a The Plasma Curve

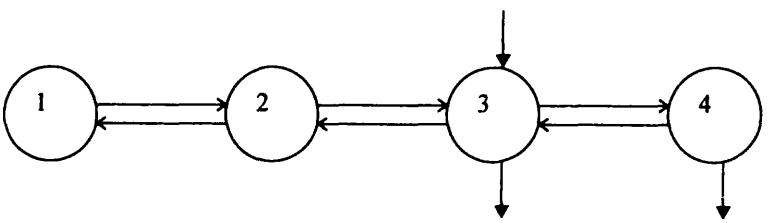


Figure 2.2b The Plasma Model

Parameter	Value	Standard Deviation	F.S.D.
k(0,4)	0.070	** Fixed **	** Fixed **
k(4,3)	2.500	** Fixed **	** Fixed **
k(3,2)	0.067	1.923 - 003	2.850 - 002
k(2,3)	1.271	2.207 - 002	1.736 - 002
k(2,1)	0.021	1.059 - 003	5.124 - 002
k(1,2)	0.098	3.077 - 003	3.138 - 002
plasma leucine	318.58	4.357 - 001	1.368 - 003
k(0,3)	1.271	2.162 - 002	1.701 - 002

Table 2.1. Typical Plasma Parameter and F.S.D. Values.

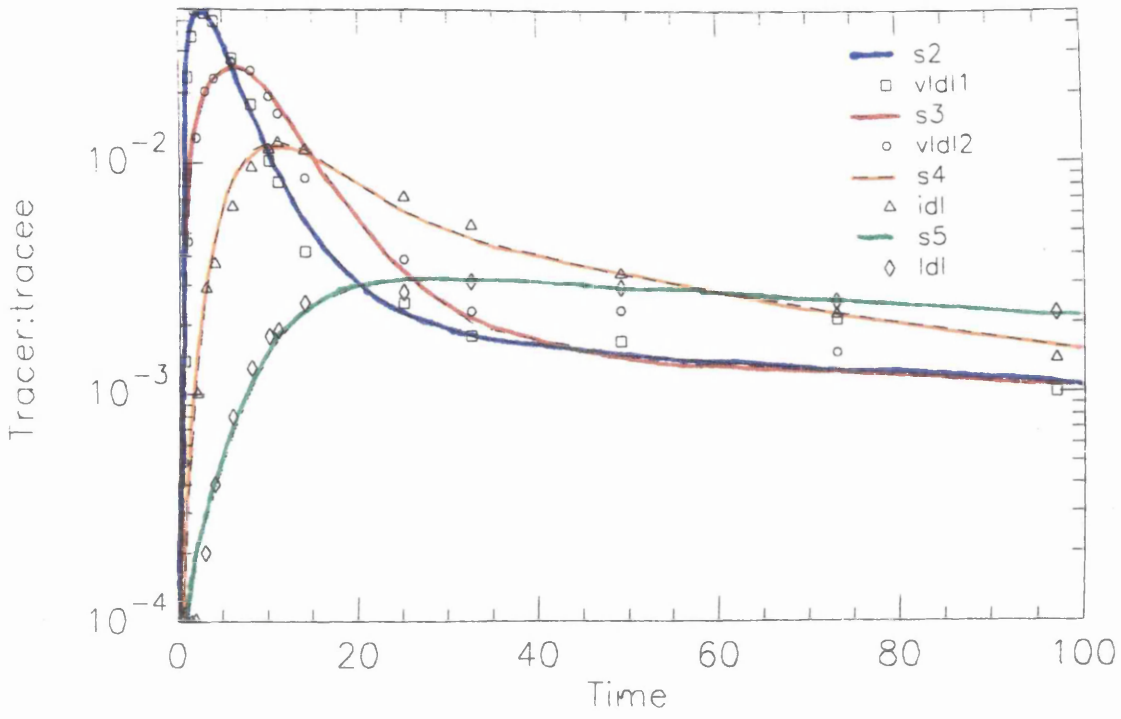


Figure 2.3 The Fits for $VLDL_1$, $VLDL_2$, IDL and LDL.

Chapter 3 Normolipidaemia - The Relationships Between VLDL, LDL and HDL Subfractions and Indices of Obesity, Insulin Resistance and Lipase Activity.

3.1 Introduction.

The 'diagnosis' of normolipidaemia, in the sense that a lipid pattern is free of associated CHD risk, can sometimes be as difficult as diagnosing hyperlipidaemia, both in the instance of the population and in that of the individual. What is normal, or average, for one population may be elevated for another. This is illustrated in the case of the West of Scotland where the average total cholesterol is 6.4mmol/l, which is 1.2mmol/l higher than the 'ideal' level of 5.2mmol/l. In the case of an individual, if he suffers from CHD it could be argued that whatever his cholesterol level is, it is too high for him. It is not uncommon for sufferers of CHD to have cholesterol levels below 6.0mmol/l. The aim of this study was to characterise the inter-relationships between various lipid and lipoprotein parameters in a group of normolipidaemic, healthy individuals, with attention being paid to differences between the sexes. In particular, the differences between lipid levels and VLDL, LDL and HDL subfractions were investigated. In addition, the relationships between indices of obesity (body mass index, waist:hip ratio), insulin resistance (insulin, glucose) and lipase activity (lipoprotein and hepatic lipase) were explored. Much of this work was combined with that done by Dr CE Tan (Tan et al 1995) on families of CHD sufferers and subsequently published. However, in the current study the definition of 'normolipidaemia' was perhaps more stringent than that used in the paper, being set at plasma cholesterol <6.0mmol/l and triglyceride <2.0mmol/l.

Additionally, a small comparative study was performed on a group of CHD sufferers with apparently normal lipid levels, looking in particular for evidence of the atherogenic lipoprotein phenotype, the insulin resistance syndrome or hyperapobetalipoproteinaemia. These three disorders have all been linked to premature CHD and to a certain extent the characteristics of each overlap. The atherogenic lipoprotein phenotype (Austin et al 1990) describes the clustering in an affected individual of small, dense LDL, elevated plasma triglycerides and low HDL cholesterol. It is thought to be inherited as a single gene trait with a dominant mode of inheritance, although the phenotype is often not expressed until the fifth decade in males and after the menopause in females. The insulin resistance syndrome (Reaven 1988), also known as syndrome X, is a plurimetabolic syndrome that includes elevated plasma triglycerides, low HDL cholesterol, hypertension, central obesity and impaired glucose tolerance. More recently a preponderance of small, dense LDL has been added to the syndrome. Hyperapobetalipoproteinaemia (Sniderman et al 1980) was first described in sufferers of CHD who were found to have elevated levels of LDL apoB despite normal levels of LDL cholesterol and total cholesterol. There is an increase in the number of LDL particles and the particles are relatively depleted in cholesterol ester such that there is an increased level of small, dense LDL. There may be associated elevations in plasma triglyceride and low HDL cholesterol levels but the plasma cholesterol is often, although not always, normal. The underlying metabolic

defect is thought to be an overproduction of VLDL apoB plus a decreased clearance of post-prandial triglycerides and free fatty acids. It appears to be inherited in a Mendelian codominant fashion and has been linked to familial combined hyperlipidaemia. The disorder is often expressed following puberty and prior to the development of CHD (Kwiterovich 1988).

3.2 Subject Selection.

3.2.1 Normolipidaemic, Healthy Subjects.

Eighty healthy subjects (41 males, 39 females) with total cholesterol <6.0mmol/l and total triglyceride <2.0mmol/l were recruited from laboratory staff, friends, family friends and colleagues from this and other hospitals. Individuals were excluded if they were taking any medication known to affect lipid metabolism, and if their lipid levels were greater than the selection criteria. None of the females were post-menopausal (as judged by cessation of menstruation) but 4 may have been peri-menopausal. However, none were taking hormone replacement therapy.

3.2.2 Normolipidaemic, CHD Sufferers.

Fifteen individuals (12 males, 3 females) were recruited from a database of patients recently admitted to the cardiology wards in this hospital. They were selected due to their normal lipid levels (cholesterol <6.0mmol/l, triglyceride <2.0mmol/l). All suffered from CHD, with a past history of myocardial infarction, coronary angioplasty or coronary artery by-pass grafting. None were on drugs known to affect lipid metabolism, in particular beta blockers or thiazide diuretics. The three females in the group were all post-menopausal and therefore not separated from the males for analysis. None was taking hormone replacement therapy.

3.3 Methods.

Venous blood samples were taken following an overnight fast. The usual endocrine and haematological samples were taken to exclude any undetected abnormality that may affect lipid levels. Blood pressure and anthropometric indices (height, weight, waist and hip) were measured as previously described. Body mass index (BMI) was calculated as weight in kg/(height in metres)^2 and waist:hip ratio was calculated as waist in cm/hip in cm. Forty subjects from the normal group (26 males, 14 females) then returned for a second visit for the measurement of post-heparin lipase levels. Post-heparin lipases were not measured in the CHD sufferers and in this group only seven subjects had LDL subfractions measured.

The following analyses were performed :- beta-quantification, glucose, insulin, non-esterified fatty acids (NEFA), apoAI, apoB, post-heparin lipases (lipoprotein lipase, LPL, hepatic lipase, HL), HDL subfractions, LDL subfractions and sequential isolation of $VLDL_1$, $VLDL_2$, IDL and LDL. In addition, the chemical compositions of $VLDL_1$,$VLDL_2$, IDL and LDL were determined. The total concentration of each lipoprotein in mg/dl was calculated as the sum of protein + free cholesterol + esterified cholesterol + triglyceride + phospholipid (all in mg/dl).

Data analysis was then performed firstly on the healthy group as a whole and then with the sexes separated. Tables 3.1 and 3.2 give the descriptive statistics for these three groups and the comparisons between the sexes using the two sample t-test. Significance is set at the higher level of $p<0.01$ to accommodate the fact that multiple comparisons were made and there was, therefore, a possibility of spuriously significant findings if the p value was set at 0.05. When indicated by the Anderson-Darling test for normality ($p<0.05$) data were transformed - total triglyceride, waist, insulin, apoAI, lipases, LDLI concentration, LDLIII concentration, %LDLI and %LDLIII were transformed to their logarithms, whilst the square root of NEFA was taken. To further explore the significant results correlation analyses were performed on certain parameters. Relationships with Pearson correlation coefficients of $r>0.3$ or $r<-0.3$ were tested for significance using regression analysis.

The group of CHD sufferers was then compared to the total healthy group. Again significance was set at $p<0.01$ for the reasons given above.

Table 3.1a Normolipidaemic, Healthy Subject Characteristics.

	-----Total	Group----	----Males	----------	--Females	----------	p value
	Mean (SEM)	Range	Mean (SEM)	Range	Mean (SEM)	Range	(males v females)
Age (years)	32.6 (0.98)	18-50	29.9 (1.19)	18-48	35.3 (1.46)	20-50	0.0056
SBP (mmHg)	119 (1.37)	92-160	121.4 (1.66)	100-150	117.0 (2.16)	92-160	NS
DBP (mmHg)	79.0 (0.98)	50-100	80.3 (1.21)	60-100	77.6 (1.52)	50-100	NS
Height (m)	1.70 (0.01)	1.44-1.93	1.77 (0.01)	1.64-1.93	1.64 (0.01)	1.44-1.88	<0.0001
Weight (kg)	68.5 (1.22)	45.8-93.2	75.1 (1.48)	52.2-93.2	61.6 (1.23)	45.8-81.0	<0.0001
Body Mass Index	23.4 (0.30)	18.2-29.8	23.9 (0.43)	18.3-29.4	23.0 (0.41)	18.2-29.8	NS
Waist (cm)	77.3 (1.02)	60-99	82.3 (1.24)	70-99	72.0 (1.14)	60-89	<0.0001
Hip (cm)	97.9 (0.77)	84-119	99.5 (0.99)	84-111	96.3 (1.14)	85-119	NS
W:H Ratio	0.79 (0.01)	0.65-0.96	0.83 (0.01)	0.72-0.92	0.75 (0.01)	0.65-0.96	<0.0001
Chol. (mmol/l)	4.54 (0.09)	2.70-5.95	4.49 (0.12)	2.70-5.90	4.62 (0.12)	3.05-5.95	NS
Trig. (mmol/l)	0.86 (0.03)	0.45-1.90	0.94 (0.05)	0.50-1.90	0.79 (0.03)	0.45-1.35	NS
VLDL (mmol/l)	0.37 (0.02)	0.05-0.90	0.42 (0.03)	0.10-0.90	0.32 (0.02)	0.05-0.65	0.0072
LDL (mmol/l)	2.82 (0.08)	1.20-4.60	2.84 (0.77)	1.60-4.60	2.79 (0.10)	1.20-4.45	NS
HDL (mmol/l)	1.35 (0.03)	0.70-2.15	1.20 (0.04)	0.70-2.00	1.50 (0.04)	1.05-2.15	<0.0001

SBP = systolic blood pressure, DBP = diastolic blood pressure, W:H Ratio = waist:hip ratio, Chol. = plasma cholesterol, Trig. = plasma triglyceride, SEM = standard error of the mean, NS = non-significant.

On account of the multiple comparisons being made the significance level is set at $p<0.01$.

Table 3.1b Normolipidaemic, Healthy Subject Characteristics (continued).

	-----Total Group----		----Males ----------		--Females ----------		p value
	Mean (SEM)	Range	Mean (SEM)	Range	Mean (SEM)	Range	(males v females)
Glucose (mmol/l)	4.84 (0.05)	3.90-5.90	4.97 (0.07)	4.20-5.90	4.69 (0.07)	3.90-5.50	0.0037
Insulin (mU/l)	7.44 (0.46)	1.26-26.6	8.16 (0.78)	2.32-26.6	6.73 (0.49)	1.26-16.3	NS
NEFA (mmol/l)	0.34 (0.03)	0.005-1.58	0.27 (0.03)	0.05-0.79	0.41 (0.05)	0.005-1.58	NS
ApoAI (mg/dl)	119 (2.09)	68-168	116 (3.01)	68-167	122 (2.85)	93-168	NS
ApoB (mg/dl)	77 (1.96)	41-120	79 (2.69)	41-112	76 (2.87)	42-120	NS
LPL (μmolFA/ml/h)	5.12 (0.35)	1.86-12.6	4.82 (0.38)	1.86-9.86	5.67 (0.70)	2.58-12.6	NS
HL (μmolFA/ml/h)	14.5 (1.15)	4.41-34.1	17.7 (1.34)	10.2-34.1	8.58 (0.87)	4.41-14.5	<0.0001
HDL_2 (mg/dl)	70.8 (4.65)	7-180	54.2 (5.0)	7-153	90.7 (6.8)	17-180	0.0001
HDL_3 (mg/dl)	246.3 (6.61)	116-392	245 (10)	116-392	247 (8.1)	182-371	NS
Total LDL (mg/dl)	246 (7.15)	196-287	254 (10.7)	150-415	238 (9.43)	150-363	NS
LDLI (mg/dl)	59.6 (3.86)	6.25-195	48.9 (4.37)	6.25-117	70.1 (5.88)	22.2-195	0.0019
LDLII (mg/dl)	148.2 (6.29)	63.7-331	160.5 (9.54)	63.7-331	136.3 (7.87)	67.8-258	NS
LDLIII (mg/dl)	38.9 (2.72)	4.51-128	46.4 (4.69)	12.6-128	31.6 (2.32)	4.51-67.8	NS
%LDLI	27.1 (3.09)	3.07-225	19.2 (1.53)	3.07-41.8	34.8 (5.68)	11.2-225	0.0001
%LDLII	59.3 (1.34)	32.6-84.6	61.8 (1.82)	33.9-84.6	56.8 (1.89)	32.6-76.4	NS
%LDLIII	16.3 (1.17)	3.01-63.0	19.0 (2.05)	5.58-63.0	13.6 (1.00)	3.01-25.0	NS

NEFA = non-esterified fatty acid, LPL = lipoprotein lipase, HL = hepatic lipase.

Table3.2a $VLDL_1$ and $VLDL_2$ Compositions (healthy subjects).

	-----Total	Group---	----Males	----------	-Females	----------	p value
	Mean (SEM)	Range	Mean (SEM)	Range	Mean (SEM)	Range	(males v females)
%$VLDL_1$ protein	11.2 (0.36)	4.0-19.9	10.1 (0.49)	4.0-19.9	12.4 (0.45)	6.7-19.2	0.0009
%$VLDL_1$ free chol.	1.6 (0.21)	0.0-7.5	1.67 (0.26)	0.0-5.9	1.45 (0.32)	0.0-7.5	NS
%$VLDL_1$ ester. chol.	10.1 (0.49)	0.0-22.3	10.6 (0.64)	2.1-19.1	9.65 (0.75)	0.0-22.3	NS
%$VLDL_1$ trig.	62.2 (1.42)	12.3-78.1	58.8 (2.50)	12.3-76.2	65.8 (0.99)	44.4-78.1	NS
%$VLDL_1$ phos.	14.9 (1.73)	0.0-72.7	18.8 (3.07)	0.0-72.7	10.7 (1.22)	0.0-38.5	NS
$VLDL_1$ Conc^n (mg/dl)	40.3 (3.30)	5.4-136.3	52.4 (5.19)	8.7-136.3	27.6 (2.89)	5.4-34.8	0.0002
%$VLDL_2$ protein	15.4 (0.38)	5.8-32.0	14.4 (0.63)	5.8-32.0	16.5 (0.37)	11.1-21.5	0.0071
%$VLDL_2$ free chol.	3.2 (0.25)	0.0-9.5	3.3 (0.36)	0.0-9.5	3.1 (0.36)	0.0-7.2	NS
%$VLDL_2$ ester. chol.	24.1 (0.72)	9.0-37.6	24.0 (1.12)	9.0-37.6	24.3 (0.92)	12-36.8	NS
%$VLDL_2$ trig.	37.9 (0.85)	12.8-52.6	35.4 (1.36)	12.8-47.7	40.5 (0.83)	27.5-52.6	0.0022
%$VLDL_2$ phos.	19.4 (1.34)	0.7-69.2	22.9 (2.41)	12.4-69.2	15.7 (0.70)	0.7-29.6	0.006
$VLDL_2$ Conc^n (mg/dl)	30.8 (1.87)	6.0-77.7	37.8 (2.90)	6.0-77.7	23.3 (1.65)	7.6-50.5	0.0002

Free chol. = free cholesterol, ester. chol. = esterified cholesterol, trig. = triglyceride, phos. = phospholipid.

On account of the multiple comparisons being made the significance level is set at $p<0.01$.

Table 3.2b IDL and LDL Compositions (healthy subjects).

	-----Total	Group---	----Males	----------	-Females	----------	p value
	Mean (SEM)	Range	Mean (SEM)	Range	Mean (SEM)	Range	(males v females)
%IDL protein	20.1 (0.26)	14.7-25.7	19.8 (0.39)	14.7-25.7	20.3 (0.36)	16.1-25.6	NS
%IDL free chol.	5.83 (0.22)	0.0-10.9	5.58 (0.37)	0.0-10.9	6.09 (0.24)	3.1-9.3	NS
%IDL ester. chol.	42.8 (0.52)	30.1-55.0	43.6 (0.70)	33.8-55.0	41.9 (0.75)	30.1-49.9	NS
%IDL trig.	13.2 (0.52)	7.3-33.4	13.4 (0.77)	7.3-33.4	13.0 (0.69)	7.5-26.5	NS
%IDL phos.	18.2 (0.41)	3.4-24.9	17.6 (0.73)	3.4-24.8	18.7 (0.36)	13.8-24.9	NS
IDL Conc^{n} (mg/dl)	40.1 (1.77)	14.4-86.7	40.1 (2.67)	14.4-86.7	40.1 (2.34)	17.8-74.6	NS
%LDL protein	24.4 (0.22)	19.8-30.7	24.6 (0.33)	20.6-30.7	24.1 (0.30)	19.8-28.0	NS
%LDL free chol.	8.8 (0.26)	4.8-14.3	8.7 (0.38)	4.8-13.8	8.9 (0.35)	4.8-14.3	NS
%LDL ester. chol.	43.4 (0.42)	35.6-55.5	43.4 (0.65)	35.6-55.5	43.3 (0.54)	37.0-52.5	NS
%LDL trig.	4.6 (0.13)	2.1-9.9	4.4 (0.16)	2.1-6.2	4.7 (0.22)	3.2-9.9	NS
%LDL phos.	18.9 (0.36)	2.0-26.1	18.8 (0.65)	2.0-26.1	18.9 (0.28)	13.1-23.7	NS
LDL Conc^{n} (mg/dl)	196.3 (5.22)	57.9-301	199.7 (7.58)	110-301	192.8 (7.22)	57.9-288	NS

Free chol. = free cholesterol, ester. chol. = esterified cholesterol, trig. = triglyceride, phos. = phospholipid.

3.4 Results.

3.4.1 Normolipidaemic, Healthy Subjects.

The females were on average five years older than the males (35.3y v 29.9y). There was no significant difference in the total lipid levels of the two groups, although the males had a tendency to higher triglycerides (p=0.021) as is reflected in their higher VLDL cholesterol levels (Table 3.1a). The total concentrations of $VLDL_1$ and $VLDL_2$ were higher in the males (Table 3.2a). Compositional analysis revealed a lower protein content in male $VLDL_1$ and lower protein and triglyceride in $VLDL_2$ in the males but there were no significant differences in the $VLDL_1$ and $VLDL_2$ triglyceride:protein ratios between the sexes. IDL and LDL compositions also showed no significant difference (Table 3.2b). The HDL level of the females was higher, specifically HDL_2 (Table 3.1b). In addition, the LDLI concentration and LDLI percentage of the females were greater.

As would be expected, the males were taller and heavier than the females but the BMI's of each group were not significantly different. The waist measurements and waist:hip ratios of the males were greater than those of the females, but there was no significant difference in the hip measurements. This suggests that the males have more central obesity than the females (Table 3.1a). Fasting glucose was significantly higher in the males, whilst insulin and NEFA measurements were also higher but not significantly so (p=0.13 and p=0.019 respectively). There was no significant difference in lipoprotein lipase between the sexes but males had significantly higher levels of hepatic lipase (Table 3.1b).

When correlation analysis was performed, first on the total group and then on each sex separately, some interesting differences were revealed. Some of the correlations for the total group were lost when the sexes were separated probably due to the reduction in numbers. As would be expected, the following parameters were correlated with each other - total cholesterol, LDL cholesterol, total LDL concentration and total apoB. In addition, LDLII concentration correlated with all of the above suggesting that it is the major determinant of total LDL in these individuals. These correlations persisted when the sexes were separated. Total triglyceride correlated with VLDL cholesterol in the whole group. $VLDL_1$ concentration correlated with VLDL cholesterol in the total group and the females, but not in the males. HDL cholesterol in all groups correlated with HDL_2 but not with HDL_3. LDLI concentration correlated positively with HDL cholesterol in the total group but this correlation was lost when the sexes were separated. Analysis of the LDL subfraction percentages showed some differing correlations between the groups (Table 3.3). %LDLI correlated significantly with HDL cholesterol and HDL_2 in all groups. %LDLI correlated negatively with LDLII concentration in the total group and the females but this was not significant in the males (p=0.550). %LDLI correlated negatively with LDLIII concentration in all the groups. %LDLII correlated negatively with LDLI concentration in the total group and the females, whilst in the males %LDLII correlated negatively with LDLIII concentration. %LDLIII correlated negatively with LDLI concentration in all the groups. In the males, %LDLIII correlated negatively with LDLII concentration, but this was not significant in the total group (p=0.07) or the females (p=0.846).

Indices of Obesity (Table 3.4).

In the total group, BMI correlated positively with waist, triglyceride, glucose, insulin, apoB and $VLDL_1$ concentration, and negatively with HDL cholesterol and HDL_2. In the males, BMI correlated with waist, waist:hip ratio, triglyceride, insulin and apoB but the correlations with glucose, $VLDL_1$ concentration, HDL cholesterol and HDL_2 lost significance. In the females, BMI showed no significant correlations. Waist:hip ratio in the total group correlated negatively with HDL cholesterol and positively with glucose, hepatic lipase and $VLDL_1$ and $VLDL_2$ concentrations. None of these correlations remained significant when the sexes were separated. In males, waist:hip ratio correlated with BMI.

Indices of Insulin Resistance (Table 3.5).

In the total group, insulin correlated positively with waist, BMI, triglyceride, glucose and $VLDL_1$ concentration. In males, only the correlation with BMI remained significant, whilst in females all correlations were lost. Glucose correlated positively with waist, waist:hip ratio, BMI, and insulin in the total group, but all these lost significance when the sexes were separated. In females, glucose correlated negatively with %LDLIII. As a possible alternative measure of insulin resistance, the insulin:glucose ratio was calculated but this showed no significant correlations and so was not used further.

Indices of Lipase Activity (Table 3.6).

Lipoprotein lipase correlated only with HDL_2 and this lost significance when the sexes were split. Hepatic lipase showed more correlations, correlating positively with waist:hip ratio and $VLDL_1$ concentration, and negatively with HDL cholesterol and HDL_2. However, again these lost significance when the sexes were analysed separately. Because lipoprotein lipase and hepatic lipase are known to have opposing actions on HDL, the LPL:HL ratio was calculated, normalised by transformation to its logarithm, and used for analysis (Brinton et al 1994). However, it added nothing to the analysis, reflecting only the positive correlation of lipoprotein lipase and the negative correlation of HL with HDL_2 ($r = 0.654$, $p<0.001$).

Table 3.3 LDL Subfraction Percentages - Pearson correlation coefficients (r).

	--------	LDLI %	---------	---------	LDLII %	---------	--------	LDLIII %	---------
	Total	Male	Female	Total	Male	Female	Total	Male	Female
Waist m	-0.331	-0.310	0.150	0.063	0.008	-0.163	0.131	0.145	-0.191
W:H Ratio	-0.262	-0.239	0.152	-0.005	-0.123	-0.171	0.112	0.264	-0.254
BMI	-0.245	-0.245	-0.186	0.132	0.014	0.198	0.165	0.133	0.151
Chol. mmol/l	0.090	0.033	0.106	0.033	0.186	-0.083	-0.175	-0.192	-0.128
Trig. mmol/l	-0.308	-0.249	-0.241	0.157	-0.110	0.353	0.075	0.155	-0.135
HDL-C mmol/l	0.589§	0.508*	0.439*	-0.396*	-0.226	-0.456*	-0.276	-0.176	-0.200
HDL_2 mg/dl	0.656§	0.546*	0.669§	-0.413*	-0.085	-0.643§	-0.35*	-0.328	-0.283
HDL_3 mg/dl	-0.007	-0.162	0.282	-0.142	-0.131	-0.160	0.095	0.372	-0.368
Glucose mmol/l	-0.165	-0.268	0.317	0.065	0.109	-0.158	-0.098	0.031	-0.445*
Insulin mU/l	-0.157	-0.301	0.106	0.220	0.435	-0.023	-0.140	-0.244	-0.105
LPL µmolFA /ml/h	0.138	-0.125	0.377	-0.291	-0.137	-0.467	-0.023	0.132	-0.055
HL µmolFA /ml/h	-0.259	0.242	-0.279	0.133	-0.168	0.208	0.345	0.032	0.396
LPL: HL	0.322	-0.167	0.510	-0.319	-0.034	-0.513	-0.290	0.070	-0.399
ApoB mg/dl	-0.306	-0.286	-0.316	0.344	0.298	0.361	-0.080	-0.128	-0.084
$VLDL_1$ Conc^n mg/dl	0.253	-0.123	-0.154	0.116	-0.120	0.221	0.167	0.192	-0.057
$VLDL_2$ Conc^n mg/dl	0.317	-0.278	-0.279	0.224	-0.049	0.360	0.191	0.263	-0.102
Total LDL mg/dl	-0.132	-0.074	-0.093	0.297	0.370	0.182	-0.169	-0.262	-0.147
LDLI mg/dl	(0.897)	(0.897)	(0.859)	-0.355*	0.049	-0.717§	-0.59§	-0.595§	-0.495*
LDLII mg/dl	-0.366*	-0.103	-0.597§	(0.722)	(0.702)	(0.722)	-0.213	-0.479*	-0.033
LDLIII mg/dl	-0.584§	-0.566§	-0.496*	-0.077	-0.474*	0.173	(0.91)	(0.902)	(0.907)

*W:H ratio = waist:hip ratio, BMI = body mass index, chol. = total plasma cholesterol, trig. = total plasma triglyceride, HDL-C = total HDL cholesterol, LPL = lipoprotein lipase, HL = hepatic lipase, apoB = total plasma apoB, * = $p<0.01$, § = $p<0.001$.*

Table 3.4 Indices of Obesity - Pearson correlation coefficients (r).

	---------- Total	-----BMI--- Males	---------- Females	---------- Total	----W:H---- Males	---------- Females
Waist (m)	0.616§	0.875§	0.361	(0.808)	(0.744)	(0.716)
Waist:Hip Ratio	0.261	0.646§	-0.150	----------	----------	----------
Body Mass Index	----------	----------	----------	0.261	0.646§	-0.150
Chol. (mmol/l)	0.180	0.247	0.140	-0.005	0.085	0.040
Trig. (mmol/l)	0.452§	0.537§	0.285	0.276	0.221	0.133
HDL-chol. (mmol/l)	-0.340*	-0.346	-0.254	-0.378*	-0.282	0.012
HDL_2 (mg/dl)	-0.289	-0.249	-0.304	-0.273	-0.268	0.116
HDL_3 (mg/dl)	0.088	0.262	-0.184	0.096	-0.022	0.307
Glucose (mmol/l)	0.343*	0.273	0.331	0.350*	0.266	0.172
Insulin (mU/l)	0.396§	0.551§	0.183	0.118	0.133	-0.025
LPL (µmolFA/ ml/h)	0.061	0.075	0.180	-0.131	0.007	-0.053
HL (µmolFA/ ml/h)	0.187	0.075	0.047	0.511*	-0.005	0.304
LPL:HL Ratio	-0.057	0.048	0.088	-0.380	0.019	-0.167
ApoB (mg/dl)	0.308*	0.435*	0.158	0.129	0.236	-0.004
$VLDL_1$ Conc^n (mg/dl)	0.354*	0.322	0.322	0.311*	0.113	0.115
$VLDL_2$ Conc^n (mg/dl)	0.271	0.290	0.142	0.341*	0.102	0.214
Total LDL (mg/dl)	0.217	0.327	0.071	0.051	0.098	-0.123
LDLI (mg/dl)	-0.126	-0.048	-0.142	-0.218	-0.158	0.076
LDLII (mg/dl)	0.242	0.256	0.186	0.048	0.036	-0.200
LDLIII (mg/dl)	0.268	0.323	0.167	0.133	0.329	-0.295

*BMI = body mass index, W:H = waist:hip ratio, chol. = total plasma cholesterol, trig. = total plasma triglyceride, HDL-chol. = total HDL cholesterol, LPL = lipoprotein lipase, HL = hepatic lipase, apoB = total plasma apoB, * = $p<0.01$, § = $p<0.001$.*

Table 3.5 Indices of Insulin Resistance - Pearson correlation coefficients (r).

	---------- Total	---Insulin--- Males	---------- Females	---------- Total	--Glucose-- Males	---------- Females
Waist (m)	0.310*	0.403	0.101	0.433§	0.291	0.320
Waist:Hip Ratio	0.118	0.133	-0.025	0.350*	0.266	0.172
Body Mass Index	0.396§	0.551§	0.183	0.343*	0.273	0.331
Chol. (mmol/l)	-0.022	0.178	-0.207	0.161	0.119	0.345
Trig. (mmol/l)	0.324*	0.362	0.214	0.210	0.085	0.212
HDL-chol. (mmol/l)	-0.275	-0.317	-0.139	-0.175	0.014	0.050
HDL_2 (mg/dl)	-0.188	-0.370	0.082	-0.298	-0.072	-0.188
HDL_3 (mg/dl)	0.146	0.109	0.212	0.110	0.126	0.164
Glucose (mmol/l)	0.398*	0.315	0.399	----------	----------	----------
Insulin (mU/l)	----------	----------	----------	0.398*	0.315	0.399
LPL (µmolFA/ ml/h)	-0.123	-0.239	0.156	0.188	0.124	0.418
HL (µmolFA/ ml/h)	0.281	0.278	0.034	0.270	0.094	0.362
LPL:HL Ratio	-0.199	-0.288	0.099	0.015	0.081	0.223
ApoB (mg/dl)	0.078	0.244	-0.119	0.150	0.104	0.169
$VLDL_1$ Conc^n (mg/dl)	0.336*	0.303	0.311	0.273	-0.005	0.369
$VLDL_2$ Conc^n (mg/dl)	0.190	0.173	0.103	0.210	0.025	0.153
Total LDL (mg/dl)	0.020	0.244	-0.248	0.284	0.220	0.308
LDLI (mg/dl)	-0.142	-0.166	-0.037	-0.041	-0.158	0.418
LDLII (mg/dl)	0.147	0.381	-0.169	0.267	0.251	0.144
LDLIII (mg/dl)	-0.113	-0.099	-0.211	0.020	0.116	-0.322

*BMI = body mass index, W:H = waist:hip ratio, chol. = total plasma cholesterol, trig. = total plasma triglyceride, HDL-chol. = total HDL cholesterol, LPL = lipoprotein lipase, HL = hepatic lipase, apoB = total plasma apoB, * = $p<0.01$, § = $p<0.001$.*

Table 3.6 Lipase Activity - Pearson correlation coefficients (r).

	---------- Total	----LPL----- Male	---------- Female	---------- Total	-----HL----- Male	---------- Female
Waist (m)	-0.002	0.117	0.229	0.386	-0.128	0.087
W:H Ratio	-0.131	0.007	-0.053	0.511*	-0.005	0.304
BMI	0.061	0.075	0.180	0.187	0.075	0.047
Chol. (mmol/l)	0.090	0.125	0.030	-0.056	-0.209	0.163
Trig. (mmol/l)	-0.074	-0.017	0.011	0.294	0.203	-0.222
HDL-chol. (mmol/l)	0.286	0.307	0.025	-0.441*	-0.187	-0.196
HDL_2 (mg/dl)	0.535*	0.446	0.770	-0.545*	-0.404	-0.469
HDL_3 (mg/dl)	0.262	0.245	0.366	-0.153	-0.083	-0.815
Glucose (mmol/l)	0.188	0.124	0.418	0.270	0.094	0.362
Insulin (mU/l)	-0.123	-0.239	0.156	0.281	0.278	0.034
LPL (µmolFA/ ml/h)	----------	----------	----------	-0.337	-0.484	0.033
HL (µmolFA/ ml/h)	-0.337	-0.484	0.033	----------	----------	----------
LPL:HL Ratio	(0.815)	(0.942)	(0.780)	(-0.76)	(-0.73)	(-0.58)
ApoB (mg/dl)	-0.236	-0.108	-0.426	0.023	-0.152	-0.038
$VLDL_1$ Conc^n (mg/dl)	-0.206	-0.342	0.316	0.557§	0.427	0.100
$VLDL_2$ Conc^n (mg/dl)	-0.189	-0.159	-0.012	0.296	-0.074	-0.230
Total LDL (mg/dl)	0.134	0.199	0.065	-0.001	-0.183	0.165
LDLI (mg/dl)	0.175	-0.027	0.354	-0.219	0.142	-0.132
LDLII (mg/dl)	-0.046	0.084	-0.196	0.110	-0.190	0.333
LDLIII (mg/dl)	0.030	0.226	-0.032	0.356	-0.008	0.445

*W:H ratio = waist:hip ratio, BMI = body mass index, chol. = total plasma cholesterol, trig. = total plasma triglyceride, HDL-chol. = total HDL cholesterol, LPL = lipoprotein lipase, HL = hepatic lipase, apoB = total plasma apoB, * = $p<0.01$, § = $p<0.001$.*

3.4.2 Normolipidaemic, CHD Sufferers.

When compared to the healthy subjects, the CHD sufferers were significantly older (54.3y v 32.6y) and had higher blood pressures (145/89mmHg v 119/79mmHg). Total cholesterol, triglyceride, LDL cholesterol and apoB levels were significantly elevated despite the lipid level inclusion criteria being the same in each group. In addition, HDL cholesterol was lower. Total LDL and LDLIII concentration were elevated whilst percentage LDLI was reduced. Compositional analysis revealed no significant differences with the exception of elevated phospholipids in IDL and LDL and reduced esterified cholesterol in LDL. Weight, body mass index, waist measurement and waist:hip ratio were elevated. Glucose and insulin measurements were also significantly higher in this group. Thus, despite having lipid levels within the 'normal range', this group of CHD sufferers appears to be a different population of individuals differing significantly from the healthy group. These findings are shown in Table 3.7, (a & b).

Correlation analysis was then performed on the CHD group and the relationships observed compared to those seen in the healthy group. In general, most of the correlations seen in the healthy group were also seen in the CHD group. However, many of these correlations were not significant, probably as a consequence of the smaller numbers analysed. As in the healthy group, total cholesterol, LDL cholesterol and total LDL correlated with each other. However, in the CHD group, LDLII concentration and apoB did not correlate with the above. Instead apoB showed a significant negative correlation with HDL cholesterol and HDL_2. Total triglyceride in this group correlated with VLDL cholesterol, $VLDL_1$ concentration and LDLIII concentration. As in the healthy group, HDL cholesterol correlated with HDL_2 but not HDL_3. As in the healthy males, %LDLIII showed a significant negative correlation with LDLII concentration. Correlations between the indices of obesity and insulin resistance and other parameters did not reach significance in this group, again this is likely to be due to the small sample size.

Table 3.7a Normolipidaemic, CHD Sufferers - subject characteristics.

	----------CHD Mean (SEM)	Sufferers------ Range	p values CHD sufferers v healthy group
Age (years)	54.3 (2.32)	37-66	<0.0001
SBP (mmHg)	145 (7.34)	110-210	0.0040
DBP (mmHg)	89 (2.51)	78-110	0.0020
Height (m)	1.71 (0.03)	1.47-1.84	NS
Weight (kg)	82.5 (4.49)	43-104	0.0095
Body Mass Index	27.8 (1.02)	19.9-34.4	0.0009
Waist (cm)	93.9 (2.73)	71-113	<0.0001
Hip (cm)	103.1 (1.89)	88-113	NS
Waist:hip Ratio	0.91 (0.02)	0.8-1.1	<0.0001
Cholesterol (mmol/l)	5.58 (0.24)	4.80-6.00	<0.0001
Triglyceride (mmol/l)	1.37 (0.11)	0.80-2.00	<0.0001
VLDL-chol. (mmol/l)	0.47 (0.07)	0.15-0.95	NS
LDL-chol. (mmol/l)	4.05 (0.13)	2.85-4.90	<0.0001
HDL-chol. (mmol/l)	1.07 (0.07)	0.80-1.65	0.0022

SBP = systolic blood pressure, DBP = diastolic blood pressure, W:H Ratio = waist:hip ratio.

On account of the multiple comparisons being made the significance level is set at $p<0.01$.

Table 3.7b Normolipidaemic, CHD Sufferers - subject characteristics (continued).

	----------CHD Sufferers------ Mean (SEM)	Range	p values CHD sufferers v healthy group
Glucose (mmol/l)	5.59 (0.24)	4.40-7.70	0.0085
Insulin (mU/l)	10.72 (0.81)	5.30-13.3	0.0006
ApoAI (mg/dl)	113 (5.45)	79-154	NS
ApoB (mg/dl)	114 (4.05)	88-147	<0.0001
HDL_2 (mg/dl)	78.3 (18.0)	9-263	NS
HDL_3 (mg/dl)	261.1 (14.4)	146-331	NS
Total LDL (mg/dl)	374 (16.1)	292-414	<0.0001
LDLI (mg/dl)	40.1 (9.65)	12.8-89.4	NS
LDLII (mg/dl)	213.7 (25.7)	148.7-300.5	NS
LDLIII (mg/dl)	120.1 (26.3)	30.2-199.1	0.0077
%LDLI	10.4 (2.21)	3.5-21.8	0.0085
%LDLII	56.9 (5.87)	39.7-75.9	NS
%LDLIII	32.7 (7.18)	7.4-55.2	NS

3.5 Discussion.

The most striking finding to emerge from this study is that even within a population defined by common criteria as normolipidaemic (plasma cholesterol <6.0mmol/l, triglyceride <2.0mmol/l) several subpopulations can be found, distinguishable by a clustering or otherwise of factors known to have an adverse effect on the risk of CHD development. In the group of healthy individuals the separation is based on sex, with males showing lower HDL cholesterol and HDL_2 levels, lower %LDLI, higher hepatic lipase, higher glucose and a tendency to central obesity. The CHD sufferers showed more extreme differences from the healthy group. Their lipid levels were at the upper end of the normal range, HDL levels were lowered, the LDL subfraction profiles showed decreased LDLI and elevated LDLIII, apoB was raised and indices of obesity and insulin resistance were elevated.

The sex differences in lipid and lipoprotein parameters in healthy individuals have been investigated before (Watson et al 1994, Tan et al 1995). Most of the findings here are in keeping with those from the other studies although in some instances the relationships are less strong. This may be due to the stricter selection criteria in the current study that effectively excludes those individuals whose slightly higher lipid levels may have more influence on parameter relationships. Correlation analysis of the healthy group reveals certain patterns. LDLII concentration appears to be the major determinant of total LDL, and HDL_2 the major determinant of total HDL. Analysis of the LDL subfractions in the total group showed that %LDLI, whilst correlated positively with HDL_2, was negatively correlated with the concentrations of LDLII and LDLIII. For %LDLII, there was a split in the relationship when the sexes were separated - in males %LDLII correlated negatively with LDLIII concentration, whilst in the females %LDLII correlated negatively with LDLI concentration.

The indices of obesity used in this study are body mass index (BMI) and waist:hip ratio. BMI gives an indication of total obesity whilst waist:hip ratio reflects the regional distribution of body fat. The distinction is relevant as studies have suggested that regional body fat distribution is a more significant risk factor for CHD development than total obesity, in particular that those individuals with more visceral or intra-abdominal obesity are at a greater risk (Despres et al 1990). Males have a tendency when gaining weight to increase their abdominal girth (becoming 'apple-shaped') whilst in females weight gain is mostly in the gluteal region ('pear-shaped'). In the current study, despite having similar BMI measurements the males had significantly greater waist measurements and waist:hip ratios. In the males, BMI and waist:hip measurement showed a positive relationship that was not seen in the females. In addition, the relationships seen between various study parameters and BMI and waist:hip ratio in the total group mostly persisted when the males were analysed alone but were not significant in the females alone. In this study, BMI showed more significant correlations with other parameters than did waist:hip ratio, perhaps because the subjects were mostly of normal weight and without the metabolic complications of abdominal obesity. As BMI increased there was an associated increase in triglyceride and decrease in HDL cholesterol. There was also a significant correlation between increasing BMI and increases in glucose and insulin, reflecting the metabolic link between obesity and insulin resistance. The males in this study had

higher glucose levels than the females and non-significant elevations in insulin. They therefore appear to be relatively more insulin resistant than the females. A predominance of small, dense LDL, as seen in these males, has also been linked with the insulin resistance syndrome. Lipase activity also showed links with obesity, as hepatic lipase correlated positively with waist:hip ratio.

When compared to the healthy group, the CHD sufferers show a clustering of adverse lipid and lipoprotein factors - elevations in total cholesterol, triglyceride, LDL cholesterol and apoB levels, low levels of HDL cholesterol and a predominance of small, dense LDL. In addition, these individuals are hypertensive and centrally obese with elevations in fasting glucose and insulin. They, therefore, show characteristics in keeping with each of the three 'disorders' of atherogenic lipoprotein phenotype (ALP), the insulin resistance syndrome (IRS) and hyperapobetalipoproteinaemia (hyperapoB). Their small, dense LDL, elevated triglyceride and apoB, and low HDL cholesterol would fit with the ALP. These factors, plus their central obesity, hypertension and elevated glucose and insulin levels are in keeping with the IRS. HyperapoB is suggested by their elevated apoB levels and the relative depletion of esterified cholesterol in LDL. As each of these disorders has been associated with an increased risk of CHD it is not difficult to see why these individuals should suffer from CHD despite normal plasma cholesterol and triglyceride levels. Of particular interest in this group were the relationships between plasma triglyceride levels and LDLIII concentration and $VLDL_1$ concentration (see below), as this influence of triglyceride was not seen in the healthy group.

The correlations seen between the subfractions of VLDL, LDL and HDL and indices of obesity, insulin resistance and lipase activity can be explained by the metabolic model shown in Figure 3.1. As noted in our subjects, elevations in BMI are associated with relative insulin resistance (seen as increases in fasting glucose and insulin levels). In the insulin resistant state, the increased intrahepatic availabilty of triglyceride is believed to result in the secretion of apoB predominantly as triglyceride-rich $VLDL_1$. $VLDL_1$ is only slowly metabolised to LDL. The persistance in plasma of triglyceride-rich VLDL allows CETP mediated neutral lipid exchange of triglyceride from VLDL to LDL and HDL_2 in exchange for cholesterol ester. This then provides suitable substrates for hepatic lipase resulting in the production of small, dense species of LDL (LDLII and LDLIII) and HDL (HDL_3). The higher levels of hepatic lipase seen in males and in those with increased waist:hip ratios increases the conversion of LDLI to LDLIII. In this study, the healthy males had a higher hepatic lipase activity than the healthy females, associated with lower LDLI and HDL cholesterol. This sex difference in hepatic lipase activity can also explain the reciprocal relationship seen between %LDLII and the concentrations of LDLI and LDLIII. In males LDLII concentration decreases as more is lipolysed to LDLIII by the action of hepatic lipase. In females the lower activity of hepatic lipase means that the majority of LDL remains as the less dense species of LDLI rather than being lipolysed to LDLII and LDLIII. Also seen in this study was the positive correlation between LDLI and HDL, in particular HDL_2, and the negative association between hepatic lipase and HDL cholesterol levels.

The study of Watson et al found hepatic lipase to be responsible for the sex differences in LDL subfraction profiles, whilst the study of Tan et al found plasma triglyceride to be the most important determinant of the LDL subfraction profile. In the healthy group, neither hepatic lipase nor plasma triglyceride showed significant correlations with the LDL subfraction profile. However, the males did have a higher hepatic lipase activity than the females and a tendency to higher triglyceride levels. The group of CHD sufferers had significantly higher triglyceride levels and in addition showed a strong relationship between plasma triglyceride and LDLIII concentration, as noted above. Thus, it could be that the CHD sufferers have reached a critical or 'threshold' triglyceride level above which the LDL subfraction profile appears to change, moving towards a predominance of LDLIII. This threshold level has been reported previously by other workers as triglyceride levels of 1.1mmol/l (Austin et al 1990), 1.3mmol/l (Tan et al 1995) and 1.6mmol/l (Superko and Krauss 1992). The mean plasma triglyceride in the CHD sufferers was 1.37mmol/l as compared to 0.86mmol/l in the healthy group, whilst the mean LDLIII concentrations in each group were 120mg/dl and 39mg/dl respectively.

To summarise, even in normolipidaemia relationships exist between the lipoprotein subfractions and indices of obesity, insulin resistance and lipase activity that can influence an individual's risk of CHD development. They are dependent on obesity and on the relative activity of hepatic lipase, both of which show differences between the sexes. Insulin resistance appears to mediate its adverse effect through the increased intrahepatic triglyceride availability and resultant production of triglyceride-rich lipoproteins, as is seen in the CHD group. In the complete group of 95 normolipidaemic individuals there appears to be three distinct subgroups with a gradient of CHD risk. At least risk are the healthy females and at most risk are those already identified as suffering from CHD. The healthy males sit between the two groups in their degree of risk. Even within this narrow range of lipid levels, the gradual increase in the levels appears to be adversely related to the existance of a clustering of adverse lipoprotein parameters. This study serves to illustrate the fact that total plasma lipid levels can be falsely reassuring when used as an indication of CHD risk.

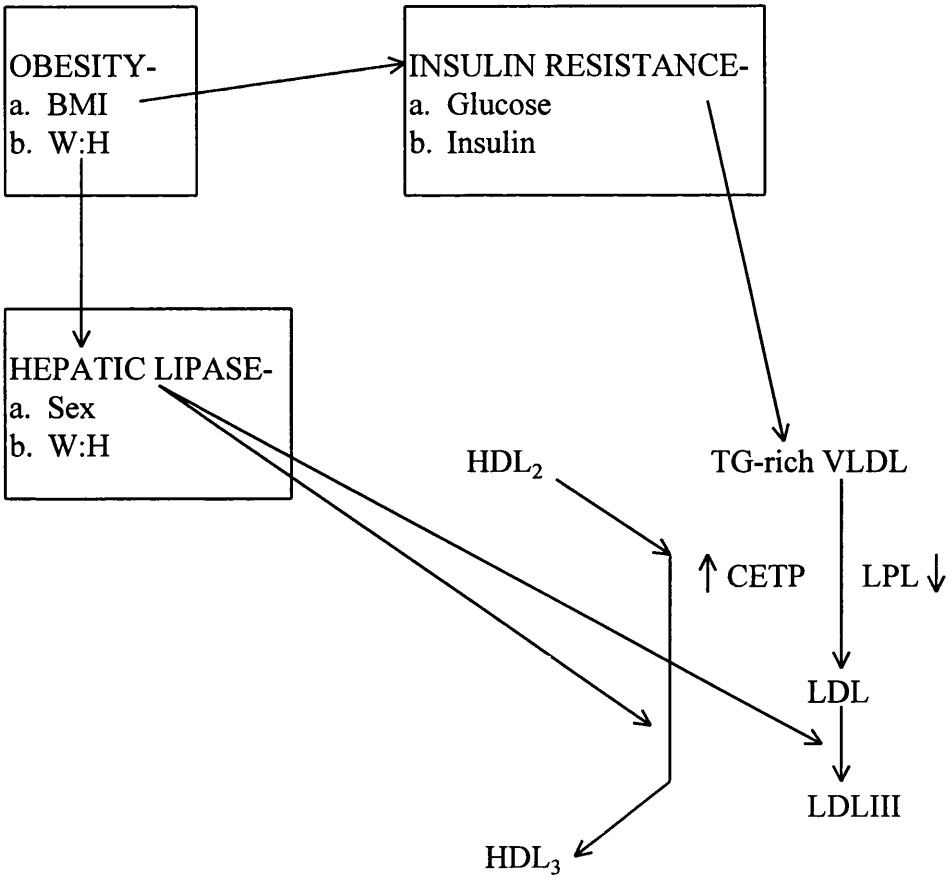


Figure 3.1 The Relationships Between the Subfractions of VLDL, LDL and HDL and Indices of Obesity, Insulin Resistance and Lipase Activity.

BMI = body mass index, W:H = waist:hip ratio, CETP = cholesterol ester transfer protein, LPL = lipoprotein lipase.

Chapter 4 Stable Isotope Turnovers in Familial Hypercholesterolaemia and Familial Defective ApoB-100.

4.1 Introduction.

Two inherited conditions, familial hypercholesterolaemia (FH) and familial defective apoB-100 (FDB), arise from a disruption of the catabolism of LDL by the LDL (or B/E) receptor, resulting in elevations in plasma LDL cholesterol and total cholesterol. Their difference lies in the manner in which LDL catabolism is disrupted. In FH there is a deficiency of functioning LDL receptors leading to reduced receptor-mediated LDL clearance, whilst in FDB it is the receptor ligand, apoB-100, that is defective thus reducing the binding of LDL to the receptor and so its clearance. Both are inherited in an autosomal dominant fashion with a frequency in the population in the heterozygous form of approximately 1:500, making them two of the most common autosomal dominant conditions reported. Each can present with the same features - elevated plasma cholesterol (specifically LDL cholesterol), tendon xanthomata and premature CHD. These features, plus a family history of affected members, have traditionally been used to define FH. However, the more recently recognised condition of FDB fits with the same definition and so the two cannot be distinguished by these criteria. Perhaps a more correct definition should include the aetiological feature in each case i.e. i). a receptor defect, or ii). a ligand defect. In the clinical context, however, this discrimination may be neither possible, requiring more complex laboratory techniques, nor relevant to patient management, as in the heterozygous form both conditions are managed similarly.

4.2 Familial Hypercholesterolaemia - a brief overview.

The medical literature of last century saw an increasing interest in xanthomata leading to the suggestion in the 1870's that they might arise from hyperlipidaemia. However, it was Muller in 1938 who first recognised the condition of Familial Hypercholesterolaemia to include the three features "xanthomata, hypercholesterolaemia and angina pectoris". Its autosomal dominant mode of inheritance was definitively described in 1964 by Khachadurian in his studies of FH families in Lebanon. The prevalence of FH is estimated at 1:500 in the heterozygous form and 1:1,000,000 in the homozygous form (Goldstein and Brown 1989). In certain populations, however, the prevalence is much greater. For example, due to the founder effect in South African Afrikaaners the prevalence is 1:100 for heterozygotes and 1:30,000 for homozygotes (Seftel et al 1980).

The most consistent manifestation of FH is an elevated plasma cholesterol level, specifically LDL cholesterol. Xanthomata and premature CHD (defined as occurring before the age of 55 years in men and 65 years in women) are highly prevalent amongst FH sufferers but not inevitable. This intra-individual variation in the clinical manifestation of FH arises for several reasons. Firstly, to date over 150 mutations of the LDL receptor gene have been reported leading to different severities of receptor deficiency. Secondly, the unaffected LDL gene and other more minor genes, e.g. apoE, HDL etc, can influence the lipid metabolism in any one individual. Thirdly,

lifestyle factors such as diet, obesity and smoking are as important in FH as in polygenic hypercholesterolaemia. It is interesting to note that in rare instances it is possible to have FH but have a normal cholesterol level. This can arise if there is an additional genetic defect of lipid metabolism that counterbalances the cholesterol elevation due to FH. This is illustrated in the case reported by Zambon et al in 1993 of an individual with heterozygous FH plus homozygous LPL deficiency. Despite having FH, his total cholesterol was 6.5mmol/l with an LDL cholesterol of only 0.6mmol/l due to the absence of LPL-mediated VLDL to LDL delipidation. This is an extreme illustration of how individuals with FH may present with a variety of phenotypes.

The treatment of heterozygous FH includes the modification of lifestyle factors, with particular attention to dietary control. However, most patients will require drug therapy. Those most commonly used are the bile acid sequestrants (resins) and the HMG-CoA reductase inhibitors (statins), either alone or in combination. These presumably act by up-regulating the functioning LDL receptors and so increasing LDL catabolism. In the homozygous form of FH treatment with resins or statins is, in theory, ineffectual as there are no functioning receptors available for up-regulation. However, this is not strictly the case as certain LDL receptor mutations retain some receptor activity, albeit markedly reduced. Furthermore, studies of individuals with complete absence of receptor function have shown some response to statins (Feher et al 1993) suggesting that these drugs can act by a mechanism additional to receptor up-regulation. The majority of homozygous FH individuals (and some with severe heterozygous FH) require more aggressive treatment with plasmapharesis or LDL-apharesis to significantly lower their cholesterol levels.

The prognosis in heterozygous FH is variable reflecting the variability in clinical presentation. However, most affected individuals will ultimately develop CHD, many prematurely. Studies have suggested that the development of CHD is happening at an increasingly early age from one generation to the next (Williams et al 1993). This anticipation phenomenon is illustrated by the case of one Africaaner family with FH - in the first generation the age of death of the one affected individual was 76 years, in the second the average age of death was 55 +/- 10 years (n=4), and in the third the average age of death was 37 +/- 8 years (n=5), (Marais 1994, unpublished observation). Much of this apparent increase in disease severity can be explained by detrimental lifestyle changes and thus, in theory, is amenable to correction.

The LDL receptor (Goldstein and Brown 1973) is a trans-membrane protein of 160kD present predominantly on hepatocytes. There are currently more than 150 mutations of the receptor known at DNA level. The majority of FH is caused by mutations in the ligand binding domain which is the functional part of the receptor. Conformational changes or promoter mutations can result in a complete absence of the protein. Alternatively, internalisation of the receptor following normal ligand binding may not occur and LDL dissociates back into the plasma, or binding and internalisation may be normal but the ligand may then fail to be released and so is degraded with the receptor protein. Depending on the mutation there may be a complete lack of LDL binding - less than 2% of normal is classified as 'receptor negative' - or a reduction in binding as compared to normal - usually less than 25% of normal, classified as 'receptor deficient'. This is an important distinction both regarding disease severity and

treatment as receptor negative individuals tend to have more severe disease and respond less well to treatment.

4.3 Familial Defective ApoB-100 - a brief overview.

The more recently discovered condition, Familial Defective ApoB-100 (FDB) was first described in 1987 by Innerarity et al when he reported on the defective binding of LDL to normal human fibroblasts. In this condition the LDL receptors are normal but the apoB moiety of the lipoproteins is abnormal resulting in defective binding of LDL to the receptors. The LDL itself is otherwise normal in composition, shape and size. As a consequence of this disrupted LDL receptor-mediated catabolism LDL accumulates in the plasma giving rise to an elevated plasma cholesterol level and its ultimate clinical sequelae.

FDB is inherited in an autosomal dominant fashion and is estimated to have a prevalence of 1:500 in the population (Innerarity et al 1990). It has been reported in various populations, mostly Caucasian and in one Chinese individual (Bersot et al 1993), but to date there are no reports of its occurrence in Finnish (Hamalainen et al 1990), Russian (Schuster et al 1992), Israeli and Japanese (Friedlander et al 1993) populations in keeping with their different ethnic origins. FDB can present with the same clinical picture as FH, with the prevalence of hypercholesterolaemia, xanthomata and premature CHD being much the same (Rauh et al 1992). However, there have more recently been suggestions in the literature that the phenotype may be generally less severe (Miserez et al 1995). For the same reasons as in FH, there is great inter-individual variation in the presentation of FDB, although the number of responsible mutations so far reported is only three as opposed to the over 150 in FH. As in FH, FDB can be treated with resins and statins that lead to the up-regulation of LDL receptors and so enhanced clearance of LDL, primarily the normal LDL. However, it is also likely that the efficacy of these drugs in FDB reflects increased clearance of LDL precursors by the LDL receptors due to the apoE moiety (Maher et al 1993).

A case of homozygous FDB has been reported (Marz et al 1993). It is interesting to note that the receptor-mediated clearance of LDL in this individual, although diminished, was not completely abolished. The larger, less dense LDL particles were being catabolised to a certain degree, possibly due to their apoE moiety, but the small, dense LDL particles remained in the plasma. This individual also had a less severe phenotype than is seen in homozygous FH individuals. These findings have been confirmed more recently (Gallagher and Myant 1995) by a study of two sibling homozygotes for FDB. In addition to the residual binding of LDL to the receptors, the level of VLDL remnants (i.e. LDL precursors) was found to be normal presumably due to normal catabolism. This in turn was stimulated by treatment with a statin and lead to a significant response to therapy.

The underlying mutation in classical FDB has been identified as a single point mutation in the receptor binding domain of apoB - a G to A mutation at nucleotide 10708 in exon 26 results in a glutamine for arginine substitution at amino acid 3500 in the mature protein (Soria et al 1989). Haplotype analysis has shown that the majority

of the FDB mutations so far identified have a common European origin (Rauh et al 1991) with the exception of an individual from Munich (Rauh et al 1993) and one from China (Bersot et al 1993) whose mutations appear to have arisen independently. A haplotype identical to that of the Chinese individual has recently been reported in a Caucasian individual in Scotland (Gaffney et al 1995). Recently Pullinger et al (1995) described a mutation at amino acid 3531 which resulted in defective apoB binding. A C to T transition at nucleotide 10800 results in a cysteine for arginine substitution at position 3531 of the mature apoB. Most recently a further mutation affecting the amino acid at position 3500 has been identified (Gaffney et al 1995). In this mutation, coined FDB_{3500W}, arginine is replaced by tryptophan resulting in reduced binding of the LDL, as shown by U937 cell growth. The binding of FDB-LDL to LDL receptors has been reported as 5-20% of normal (Myant 1993) for FDB_{3500} and 27% of normal for FDB_{3531} (Pullinger et al 1995). The relative plasma concentrations of FDB-LDL to normal LDL are 70%:30% and 60%:40% respectively due to the more efficient clearance of the normal LDL. It is possible to separate normal and FDB-LDL by immunoaffinity methods.

Thus, FH and FDB are both conditions of disrupted receptor-mediated LDL catabolism that can present with identical clinical phenotypes. Despite their different aetiology, the prognosis is similar and they both respond to treatment with agents that up-regulate the LDL receptors. In the homozygous form the conditions differ to a greater degree with homozgous FH being more severe than homozygous FDB. Cases of double heterozygotes for FH and FDB have been reported and studies of these individuals should prove interesting (Rubinsztein et al 1993). The aim of this study was to compare the metabolism of apoB-100 in heterozygotes for FH and FDB.

4.4 Subject Selection.

4.4.1 Heterozygous Familial Hypercholesterolaemic Subjects.

Five unrelated male subjects were recruited from the Lipoprotein Clinic at Glasgow Royal Infirmary based on criteria adopted by the Simon Broome Register (Table 4.1). Subjects all screened negative for FDB.

4.4.2 Heterozygous Familial Defective ApoB-100 Subjects.

Two unrelated male subjects were identified as heterozygotes for the mutation FDB_{3500w} following screening of all attenders at the Lipoprotein Clinic, courtesy of the DNA Laboratory, Pathological Biochemistry. One subject (JR) is Caucasian and the other (NS) is of Asian origin. To our knowledge this is the first report of FDB in an Asian individual. Haplotype analysis suggested two separate mutations.

The characteristics of the seven subjects are given in Table 4.2 and their lipid and lipoprotein levels in Table 4.3. Biochemical and haematological screening excluded hepatic, renal and endocrine dysfunction and in each case lipid-lowering medication was stopped four weeks prior to the study. No subject was taking drugs known to

affect lipid metabolism. Each subject gave signed informed consent and the study was approved by the Ethical Committee of Glasgow Royal Infirmary.

Table 4.1 The Simon Broome Register Definition of Familial Hypercholesterolaemia.

Definite Familial Hypercholesterolaemia
(a) Cholesterol level above 7.5mmol/l or LDL level above 4.9mmol/l, *plus*
(b) Tendon xanthomata in patient or first or second-degree relative.

Possible Familial Hypercholesterolaemia
(a) As (a) above, *plus* one of (b) or (c) below
(b) Family history of myocardial infarction below the age of 50 years in second-degree relative or below the age of 60 years in first-degree relative
(c) Family history of raised cholesterol levels above 7.5mmol/l in first or second-degree relative.

Table 4.2 FH and FDB Subject Characteristics.

Subject	Type	Age (years)	Weight (kg)	ApoE	Physical Signs	Family History
AC	FH	34	77	3/2	-	father : died 35y MI
WT	FH	32	80	3/3	CABG 30y.	father : MI 50y
RM	FH	52	82	3/3	TX, corneal arcus.	father : died 52y MI
MM	FH	42	78	3/3	TX, corneal arcus.	son : 13y chol. 7.6
RH	FH	28	100	3/3	Corneal arcus.	mother & sister: hyperchol.
JR	FDB	58	83	3/3	-	1 sibling & 2 children with FDB
NS	FDB	42	52	4/3	TX	2 siblings & 1 child with FDB

MI = myocardial infarction, TX = tendon xanthomata, CABG = coronary artery by-pass graft.

Table 4.3 FH and FDB Subject Lipid Profiles.

Subject	Cholesterol (mmol/l)	Triglyceride (mmol/l)	VLDL-chol. (mmol/l)	LDL-chol. (mmol/l)	HDL-chol. (mmol/l)
AC	7.75	1.90	1.10	5.70	0.95
WT	8.45	2.10	1.15	6.20	1.10
RM	8.40	1.30	0.80	6.15	1.45
MM	9.40	1.50	1.15	7.75	0.90
RH	14.20	3.10	1.35	11.90	0.95
JR	8.35	1.60	0.90	6.35	1.10
NS	7.05	0.75	0.10	5.55	1.40

4.5 Methods.

Following a ten hour overnight fast each individual underwent a turnover study of apoB kinetics using a primed constant infusion of tri-deuterated leucine at a dose of 0.7mg/kg body weight bolus followed by an infusion of 0.7mg/kg/h for 10 hours. Subjects remained fasting throughout the day and venous blood samples were taken according to the protocol described in Chapter 2. The methodology for sample preparation, GC-MS measurement of isotopic enrichment and kinetic analysis by multicompartmental modelling is as given previously. The results obtained were compared to those of a similar study of 15 normal individuals (cholesterol <6.0mmol/l, triglyceride <2.0mmol/l) carried out previously in this laboratory using the same methodology (Packard, unpublished data). Comparisons between the FH and normal subjects were performed using the two sample t-test. Due to the small sample size of FDB subjects, no statistical analysis was performed.

4.6 Results.

4.6.1 Familial Hypercholesterolaemia.

1. Lipids and Lipoproteins (Table 4.4).

The five heterozygous FH individuals had significantly higher total cholesterol and LDL cholesterol levels than the normals as would be expected in this condition. In addition, their triglyceride and VLDL levels were higher. In keeping with other work, HDL had a tendency to be lower but this did not reach significance.

Table 4.4 Lipids and Lipoproteins - normal v FH subjects.

	Cholesterol (mmol/l)	Triglyceride (mmol/l)	VLDL-chol. (mmol/l)	LDL-chol. (mmol/l)	HDL-chol. (mmol/l)
Normal					
mean (SEM)	4.90 (0.20)	1.00 (0.07)	0.50 (0.04)	3.05 (0.15)	1.35 (0.06)
FH					
mean (SEM)	9.60 (1.20)	2.00 (0.30)	1.05 (0.10)	7.45 (1.10)	1.05 (0.10)
p value	**p=0.02**	**p=0.03**	**p=0.003**	**p=0.02**	**NS**

SEM = standard error of mean, NS = not significant.

Compositional analysis (Table 4.5, a & b) of the lipoproteins revealed significant ($p<0.05$) increases in $VLDL_1$ and $VLDL_2$ free and esterified cholesterol, with significant decreases in the protein content. In IDL there were decreases in both protein and triglyceride. The composition of LDL in our subjects was essentially normal. Here the compositions were compared to results of a study of eighty normolipaemic subjects (cholesterol <6.0mmol/l and triglyceride <2.0mmol/l) as compositional analysis of the 15 normal subjects used for the kinetic comparisons was not available.

Table 4.5a Percentage Compositions of $VLDL_1$ and $VLDL_2$ - normal v FH subjects.

	------ Prot	------- FC	$VLDL_1$ EC	------- TG	---- PL	------ Prot	------- FC	$VLDL_2$ EC	------- TG	---- PL
Normal										
mean	**11.2**	**1.6**	**10.1**	**62.2**	**14.9**	**15.4**	**3.2**	**24.1**	**37.9**	**19.4**
(SEM)	**(0.4)**	**(0.2)**	**(0.5)**	**(1.4)**	**(1.7)**	**(0.4)**	**(0.2)**	**(0.7)**	**(0.8)**	**(1.3)**
AC	6.5	3.3	20.4	57.9	11.9	11.4	8.0	41.2	32.4	16.6
WT	7.0	4.3	12.2	62.3	14.2	13.0	3.9	25.5	39.2	18.3
RM	7.4	4.2	12.8	64.0	11.6	14.0	7.1	31.2	29.5	18.1
MM	6.4	2.7	16.0	61.6	13.3	13.4	4.8	38.3	25.3	18.3
RH	3.9	5.4	15.8	59.9	15.1	13.3	11.9	32.5	21.7	20.6
FH										
mean	**6.2**	**4.0**	**15.4**	**61.1**	**13.2**	**13.0**	**7.1**	**33.7**	**27.7**	**18.4**
(SEM)	**(0.6)**	**(0.5)**	**(1.5)**	**(1.0)**	**(0.7)**	**(0.4)**	**(1.4)**	**(2.8)**	**(3.2)**	**(0.6)**
p value	**0.000**	**0.005**	**0.03**	**NS**	**NS**	**0.001**	**0.05**	**0.03**	**NS**	**NS**

Prot = protein, FC = free cholesterol, EC = esterified cholesterol, TG = triglyceride, PL = phospholipid.

Table 4.5b Percentage Compositions of IDL and LDL - normal v FH subjects.

	------ Prot	------- FC	IDL --- EC	------- TG	---- PL	------ Prot	------- FC	LDL --- EC	------- TG	---- PL
Normal										
mean	**20.1**	**5.9**	**42.3**	**13.7**	**18.2**	**24.4**	**8.8**	**43.4**	**4.6**	**18.9**
(SEM)	**(0.3)**	**(0.2)**	**(0.5)**	**(0.5)**	**(0.4)**	**(0.2)**	**(0.3)**	**(0.4)**	**(0.1)**	**(0.4)**
AC	19.5	7.8	48.4	8.4	15.9	22.5	10.4	42.1	5.5	16.5
WT	18.4	5.8	43.6	11.9	20.4	22.9	9.8	41.6	4.8	20.9
RM	18.4	10.6	44.0	7.9	19.2	24.1	14.5	39.0	3.6	18.8
MM	18.6	8.4	49.1	6.7	17.3	24.4	15.9	34.2	4.0	21.5
RH	17.6	12.8	39.0	7.9	22.7	26.9	20.1	25.5	5.8	21.8
FH										
mean	**18.5**	**9.1**	**44.8**	**8.6**	**19.1**	**24.2**	**14.1**	**36.5**	**4.7**	**19.9**
(SEM)	**(0.3)**	**(1.2)**	**(1.8)**	**(0.9)**	**(1.2)**	**(0.8)**	**(1.9)**	**(3.1)**	**(0.4)**	**(1.0)**
p value	**0.003**	**NS**	**NS**	**0.003**	**NS**	**NS**	**0.05**	**NS**	**NS**	**NS**

2. ApoB Pool Sizes (Table 4.6).

The total apoB in the FH subjects was significantly greater than normal. In the subfraction apoB pool sizes only IDL apoB mass was significantly increased, although in the case of LDL all the FH pool sizes were at or above the upper limit of the normal range. For the $VLDL_2$ apoB two of the five FH subjects had pool sizes within the normal range whilst the rest were above the upper limit. The FH $VLDL_1$ pool sizes all fell into the normal range with the exception of one which was increased.

3. Kinetic Analysis.

Table 4.7 (a & b) gives the kinetic results for all seven subjects and the mean values for the 15 normal subjects. The full set of rate constants and calculated leucine pool sizes is given in Appendix 3. Figure 4.1 summarises the kinetic findings. Figures 4.2 and 4.3 show VLDL, IDL and LDL tracer mass curves from the two subject groups.

The kinetics of $VLDL_1$ in the FH subjects are largely as normal. The production rates and fractional rates of direct catabolism (FDC) all fall within the normal range. However, the fractional rate of transfer (FTR) of apoB to $VLDL_2$ is significantly decreased. $VLDL_2$ shows both a decreased FDC and a decreased FTR. The production rate is again within the normal range, with one exception (RH) which is increased. The production rate of IDL in FH is as normal. The FDC values all tend towards the lower limit of the normal range but this does not reach statistical significance ($p=0.07$). As with $VLDL_1$ and $VLDL_2$, the IDL FTR is decreased. LDL shows normal production rates with a significantly decreased fractional catabolic rate (FCR).

4. LDL Subfractions.

The LDL subfraction profiles of these subjects were largely determined by the individual triglyceride level - those with moderately elevated triglycerides tended to have an increased percentage of small, dense LDL III, whilst those with lower triglycerides had profiles normal for males i.e. a predominance of LDL II. These observations are in keeping with previously reported work in both normal and FH individuals (Tan et al 1995, Caslake 1996).

4.6.2 Familial Defective ApoB-100.

These results were compared to those of the normal and FH subjects but not analysed statistically due to the small sample size. Both subjects had an elevated plasma cholesterol level, with associated increases in LDL cholesterol. Compositional analysis of the lipoproteins gave no differences from normal. The total apoB levels were both above the normal range, with specific increases in the LDL apoB pool sizes (Table 4.6). In the kinetic analysis (Table 4.7) the most marked abnormality was a reduced LDL FCR as would be expected. There was a suggestion of reduced FTR's for $VLDL_1$, $VLDL_2$ and IDL with both values in each case being at or below the lower limit of the normal range. In both subjects the production rates and FDC's of each lipoprotein were normal. The LDL subfraction profiles of the two subjects showed a predominance of LDL II (75% and 59%), with some LDL I (16% and 32%) and little LDL III (9% and 9%). Such profiles are normal for males.

Table 4.6 ApoB Pool Sizes - normal, FH and FDB subjects.

	Total ApoB (mg/dl)	**$VLDL_1$ Pool (mg)**	**$VLDL_2$ Pool (mg)**	**IDL Pool (mg)**	**LDL Pool (mg)**
Normal					
mean (SEM)	**60 (3.5)**	**42 (6.3)**	**93 (13)**	**269 (40)**	**1420 (99)**
range	**42-84**	**18-100**	**41-220**	**71-724**	**860-2248**
AC	98	94	237	441	2232
WT	115	159	254	365	2899
RM	104	41	183	610	2583
MM	127	97	198	470	3193
RH	210	39	713	974	6691
FH					
mean (SEM)	**131 (21)**	**86 (22)**	**317 (100)**	**572 (108)**	**3519 (809)**
p value	**p=0.03**	**p=0.13**	**p=0.09**	**p=0.05**	**p=0.06**
JR	120	75	197	486	3217
NS	95	14	51	302	1605

Table 4.7a Kinetic Results - $VLDL_1$ and $VLDL_2$, normal, FH and FDB subjects.

	$VLDL_1$ PROD (mg/d)	$VLDL_1$ FDC (pool/d)	$VLDL_1$ FTR (pool/d)	$VLDL_2$ PROD (mg/d)	$VLDL_2$ FDC (pool/d)	$VLDL_2$ FTR (pool/d)
Normal						
mean (SEM)	**686 (84)**	**6.17 (1.84)**	**13.5 (2.00)**	**176 (26)**	**1.26 (0.33)**	**6.60 (0.69)**
range	**264-1508**	**0.05-27.2**	**5.81-37.7**	**21-358**	**0.06-3.90**	**3.6-12.7**
AC	912	7.1	2.4	127	0.16	1.3
WT	1254	1.0	7.0	86	0.80	3.6
RM	862	16.1	4.7	308	0.50	2.2
MM	727	5.3	1.6	220	0.11	2.1
RH	327	1.2	6.5	771	0.24	1.2
FH						
mean (SEM)	**816 (150)**	**6.14 (2.75)**	**4.44 (1.07)**	**302 (123)**	**0.36 (0.13)**	**2.08 (0.43)**
p value	**NS**	**NS**	**p=0.001**	**NS**	**p=0.02**	**p=0.0000**
JR	395	2.1	3.1	156	0.3	1.6
NS	418	16.8	0.6	293	5.6	3.8

PROD = production rate, FDC = fractional rate of direct catabolism, FTR = fractional transfer rate.

Table 4.7b Kinetic Results - IDL and LDL.

	IDL PROD (mg/d)	IDL FDC (pool/d)	IDL FTR (pool/d)	LDL PROD (mg/d)	LDL FCR (pool/d)
Normal					
mean (SEM)	**152 (31)**	**1.00 (0.24)**	**2.47 (0.36)**	**117 (28)**	**0.49 (0.04)**
range	**3-381**	**0.02-3.30**	**1.01-6.50**	**1-326**	**0.26-0.94**
AC	92	0.22	0.66	35	0.15
WT	0	1.10	1.20	101	0.21
RM	308	0.40	0.80	62	0.20
MM	154	0.25	0.95	0	0.14
RH	52	0.10	0.90	144	0.15
FH					
mean (SEM)	**121 (53)**	**0.41 (0.18)**	**0.90 (0.09)**	**68 (25)**	**0.17 (0.01)**
p value	**NS**	**(p=0.07)**	**p=0.0007**	**NS**	**p=0.0000**
JR	140	0.5	0.5	184	0.13
NS	107	0.1	1.2	71	0.21

FCR = fractional catabolic rate.

4.7 Discussion.

Using the combination of stable isotope techniques and multicompartmental modelling, the kinetics of apoB-100 was studied in subjects heterozygous for familial hypercholesterolaemia (n=5) and familial defective apoB-100 (n=2). For the FH individuals, the results were largely in agreement with those obtained using radio-isotope techniques and other methods of data analysis despite the limitations of a small sample size and some inter-individual heterogeneity. However, the labour-intensive nature of stable isotope studies precludes studies of larger numbers. In the case of FDB, there is a minimal amount of literature on apoB kinetics for comparison and certainly none on individuals with the specific mutation FDB_{3500W}. Furthermore, subject NS is the first case of FDB to be reported in an Asian individual.

4.7.1 Familial Hypercholesterolaemia.

The lipid and lipoprotein values in these five FH subjects are largely in keeping with those in the current literature although two subjects had minimally raised triglycerides. However, their clinical and biochemical characteristics were more suggestive of FH than familial combined hyperlipidaemia. Moderate elevations in triglyceride levels have been reported elsewhere in FH subjects. The compositional findings of an increased cholesterol content in VLDL, especially $VLDL_2$, and triglyceride depletion in IDL was also seen by James et al (1989) in his study of FH homozygotes and perhaps reflects a prolonged residence time of these lipoproteins in the plasma and so increased exposure to neutral lipid exchange.

The kinetic abnormalities found in this study are i). reduced fractional transfer rates of apoB down the delipidation chain from $VLDL_1$, through $VLDL_2$, and IDL to LDL, ii). reduced fractional rates of direct clearance of $VLDL_2$ and IDL, and iii). a reduced LDL fractional catabolic rate.

The reduced $VLDL_2$ and IDL FTR's are in keeping with other studies, but the metabolism of $VLDL_1$ has previously been reported as normal (Shepherd and Packard 1989). This could perhaps be a reflection of the moderately increased triglyceride levels of two out of the five FH subjects in this study - large, triglyceride-rich VLDL ($VLDL_1$) tend to pass slowly down the delipidation cascade as compared to the less triglyceride-rich $VLDL_2$. Classically the delipidation of $VLDL_2$ and IDL to LDL is mediated by hepatic lipase and, therefore, should not be influenced by defects in LDL receptor function. It is possible that the enlarged IDL and LDL pool sizes seen in FH have a negative feedback effect on hepatic lipase action. However, a more interesting possibility is that the LDL receptor acts in a way complimentary to hepatic lipase by approximating the lipoproteins to the enzyme and thus making them more accessible for delipidation (James et al 1989). Both the LDL receptors and hepatic lipase are found predominantly on sinusoidal hepatocytes. In the event of reduced LDL receptor function this cooperation would be diminished. Recently, an analogous situation has been seen with lipoprotein lipase (LPL) and the LDL receptor-related protein (LRP). LPL, in addition to its enzyme action, appears to have a role as a ligand and promotes the binding of triglyceride-rich lipoproteins to the LRP (Applebaum-Bowden 1995).

The observed reduction in direct catabolism of $VLDL_2$ and IDL reflects inefficient clearance of these LDL precursors by the LDL receptor which, in health, would ordinarily recognise the apoE moiety on these lipoproteins. This is illustrated by the findings in the FDB subjects who had normal clearance rates for VLDL and IDL (see below).

In any study of apoB kinetics in FH the most consistent finding is a reduced LDL FCR. In heterozygotes this is usually reduced to about one half of normal, whilst in homozygotes it is about one third. This is a direct consequence of the deficient LDL receptor function, resulting in reduced LDL clearance from the plasma and thus its accumulation. This is reflected in the elevated LDL apoB pool sizes seen in this study with all individuals having LDL pool sizes at or above the upper limit of the normal range.

Most other studies of FH kinetics, including those using endogenous tracers, have found increases in the production rates of various apoB containing lipoproteins. The fact that this was not seen in the present study may be due to the small sample size, the subjects selected or to different methods of kinetic analysis and modelling. In 1977, in their radio-isotope study of homozygous FH, Soutar et al reported an increase in LDL apoB production (with normal VLDL apoB production). She found that the total amount of apoB could not be accounted for by that produced as VLDL alone and so introduced the concept of direct LDL synthesis. This was further elaborated on by Janus et al (1980) when he found that direct LDL synthesis could account for up to 70% of LDL apoB production in FH. However, in some FH subjects with additional triglyceride elevations he reported an increase in VLDL apoB production and a lesser amount of direct LDL synthesis. This inverse relationship between direct LDL synthesis and triglyceride level was also reported by Gaw et al (1995). When hepatic triglyceride is plentiful apoB is mainly secreted as triglyceride-rich lipoproteins (VLDL). However, when hepatic triglyceride is low an increased amount of apoB is secreted directly as LDL. In our subjects, direct LDL synthesis only accounted for 0-11% of total apoB secretion with the majority of apoB being secreted as VLDL, reflecting the moderately raised triglyceride levels in these subjects. Cummings et al (1995) reported an increase in total VLDL apoB production in heterozygous FH, but did not differentiate between $VLDL_1$ and $VLDL_2$. Although this study was performed using an endogenous tracer, the results were analysed using a monoexponential function rather than multicompartmental modelling and this may explain the differing findings. These increases in apoB production appear initially to be unrelated to the LDL receptor deficiency. However, in FH, despite the suppressed activity of HMG-CoA reductase, there is an elevation in hepatic cholesterol (Fisher et al 1994). Since receptor-mediated uptake is deficient, this is likely to be due to receptor-independent, and thus unrestrained, uptake. This increase in the intrahepatic availability of cholesterol stimulates an increased production of apoB and apoB containing lipoproteins and thus further exacerbates the elevated LDL levels due to reduced clearance.

Thus, the effects of the LDL receptor deficiency of FH appear to have consequences more far reaching than LDL clearance alone, giving us several reasons for the atherogenicity of this condition. First, LDL accumulates in the plasma due to reduced

clearance. Secondly, the delayed transfer of apoB down the delipidation chain and reduced clearance leads to the accumulation in the plasma of LDL precursors which are in themselves atherogenic. Finally, unrestrained receptor-independent lipoprotein uptake results in an elevated hepatic cholesterol which in turn stimulates increased lipoprotein production. In addition to these three problems, the effect of prolonged residence of the lipoproteins in the plasma is increased exposure to neutral lipid exchange and thus formation of particles of a more atherogenic nature, as can be seen by the increased cholesterol to triglyceride content of VLDL and IDL.

4.7.2 Familial Defective ApoB-100.

The lipid and lipoprotein findings in the two FDB subjects are in keeping with those reported in the literature i.e. elevations in total cholesterol and LDL cholesterol, with normal lipoprotein compositions. The reduced LDL FCR is also as expected and reflected in the increased LDL apoB pool size. In contrast to FH, there was no reduction of VLDL and IDL direct catabolism, suggesting that their clearance in FDB is mediated by apoE interacting with normal LDL receptors (an event that is impaired in FH by the receptor deficiency). However, there was a tendency for the transfer of apoB down the delipidation chain to be retarded in FDB, a finding similar to that in FH and possibly explained by the same disruption of receptor-lipase interaction that is in this case being upset by the apoB moiety. The normal production rate of VLDL seen in this study is in keeping with that found in another study (Zulewski et al 1996). However, we did not see the reduced IDL production reported by these investigators.

Thus, despite the different aetiology, much of the pathology of FDB can be explained by the same mechanisms as in FH, stemming from reduced LDL clearance and delayed progress of apoB down the delipidation chain. However, perhaps the normal apoB production rates can account for the less severe phenotype seen in some FDB subjects. In addition, the presence of functioning receptors that can clear LDL precursors by virtue of their apoE moiety possibly lessens the severity of the situation. This is reflected in the normal LDL subfraction profiles seen in our two subjects.

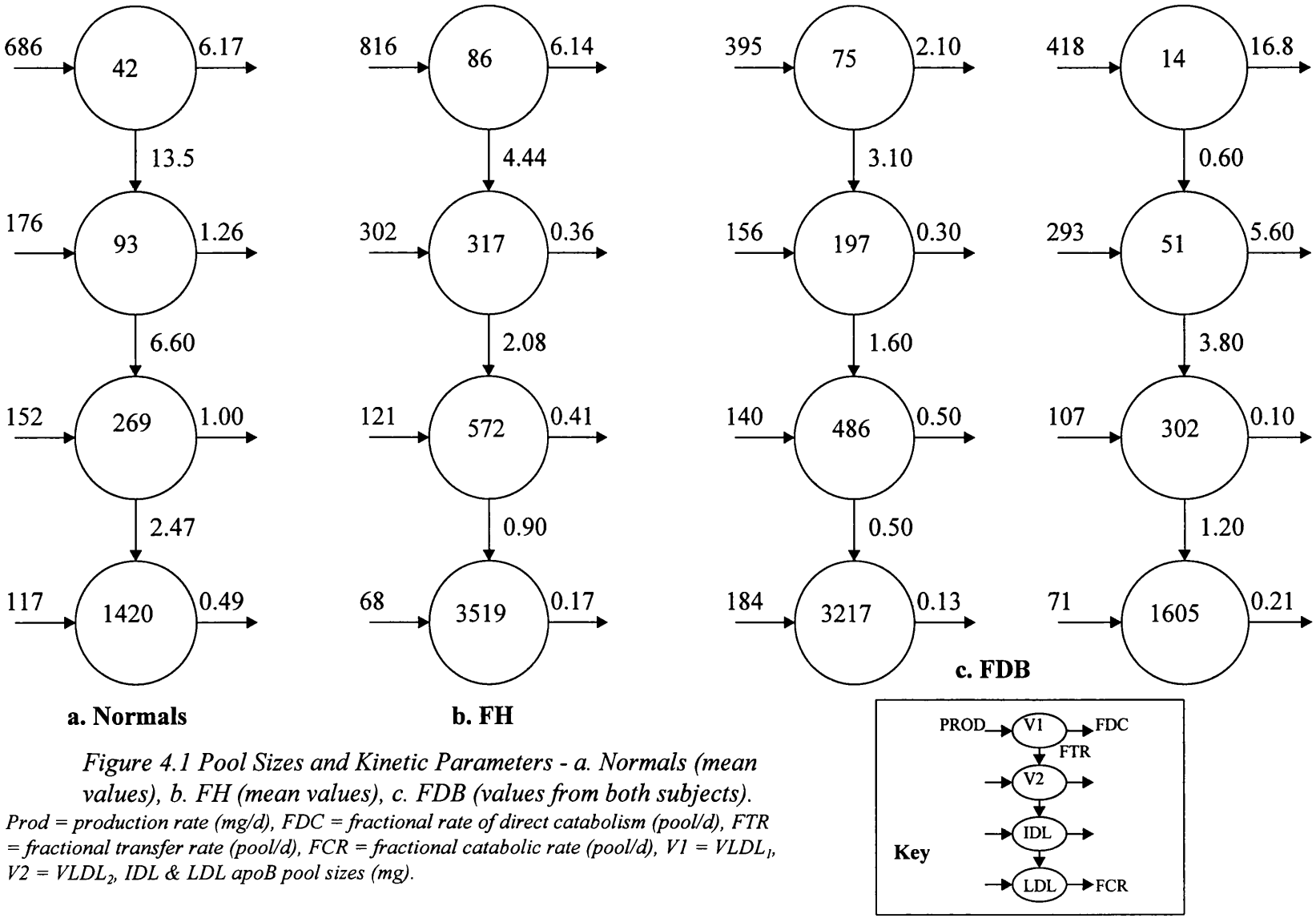


Figure 4.1 Pool Sizes and Kinetic Parameters - a. Normals (mean values), b. FH (mean values), c. FDB (values from both subjects).

Prod = production rate (mg/d), FDC = fractional rate of direct catabolism (pool/d), FTR = fractional transfer rate (pool/d), FCR = fractional catabolic rate (pool/d), V1 = $VLDL_1$, V2 = $VLDL_2$, IDL & LDL apoB pool sizes (mg).

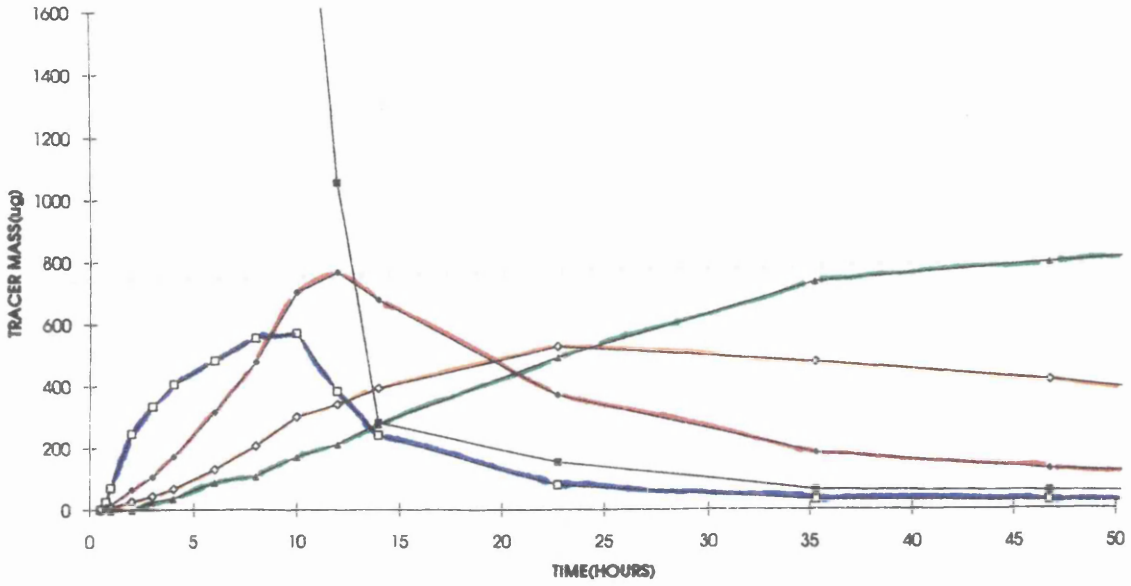


Figure 4.2a Tracer Mass Curves for an FH Subject (0-50 hours).

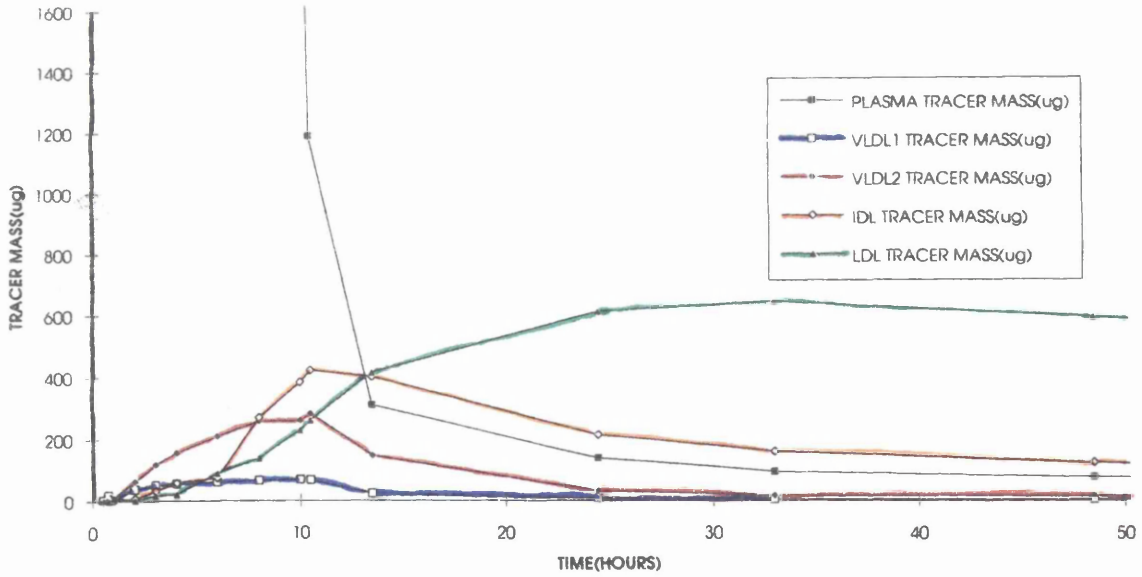


Figure 4.2b Tracer Mass Curves for an FDB Subject (0-50 hours).

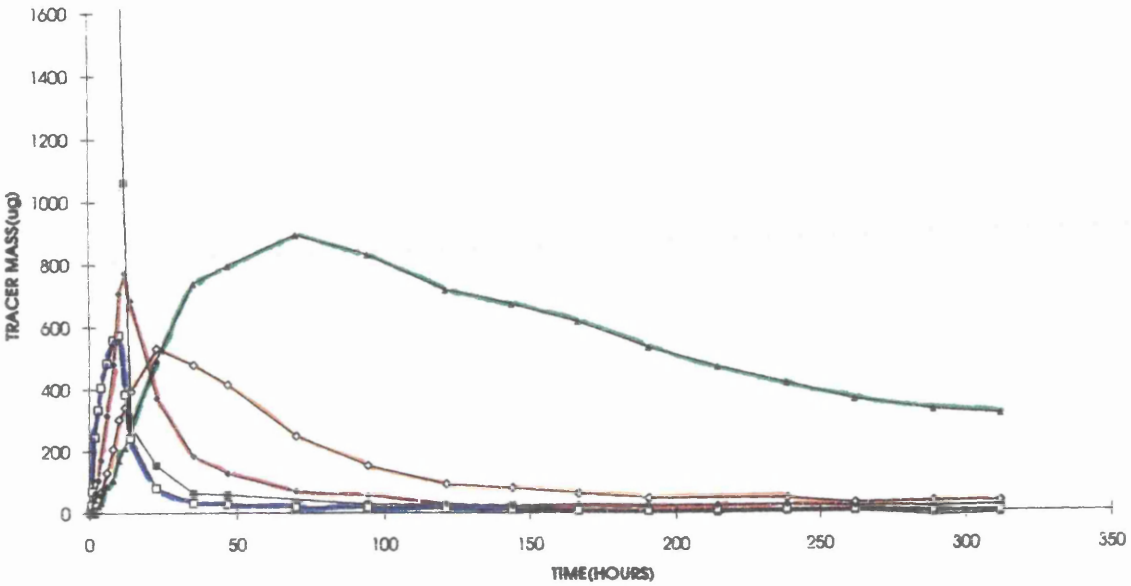


Figure 4.3a Tracer Mass Curves for an FH Subject (0-350 hours).

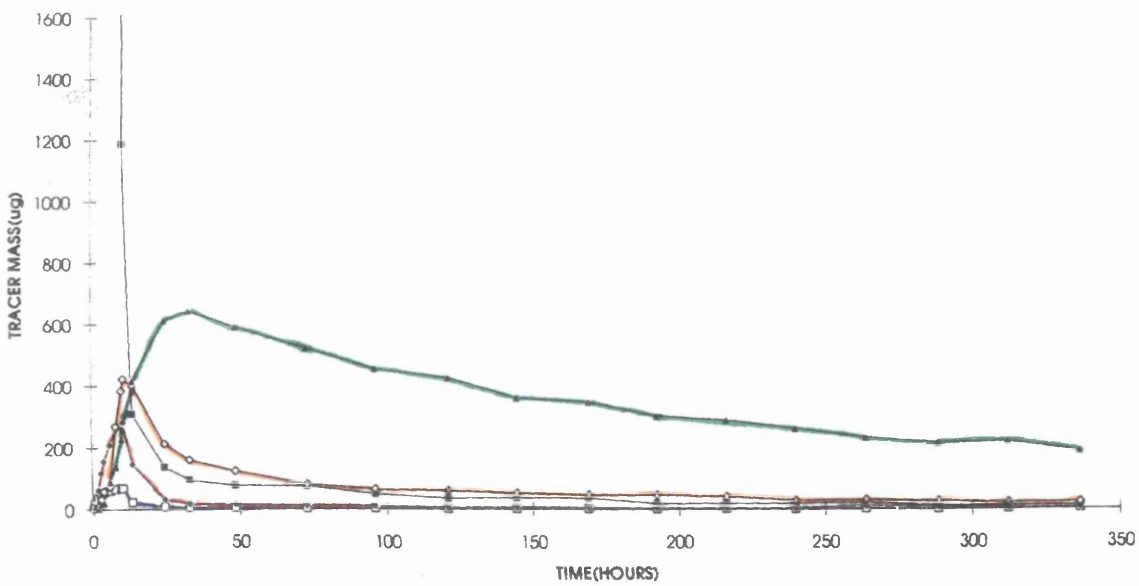


Figure 4.3b Tracer Mass Curves for an FDB Subject (0-350 hours).

Chapter 5 Stable Isotope Turnovers in a Case of Hypobetalipoproteinaemia and a Case of Analbuminaemia.

5.1 Introduction.

Hypobetalipoproteinaemia is associated with very low levels of apoB and cholesterol, whilst in analbuminaemia subjects have very high levels of apoB and total cholesterol. The metabolism of apoB in the two conditions is, therefore, likely to be starkly contrasting. To illustrate these extremes of apoB metabolism, stable isotope turnover studies were performed in two individuals, one with hypobetalipoproteinaemia and one with analbuminaemia.

5.2 Hypobetalipoproteinaemia.

Familial hypobetalipoproteinaemia is a rare genetic condition, first described by Salt et al in 1960. It is inherited in an autosomal codominant fashion. Homozygotes are often from consanguinous matings. The condition is characterised by hypocholesterolaemia. Heterozygotes can be diagnosed by the following criteria: "(1) LDL abnormally low but present and identifiable immunochemically, while concentrations of VLDL and HDL are normal, (2) absence of diseases to which hypobetalipoproteinaemia may be secondary, and to be certain (3) detection of a similar pattern in a first-degree relative" (Fredrickson et al 1972). The heterozygous condition is often asymptomatic, and, therefore, frequently not detected, whilst homozygotes may have a more striking presentation with many of the features of classical abetalipoproteinaemia although somewhat moderated. However, abetalipoproteinaemia is a distinct and more severe condition that arises from a different genetic mutation and is inherited in an autosomal recessive fashion. The genetic cause of abetalipoptoteinaemia is thought not to be linked to the apoB gene (Talmud et al 1988) but to a defect in microsomal triglyceride transfer protein (Wetterau et al 1992). A subset of hypobetalipoproteinaemic individuals has been shown to have various truncations of apolipoprotein B (Aguilar-Salinas et al 1995). To date about 25 such truncations of varying length have been described.

Hypobetalipoproteinaemia primarily affects four body systems - gastro-intestinal, neuromuscular, ocular and haematological - in addition to the abnormalities in lipids and lipoproteins (Herbert et al 1978). Due to a limited ability to synthesise chylomicrons there may be fatty intolerance with associated steatorrhoea. In homozygotes, jejunal biopsies show lipid accumulation in the enterocytes, there is increased faecal fat excretion and there may be mild hepatic steatosis. Absorption of the fat soluble vitamins A, D, E and K is reduced with corresponding reductions in their plasma levels, with the exception of vitamin D which is usually normal (Innerarity 1990). Neuromuscular abnormalities are not consistent and again are often absent in heterozygotes. They are presumed to be due to the reduced levels of vitamin E, and include diminished deep tendon reflexes and ataxia. The main ocular manifestation in homozygotes is retinitis pigmentosa, with heterozygotes possibly

only having minor retinal changes. The characteristic haematological finding in homozygotes is acanthocytosis which may be the result of an altered phospholipid content of the red blood cell membrane. Heterozygotes can show a mild degree of acanthocytosis. The extent of the hypolipidaemia in affected individuals is variable. Heterozygotes generally have low but detectable amounts of VLDL and LDL with normal or elevated levels of HDL. The lipoprotein composition is mostly normal. VLDL and LDL in the homozygote are markedly reduced and sometimes undetectable. There is also an absence of post-prandial chylomicronaemia.

5.3 Analbuminaemia.

Analbuminaemia is a rare ($<1:10^6$) autosomal recessive condition that was first described in 1954 by Benhold et al. It is characterised by the absence or virtual absence of albumin from the plasma. A recent literature search identified less than 30 reported cases worldwide. Affected subjects are often asymptomatic or may complain only of mild fatigue, postural hypotension and dependent oedema. Identification of an affected individual is, therefore, usually incidental which may go some way to explaining the apparent rarity of the condition, although the relative ease and frequency with which plasma albumin is measured would tend to identify more cases if it were more prevalent. Heterozygotes tend to have low-normal plasma albumin levels and are completely asymptomatic. Consanguinuity has been noted in approximately half of the reported cases. The underlying defect in this condition appears to be one of reduced albumin synthesis rather than increased catabolism. Indeed studies of affected individuals who have been infused with exogenous albumin have shown that the catabolism of the albumin is in fact reduced so resulting in a longer half life (Russi and Weigand 1983).

Despite normally being the most abundant plasma protein and having many major functions, including maintenance of colloid-oncotic pressure and transport of many metabolites, hormones, fatty acids and anions, it is, perhaps, amazing that the absence of albumin from the plasma can result in so benign a condition. This may be explained in part by the compensatory increase in other plasma proteins that is seen in affected individuals, and by the relative reduction in capillary hydrostatic pressure (Baldo-Enzi et al 1987). In particular, there is a marked increase in the levels of lipoproteins with an associated increase in plasma cholesterol but not in triglyceride. However, of note is the fact that despite these increased levels of cholesterol and lipoproteins affected individuals do not invariably suffer from CHD.

An animal model for analbuminaemia, the Nagase analbuminaemic rat, was identified as a mutant amongst Sprague-Dawley hypercholesterolaemic rats (Nagase et al 1979). Much of the research into analbuminaemia has been performed on these rats due to the limited number of affected humans available for study. Van Tol et al (1991) showed that the majority of cholesterol in these rats was carried on HDL which was present in the plasma at high levels. They also noted an increase in lecithin:cholesterol acyltransferase activity which may explain the elevated HDL levels. Catanozzi et al (1994) found increased triglyceride and VLDL synthesis in Nagase rats, while Joles et al (1993) found that the hyperlipidaemia in these rats was in part due to extrahepatic lipogenesis.

The precise genetic mutation responsible for analbuminaemia has not yet been identified and in fact it appears that several different mutations can lead to reduced albumin synthesis. A review of five cases that had been studied at DNA level (Watkins et al 1994) identified different single nucleotide insertions producing stop codons in four of the subjects and an exon-splicing defect in the fifth.

5.4 Subject Selection.

5.4.1 Hypobetalipoproteinaemic Subject.

The original subject, a twenty year old caucasian girl (JR), was referred to this department from The Institute of Neurology, The National Hospital, London with a diagnosis of HARP syndrome (Amrolia 1992, unpublished report). HARP syndrome is a recently described condition of hypoprebetalipoproteinaemia, acanthocytosis, retinitis pigmentosa and pallidal degeneration (Higgins et al 1992). She presented with a two year history of facial dystonia, dysarthria and dysphagia associated with poor dietary intake. Examination revealed a slightly educationally subnormal girl of small, underweight stature. There was dystonic dysarthria with diminished palatal and tongue movements and gag reflex. Retinitis pigmentosa was seen on fundoscopy. Investigations showed hypolipidaemia with a total plasma cholesterol of 2.70mmol/l, triglycerides of 0.35mmol/l and a reduced LDL cholesterol of 1.00mmol/l. Peripheral blood films showed acanthocytosis. Vitamins A and E were normal on supplementation. An MRI scan confirmed degeneration of the pallidal nuclei. A fat absorption test was performed which showed interestingly that fatty acids were being absorbed, although not as chylomicrons but as non-esterified fatty acids. The subsequent VLDL apoB rise was in keeping with hepatic VLDL production, with the fatty acid content of the VLDL triglycerides reflecting those of the fat load. Electron microscopy of duodenal biopsy specimens revealed patchy lipid deposition in the enterocytes. Video fluoroscopy of swallowing was performed and this showed spasticity of the tongue and muscles of mastication.

Both the mother and the sister of the proband had similar lipid biochemistry but were otherwise healthy and without the neurological manifestations described above. The father was normolipaemic and there was no suggestion of consanguinuity in the family history. The original intention was to study apoB metabolism in both the proband and her mother (LR). However, due to technical difficulties with the proband (recurrent blocking of intravenous cannulae) it was felt unfair to persist and the stable isotope study was performed on the mother only. The characteristics of the proband (JR) and her mother (LR) are given in Table 5.1.

5.4.2 Analbuminaemic Subject.

This patient (AK) was a 60 year old black South African gentleman who was incidentally found to have analbuminaemia. His only complaint was of occasional ankle oedema following prolonged immobility which was first noted seven years previously. Importantly he gave no symptoms suggestive of CHD. He was descended from the Griqua and Tswana tribes with no known consanguinuity and no family history of note. Physical examination revealed bilateral circumferential arcus

cornealis, mild bilateral pitting oedema to the knees and unusually white and opaque fingernails. More interestingly, he had an unusual body fat distribution with excess adipose tissue over his buttocks and thighs but a slim upper body and face. To his knowledge he has always had this body shape and is the only member of his family of this build. This body shape is often seen amongst the Griqua bushmen. However, it also resembles partial lipodystrophy, a condition which has possibly been reported in two other analbuminaemic subjects (Gordon et al 1959, Montgomery et al 1962).

Investigations revealed a serum albumin level of <9g/l (normal range 35-50g/l). Liver biochemistry, urea, creatinine and electrolytes were normal. Total plasma cholesterol was 14.80mmol/l with an LDL cholesterol of 11.30mmol/l and an HDL cholesterol of 3.20mmol/l. Plasma triglycerides were 0.70mmol/l. ApoAI was elevated at 218mg/dl and total plasma apoB was also elevated at 229mg/dl. An ECG showed only left axis deviation and a chest x-ray was normal. Ultrasound scanning of the carotid and femoral arteries was performed and this showed a normal intimal-medial thickness with a smooth regular surface. There was no evidence of atherosclerosis. The characteristics of this subject are summarised in Table 5.2.

Table 5.1 Characteristics of Hypobetalipoproteinaemic Proband (JR) and Mother (LR).

	JR	LR		JR	LR
Age (years)	20	41	**ApoE phenotype**	3/3	3/3
Height (cm)	158	154	**Apo AI (mg/dl)**	114	138
Weight (kg)	48.2	53	**Apo B (mg/dl)**	27	23
Body Mass Index	19.3	22.3	**Lp(a) (mg/dl)**	1	1
Cholesterol (mmol/l)	2.70	3.20	**HDL_2 (mg/dl)**	92	148
Triglyceride (mmol/l)	0.35	0.40	**HDL_3 (mg/dl)**	191	201
VLDL-chol. (mmol/l)	0.20	0.20	**%LDLI**	31	28
LDL-chol. (mmol/l)	1.00	1.05	**%LDLII**	60	58
HDL-chol. (mmol/l)	1.50	1.95	**%LDLIII**	9	14

Table 5.2 Characteristics of Analbuminaemic Subject (AK).

	AK		AK
Age (years)	60	**Albumin (g/l)**	<9
Height (cm)	171	**Cholesterol (mmol/l)**	14.80
Weight (kg)	86	**Triglyceride (mmol/l)**	0.70
Body Mass Index	29.4	**VLDL-chol. (mmol/l)**	0.30
Apo AI (mg/dl)	218	**LDL-chol. (mmol/l)**	11.30
Apo B (mg/dl)	229	**HDL-chol. (mmol/l)**	3.20

5.5 Methods.

Stable isotope turnover studies were performed on the two subjects using the methodology described in Chapter 2. The hypobetalipoproteinaemic subject received a primed constant infusion of tri-deuterated leucine with the size and duration of the infusion being reduced to 500ml over eight hours to accommodate the small size of the subject. The dose of leucine remained the same at 0.7mg/kg body weight bolus, followed by 0.7mg/kg/hour infusion. In addition, VLDL was separated ultracentrifugally as total VLDL rather than as its subfractions $VLDL_1$ and $VLDL_2$. This was to allow for the low levels of apoB in the VLDL which could not have been reliably measured on the separated subfractions. To allow for this change when doing the kinetic analysis, the model was altered to a 12 compartment model with three compartments for total VLDL. No modifications were made to the methodology for the analbuminaemic individual who received a bolus dose of leucine at 7mg/kg body weight. However, this work was carried out in the Lipid Laboratory of Groote Schuur Hospital, Cape Town, South Africa, made possible by the Sue M^{c}Carthy Travelling Scholarship of the British Hyperlipidaemia Association. Ethical approval was granted by the Ethical Committee of Groote Schuur Hospital. The lipid and lipoprotein results were compared to those of the normolipidaemic subjects described in Chapter 3, while the kinetic analyses were compared to those of 15 normolipidaemic subjects whose apoB metabolism was studied previously in this department using the same methodology (Packard, unpublished results).

5.6 Results.

5.6.1 Hypobetalipoproteinaemic Subject.

1. Lipids and Lipoproteins.

The total plasma cholesterol in this subject varied from 2.60mmol/l to 3.20mmol/l and LDL cholesterol varied from 0.90mmol/l to 1.10mmol/l. These levels are lower than or at the lower limit of the normal range. However, HDL cholesterol tended to be on the high side of normal, ranging between 1.60mmol/l and 1.95mmol/l. The LDL:HDL ratios were 0.5-0.6. Triglyceride and VLDL cholesterol levels were also low at 0.25-0.40mmol/l and 0.05-0.20mmol/l respectively. These results are similar to those seen in the original proband (JR). Compositional analysis of the VLDL, IDL and LDL subfractions was essentially normal. Both the mother and daughter had LDL subfraction profiles of predominantly LDLII with little LDLIII, the percentage amounts being approximately 30% LDLI, 60% LDLII and 10% LDLIII (Table 5.1). Such profiles are normal for pre-menopausal women. Apo AI, HDL_2 and HDL_3 levels were at the upper end of normal in keeping with the elevated HDL cholesterol. Measured apoB levels were low at 23mg/dl (LR) and 27mg/dl (JR).

2. ApoB Pool Sizes (Table 5.3).

The total apoB concentration and apoB pool sizes were all markedly reduced with values of about 25% of normal. Total VLDL apoB mass was 14mg, IDL apoB mass was 44mg and LDL apoB mass was 234mg. The total calculated apoB concentration was 14.2mg/dl.

3. Kinetic Analysis.

Total apoB production in this individual was low at 123mg/day. The majority of the apoB (>80%) was produced as VLDL, with the remainder being produced directly as LDL. Both VLDL and LDL production rates were low. Direct IDL synthesis was negligible. The direct catabolic rates of VLDL and IDL were low at 0.01 pools/day and 0.08 pools/day respectively, whilst the transfer rates of VLDL apoB to IDL apoB and IDL apoB to LDL apoB were within the normal range at 6.2 pools/day and 1.9 pools/day respectively. The fractional catabolic rate of LDL was normal at 0.55 pools/day. These findings are summarised in Table 5.4 and the tracer mass curves are shown in Figure 5.1. The complete set of rate constants and calculated leucine pool sizes is given in Appendix 4.

5.6.2 Analbuminaemic Subject.

1. Lipids and Lipoproteins.

The total plasma cholesterol was elevated at 14.80mmol/l, with an elevated LDL cholesterol of 11.30mmol/l and an elevated HDL cholesterol of 3.20mmol/l. This gave an LDL:HDL ratio of 3.5. Plasma triglyceride and VLDL cholesterol on the other hand were normal at 0.70mmol/l and 0.30mmol/l respectively. Compositional analysis showed a tendency towards elevated percentage free cholesterol and decreased percentage phospholipid in $VLDL_1$, $VLDL_2$, IDL and LDL. LDL subfraction analysis revealed a predominance of the large LDL species. Apo AI was elevated in keeping with the elevated HDL cholesterol. Measured total apoB was also elevated.

2. ApoB Pool Sizes (Table 5.3).

The $VLDL_1$ apoB mass in this individual was normal at 60mg. However, the pool sizes of $VLDL_2$, IDL and LDL were all grossly elevated at 626mg, 3369mg and 4710mg respectively. The total calculated apoB concentration was also elevated at 255mg/dl.

3. Kinetic Analysis.

Initial inspection of the tracer mass curves for the four subfractions suggested normal $VLDL_1$ apoB metabolism but a massively increased direct production of $VLDL_2$ with little $VLDL_1$ to $VLDL_2$ apoB transfer. This was in fact borne out by the multicompartmental modelling which showed 85% of total apoB production being produced directly as $VLDL_2$ with the remaining 15% being produced as $VLDL_1$.

Direct synthesis of IDL and LDL was negligible. The production rate of $VLDL_2$ was elevated at 8146mg/day whilst that of $VLDL_1$ was normal at 1437mg/day. The fractional transfer rate of apoB from $VLDL_1$ to $VLDL_2$ was reduced at 1.3pools/day but the rate of direct catabolism was within the normal range (although towards the upper limit) so maintaining the normal $VLDL_1$ apoB pool size. $VLDL_2$ direct catabolism was elevated at 6.6pools/day but the transfer rate of apoB to IDL was normal at 6.7pools/day. IDL direct catabolism was normal (0.94pools/day) but the transfer rate to LDL was reduced (0.27pools/day). The fractional catabolic rate of LDL was also reduced at 0.20pools/day. These findings are summarised in Table 5.4 and the tracer mass curves are shown in Figure 5.2. The complete set of rate constants and calculated leucine pool sizes is given in Appendix 4.

Table 5.3 ApoB Pool Sizes for Hypobetalipoproteinaemic Subject (LR) and Analbuminaemic Subject (AK).

Subfraction	Subject LR	Subject AK	Normal Range
Total VLDL (mg)	14	------	------
$VLDL_1$ (mg)	------	60	18-100
$VLDL_2$ (mg)	------	626	41-220
IDL (mg)	44	3369	71-724
LDL (mg)	234	4710	860-2248
Total apoB (mg/dl)	14.2	225	42-84

Table 5.4 Kinetic Results for Hypobetalipoproteinaemic Subject (LR) and Analbuminaemic Subject (AK).

Subfraction	Kinetic Parameter	Subject LR	Subject AK	Normal Range
Total VLDL	Production (mg/d)	99.4	------	------
	FDC (pools/d)	0.01	------	------
	FTR (pools/d)	6.2	------	------
$VLDL_1$	Production (mg/d)	------	1437	264-1508
	FDC (pools/d)	------	22.6	0.05-27.2
	FTR (pools/d)	------	1.3	5.81-37.7
$VLDL_2$	Production (mg/d)	------	8146	21-358
	FDC (pools/d)	------	6.6	0.06-3.90
	FTR (pools/d)	------	6.7	3.6-12.7
IDL	Production (mg/d)	0	0	3-381
	FDC (pools/d)	0.08	0.94	0.02-3.30
	FTR (pools/d)	1.9	0.27	1.01-6.50
LDL	Production (mg/d)	23.3	0	1-326
	FCR (pools/d)	0.55	0.20	0.26-0.94
Total ApoB	Production (mg/d)	122.7	9583	561-2209

FDC = fractional rate of direct catabolism, FTR = fractional transfer rate, FCR = fractional catabolic rate.

5.7 Discussion.

Stable isotope turnover studies of apoB kinetics were performed in two very different subjects with plasma cholesterol and apoB levels at opposite extremes. Hypobetalipoproteinaemia is associated with low levels of plasma cholesterol and apoB, whilst in analbuminaemia the converse is true. Comparison of the results, both to normolipidaemic subjects and to each other, has highlighted some interesting findings.

5.7.1 Hypobetalipoproteinaemia.

It is unfortunate that a stable isotope study of the apoB kinetics in the original proband (JR) was not possible, but the similarity in the lipid and lipoprotein levels between her and her mother allows some inference of her apoB kinetics. What is not entirely understood is the reason why JR should be symptomatic while her mother, and sister, are not. As her father is unaffected it is not possible that JR is a homozygote for hypobetalipoproteinaemia and her mother a heterozygote so explaining the more severe phenotype. A possibility is that JR's poor dietary intake in fact preceded the development of neurological symptoms and in some way exacerbated the hypobetalipoproteinaemia to such a degree that the neurological symptoms became manifest and in turn further worsened the dietary intake. Whatever the reason, the neurological symptoms seem more extreme than is usually seen in heterozygous hypobetalipoproteinaemia and are out of keeping with the degree of fat malabsorption and lipid abnormality.

The proband's mother (LR) proved interesting to study. Her low plasma lipids, low LDL cholesterol and VLDL cholesterol, elevated HDL cholesterol and normal lipoprotein composition are in keeping with previous reports of such subjects (Herbert et al 1978). In an early study of LDL kinetics in three heterozygotes for this condition Levy et al (1970) found that LDL synthesis was reduced but LDL catabolism was normal. From these findings they suggested that the disorder was secondary to reduced LDL synthesis rather than increased catabolism. Subject LR had LDL production and catabolic rates that fell within the normal range, but the production rate was at the lower end of the normal range whilst the catabolic rate was normal. The production rate of VLDL was also low. In this subject the transfer of apoB down the delipidation chain appeared to be occurring at a normal rate. However, the direct catabolism of VLDL and IDL was low to low-normal. Thus the primary abnormality in this subject appears to be one of reduced apoB synthesis. More recently studies have been performed on hypobetalipoproteinaemic subjects with various truncations of apoB. Using endogenous tracer methodology, Aguilar-Salinas et al (1995) found decreased apoB production (both as VLDL and LDL apoB) in hypobetalipoproteinaemic subjects heterozygous for an apoB truncation. These subjects had normal apoB transfer down the delipidation chain and normal catabolism of VLDL, IDL and LDL. This was further elaborated on by Parhofer et al (1996) when he found a positive linear correlation between the length of the truncated apoB and its secretion rate, such that the shorter the apoB molecule the lower was its production rate. Extrapolation of the linear regression suggested apoB secretion would be zero at a truncation length of apoB-28 which is in keeping with the observation that

apoB molecules shorter than apoB-28 are not seen in the plasma. This is perhaps due to the inability of short forms of apoB to acquire enough lipid prior to secretion and they are therefore degraded intracellularly. Interestingly this study also found increased catabolic rates for the longer apoB truncations, presumably because they still contain the LDL receptor recognition region, although this does not explain why they should bind to the receptor with a greater affinity than apoB-100. Unfortunately, the apoB of subject LR was not examined for a truncation. It may be that her apoB is of normal length and that the underlying genetic defect lies in the promoter-enhancer region of the apoB gene (Innerarity 1990). This would fit with the findings of reduced apoB production rates but normal catabolism. However, of note is the fact that the proband (JR) does not produce chylomicrons and therefore presumably cannot synthesise apoB-48, despite having low but measurable amounts of apoB-100 in her plasma. This finding remains to be explained.

5.7.2 Analbuminaemia.

This subject (AK) is one of less than thirty individuals identified worldwide as having analbuminaemia. His incidental finding and benign clinical course are in keeping with the others. His strikingly low serum albumin level of <9g/l and grossly elevated plasma cholesterol are characteristic of the condition. As with the other affected individuals, his LDL and HDL cholesterol levels were elevated while triglyceride and VLDL cholesterol were normal. Plasma apoB was also elevated in keeping with the elevated LDL.

To our knowledge stable isotope turnover studies of apoB kinetics have not been performed previously in analbuminaemic subjects and this is, therefore, the first reported case of such work. This subject had normal production and catabolic rates of $VLDL_1$ with a resultant normal $VLDL_1$ pool size. This is reflected in his normal plasma triglyceride level. The pool sizes of $VLDL_2$, IDL and LDL were all elevated. However, the most striking finding was the greatly increased direct production rate of $VLDL_2$. This was increased by approximately 20 times the upper limit of the normal range. Little $VLDL_2$ came from transfer from $VLDL_1$ as is reflected in the low $VLDL_1$ fractional transfer rate. The $VLDL_2$ fractional rate of direct catabolism was elevated, possibly reflecting increased substrate availability for clearance e.g. by apoE-mediated receptor clearance. However, the catabolism was not elevated to such an extent that the pool size was reduced to normal. The production of predominantly $VLDL_2$ rather than $VLDL_1$ is in keeping with the nature of this subject's LDL subfraction profile. He had a predominance of the larger, less dense species which are known to be preferentially formed from the delipidation of $VLDL_2$.

The transfer of apoB to IDL occured at a normal rate and this was the source of apoB in the IDL and LDL fractions as direct IDL and LDL synthesis was practically zero. The pool sizes of IDL and LDL were similar, with the relative increases as compared to normal being greater for IDL. In addition the transfer of apoB from IDL to LDL was found to be reduced despite the findings of an increased LDL pool size but negligible LDL direct production. This may in fact reflect a methodological error at the stage of ultracentrifugal separation of the lipoproteins. Due to the large amount of lipoprotein present in the LDL density range there is some overflow of LDL into the

IDL density range (Marais 1996, unpublished observation). It is, therefore, possible that some of the measured IDL apoB is in fact LDL apoB. The fractional rate of direct catabolism of IDL was normal. However, the fractional catabolic rate of LDL was reduced below the lower limit of normal, almost to the levels seen in familial hypercholesterolaemia. This could perhaps be a compensatory mechanism to keep LDL in the plasma and so help to maintain the plasma oncotic pressure in the absence of albumin.

The effects of albumin on lipid synthesis, apoB secretion and LDL catabolism have been studied recently in HepG2 cells (Cianflone et al 1994). This study found that as the extracellular albumin concentration was reduced the secretion of apoB was increased. In addition, reductions in albumin concentration were associated with increased cholesterol ester synthesis but no change in triglyceride synthesis. Finally, a decreased albumin concentration resulted in a decrease in LDL catabolism. Although caution is always required when extrapolating from in vitro studies to in vivo work, these findings are similar to those seen in our analbuminaemic subject. In parallel with this is the observation that the lipoprotein compositions showed an increased percentage of free cholesterol with a decreased percentage of phospholipid. Increased free cholesterol in the lipoprotein coat stiffens the phospholipid lattice so making it more difficult for apolipoproteins to insert into the coat. This in turn may impede the lipoprotein clearance. Alternatively, the lipoproteins could be cholesterol-rich due to increased hepatic uptake of cholesterol-rich $VLDL_2$ (as is seen in the increased catabolic rate of $VLDL_2$) in a sense reflecting a degree of futile cycling of $VLDL_2$. However, if this were the case the increase would be expected in cholesterol ester rather than in free cholesterol.

A very interesting aspect of this condition is that, despite gross elevations in plasma and LDL cholesterol, these subjects do not invariably go on to develop CHD. Indeed, our subject at the age of 60 years was completely asymptomatic for CHD and had no ECG or ultrasound findings suggestive of its development. This observation is not entirely explained but may be in part due to the following. Firstly, the increase in HDL cholesterol means that despite an elevated LDL cholesterol the LDL:HDL ratio was only minimally raised, in this case at 3.5, thus exerting some protective effect. Secondly, the nature of the LDL itself (a large, less dense species) is known to be less atherogenic than the small, dense species that is often seen in cases of hypercholesterolaemia. Thirdly, the relatively low blood pressures seen in these individuals may go some way to reducing the predisposition to atherosclerosis development.

In summary, the main kinetic findings in this subject with analbuminaemia were i). normal $VLDL_1$ metabolism, ii). greatly elevated $VLDL_2$, IDL and LDL pool sizes, iii). grossly elevated $VLDL_2$ production, and iv). reduced LDL catabolism. These findings are reflected in the elevated plasma cholesterol and apoB levels and the normal plasma triglyceride level.

Thus, in hypobetalipoproteinaemia there is a reduction in apoB production, secondary to a defect in the apoB gene, which results in low levels of lipids and apoB containing lipoproteins in the plasma. By contrast, in analbuminaemia there is a greatly increased apoB production as part of a physiological compensatory mechanism to maintain plasma oncotic pressure. This in turn is associated with grossly elevated levels of plasma cholesterol and LDL apoB. Despite these extreme aberrations in apoB metabolism, both these conditions run a relatively benign course but add insight into apoB metabolism in normolipidaemia. The kinetic findings are summarised in Figure 5.3.

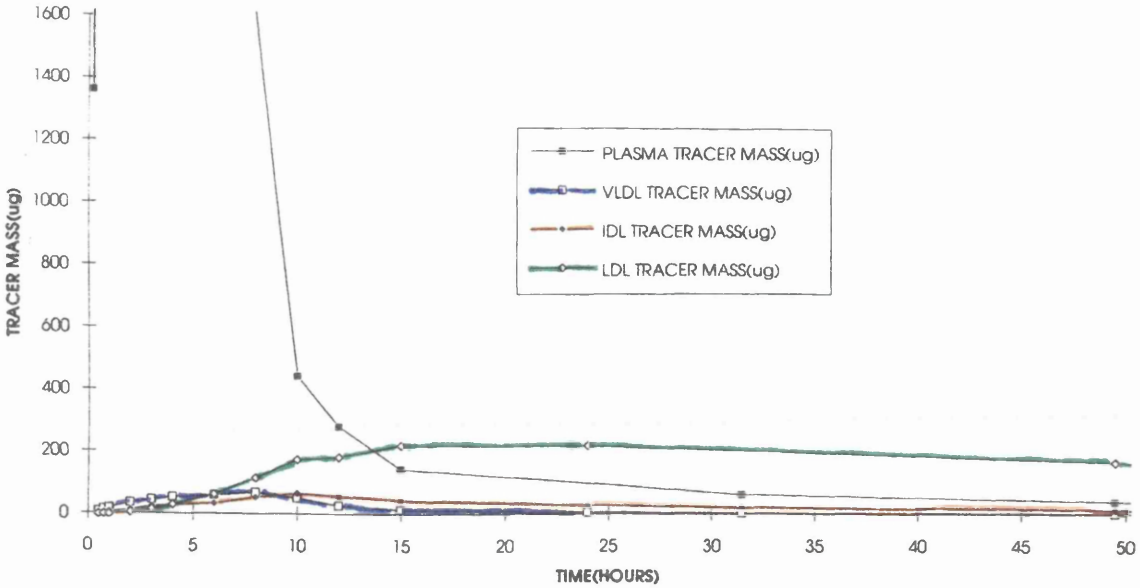


Figure 5.1 Tracer Mass Curves for Hypobetalipoproteinaemic Subject (LR), 0-50 hours.

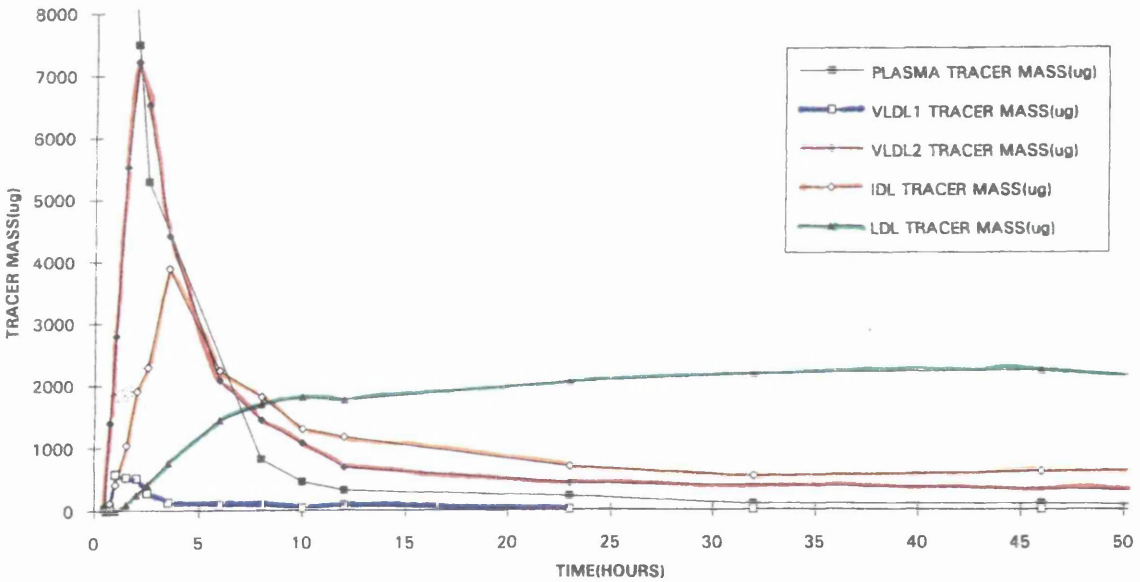
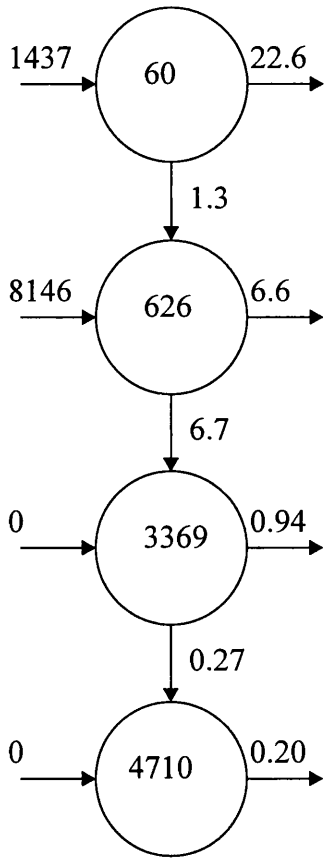
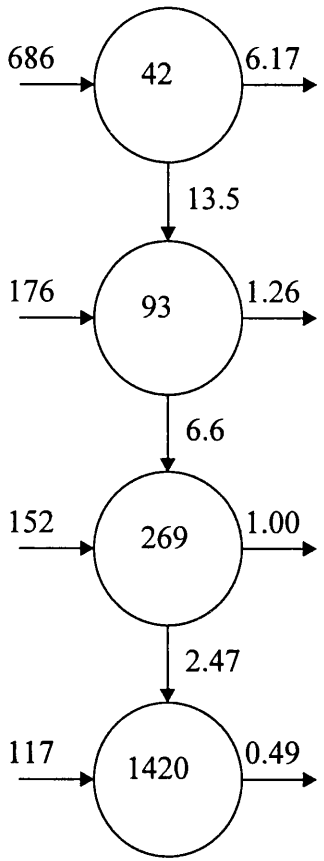


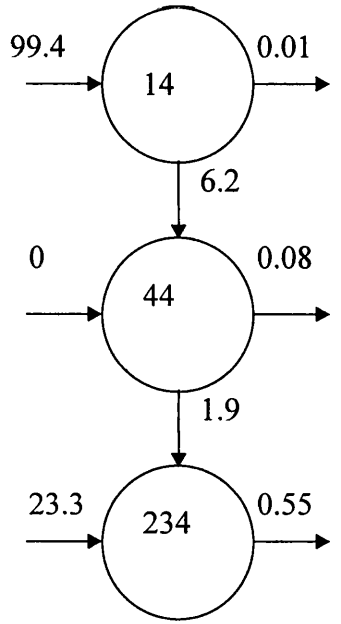
Figure 5.2 Tracer Mass Curves for Analbuminaemic Subject (AK), 0-50 hours.



a. Analbuminaemic Subject



b. Normal Subjects



c. Hypobetalipoproteinaemic Subject

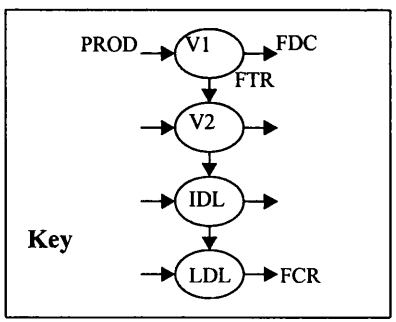


Figure 5.3 Pool Sizes and Kinetic Parameters - a. Analbuminaemic Subject, b. Normals (mean values), c. Hypobetalipoproteinaemic Subject.
Prod = production rate (mg/d), FDC = fractional rate of direct catabolism (pool/d), FTR = fractional transfer rate (pool/d), FCR = fractional catabolic rate (pool/d), V1 = $VLDL_1$, V2 = $VLDL_2$, IDL & LDL apoB pool sizes (mg).

Chapter 6 Stable Isotope Turnovers in Mixed Hyperlipidaemia.

6.1 Introduction.

Mixed hyperlipidaemia is perhaps one of the more common forms of hyperlipidaemia to present at Lipoprotein Clinics. Typically the patient has moderate elevations in both cholesterol and triglycerides, giving a Fredrickson type IIb or occasionally type III pattern, often with an associated reduction in HDL cholesterol. ApoB may also be elevated. The aetiology of mixed hyperlipidaemia is multifactorial. The hypercholesterolaemia may be inherited in a polygenic fashion, with the hypertriglyceridaemia being secondary to, for example, obesity, insulin resistance or alcohol excess. Alternatively, it may be inherited as familial combined hyperlipidaemia (FCHL) as described by Goldstein et al in 1973. This condition is thought to affect 0.5-2.0% of the population in the Western world and is found in up to 10% of those with premature CHD. The presenting phenotype may be a pure hypercholesterolaemia, a pure hypertriglyceridaemia or a mixed hyperlipidaemia. In addition, more than one phenotype is present within a family and an affected individual's phenotype may change during his lifetime. It was originally suggested that the mode of inheritance of FCHL was autosomal dominant with reduced penetrance, but it now appears that the genetic basis is more complex. FCHL is difficult to diagnose due to its lack of clinical marker, so requiring family studies for true identification. However, in a clinical setting this is not necessarily relevant as treatment strategies remain the same and not all patients with mixed hyperlipidaemia will have FCHL. The treatment of mixed hyperlipidaemia is discussed in the following chapter.

Much of the kinetic work on mixed hyperlipidaemia has been performed using subjects with FCHL. The most consistent finding is that of increased hepatic VLDL apoB production (Janus et al 1980, Kissebah et al 1981, Cortner et al 1991, Venkatesan et al 1993) with the VLDL composition being normal. In addition, some workers have found an increase in the formation of LDL from VLDL (Janus et al 1980, Kissebah et al 1981). Other metabolic findings include reduced lipoprotein lipase activity (Babirak et al 1992), reduced chylomicron remnant clearance (Cabezas et al 1993) and impaired fatty acid metabolism (Cabezas et al 1993). This chapter aims to characterise the apoB kinetics in subjects with mixed hyperlipidaemia.

6.2 Subject Selection.

Seven subjects (six males and one post-menopausal female) with mixed hyperlipidaemia (cholesterol 6.0-9.0 mmol/l, triglyceride 2.0-4.0 mmol/l) were recruited from the Lipoprotein Clinic at Glasgow Royal Infirmary. Secondary causes for the hyperlipidaemia were excluded by the relevant biochemical and haematological tests. In addition, no subject had the apoE 2/2 phenotype. All lipid-lowering medication was stopped four weeks prior to the commencement of the study and no subject was taking medication known to affect lipoprotein metabolism. The characteristics and lipid profiles of the seven subjects are given in Tables 6.1 and 6.2.

These subjects were not classified as FCHL as family studies proved impractical - many of the subjects had fewer than 3 first degree relatives in the area available for screening. Each subject gave signed informed consent and the study was approved by the Ethical Committee of Glasgow Royal Infirmary.

Table 6.1 Mixed Hyperlipidaemic Subject Characteristics.

Subject	Age (years)	Sex	Weight (kg)	Body Mass Index	ApoE Phenotype
RA	50	M	74	25.6	3/3
PS	37	M	72	23.9	3/3
JC	45	M	70	25.4	4/3
RS	39	M	90	27.8	4/3
DM	40	M	84	29.1	3/3
LB	52	F	73	31.2	4/4
PC	53	M	91	28.1	3/3

Table 6.2 Mixed Hyperlipidaemic Subject Lipid Profiles.

Subject	Cholesterol (mmol/l)	Triglyceride (mmol/l)	VLDL-chol. (mmol/l)	LDL-chol. (mmol/l)	HDL-chol. (Mmol/l)
RA	6.20	2.90	0.85	3.95	1.40
PS	6.45	2.60	0.80	4.60	1.05
JC	6.40	2.25	0.75	4.70	0.95
RS	6.35	2.60	1.15	4.55	0.70
DM	7.90	3.55	1.90	5.10	0.90
LB	6.60	2.95	1.25	4.45	0.90
PC	7.00	3.60	1.45	4.60	0.95

6.3 Methods.

Following a ten hour overnight fast each individual underwent a turnover study of apoB kinetics using a bolus of tri-deuterated leucine at a dose of 7mg/kg body weight. Subjects remained fasting throughout the day and venous blood samples were taken according to the protocol as described in Chapter 2. The methodology for sample preparation, GC-MS measurement of isotopic enrichment and kinetic analysis by multicompartmental modelling is as given previously. The results obtained were compared to those of a similar study of 15 normal individuals (cholesterol <6.0mmol/l, triglyceride <2.0mmol/l) carried out previously in this laboratory using the same methodology (Packard, unpublished data). Comparisons between the mixed hyperlipidaemic and normal subjects were performed using the two sample t-test.

6.4 Results.

1. Lipids and Lipoproteins.

As would be expected due to the sample selection, there were significant elevations in the cholesterol ($p<0.0001$), triglyceride ($p<0.0001$), VLDL cholesterol ($p=0.006$) and LDL cholesterol ($p<0.0001$) levels of the seven mixed hyperlipidaemic subjects as compared to those of the normal subjects. In addition, HDL cholesterol was significantly lower ($p=0.0005$). The lipoprotein compositions (Table 6.3, a & b) were compared to the results of the study of eighty normolipaemic subjects (cholesterol <6.0mmol/l and triglyceride <2.0mmol/l) reported in Chapter 3 as compositional analysis of the 15 normal subjects used for the kinetic comparisons was not available. Percentage free cholesterol was significantly ($p<0.05$) increased in $VLDL_1$ and $VLDL_2$, but decreased in IDL and LDL. The esterified cholesterol content of $VLDL_1$ was increased, with reductions in the protein content. The percentage phospholipid in IDL and LDL was elevated. Analysis of the triglyceride:protein and cholesterol:protein ratios in $VLDL_1$ and $VLDL_2$ showed that these lipoproteins, particularly $VLDL_1$, in the mixed hyperlipidaemic subjects were relatively lipid enriched (Table 6.4). The $VLDL_1$ triglyceride:protein, free cholesterol:protein and esterified cholesterol:protein ratios were all significantly increased, whilst in $VLDL_2$ only the free cholesterol:protein ratio was increased. In $VLDL_1$ the free cholesterol:phospholipid ratio is increased as compared to normal.

2. ApoB Pool Sizes (Table 6.5).

The apoB pool sizes in the seven subjects were all significantly elevated with the exception of IDL which narrowly missed significance ($p = 0.08$). The total apoB concentration was also elevated. The total apoB and $VLDL_1$ pool sizes all fell above the upper limit of the normal range. Two of the seven subjects had $VLDL_2$ pool sizes within the normal range, whilst only one LDL pool size came within normal limits. The IDL pool sizes all fell within the normal range but most tended towards the upper limit.

Table 6.3a Percentage Compositions of $VLDL_1$ and $VLDL_2$ - normal v mixed hyperlipidaemic subjects.

	------ Prot	------- FC	$VLDL_1$ EC	------- TG	---- PL	------ Prot	------- FC	$VLDL_2$ EC	------- TG	---- PL
Normal										
mean	**11.2**	**1.6**	**10.4**	**62.2**	**14.9**	**15.4**	**3.2**	**24.1**	**37.8**	**19.4**
(SEM)	**(0.4)**	**(0.2)**	**(0.5)**	**(1.4)**	**(1.7)**	**(0.4)**	**(0.2)**	**(0.7)**	**(0.8)**	**(1.3)**
RA	6.5	2.2	10.7	65.5	15.1	12.3	3.5	18.3	47.6	18.2
PS	7.0	4.2	10.5	62.4	15.9	12.0	4.8	21.6	41.0	20.6
JC	8.3	2.8	14.8	59.0	15.0	18.0	3.5	29.1	31.2	18.2
RS	8.9	2.6	15.7	57.3	15.5	14.0	3.8	31.8	30.3	20.0
DM	6.1	4.4	15.6	58.7	15.1	11.9	4.9	33.9	30.2	19.2
LB	6.1	3.6	10.8	63.4	16.1	12.2	5.4	23.6	36.5	22.1
PC	6.5	4.2	10.9	62.8	15.6	13.9	3.2	24.9	38.2	19.9
Mixed HLP										
mean	**7.1**	**3.4**	**12.7**	**61.3**	**15.5**	**13.5**	**4.2**	**26.2**	**36.4**	**19.7**
(SEM)	**(0.4)**	**(0.3)**	**(0.9)**	**(1.1)**	**(0.2)**	**(0.8)**	**(0.3)**	**(2.1)**	**(2.4)**	**(0.5)**
p value	**<0.001**	**0.001**	**0.04**	**NS**	**NS**	**NS**	**0.03**	**NS**	**NS**	**NS**

Prot = protein, FC = free cholesterol, EC = esterified cholesterol, TG = triglyceride, PL = phospholipid, Mixed HLP = mixed hyperlipidaemic subjects, SEM = standard error of mean, NS = not significant.

Table 6.3b Percentage Compositions of IDL and LDL - normal v mixed hyperlipidaemic subjects.

	------ Prot	------- FC	IDL --- EC	------- TG	---- PL	------ Prot	------- FC	LDL --- EC	------- TG	---- PL
Normal										
mean	**20.1**	**5.8**	**42.8**	**13.7**	**18.2**	**24.4**	**8.8**	**43.4**	**4.6**	**18.8**
(SEM)	**(0.3)**	**(0.2)**	**(0.5)**	**(0.5)**	**(0.4)**	**(0.2)**	**(0.3)**	**(0.4)**	**(0.1)**	**(0.4)**
RA	20.4	5.2	37.2	16.5	20.7	23.7	5.9	44.5	5.4	20.5
PS	15.8	6.1	42.0	11.2	24.9	22.1	9.4	43.9	3.8	20.8
JC	24.2	4.0	44.8	8.0	18.9	27.0	7.4	42.2	3.9	19.4
RS	19.1	4.6	48.5	6.9	21.0	23.9	7.2	44.0	4.0	20.8
DM	17.7	5.3	45.7	10.6	20.7	24.6	8.1	41.8	5.7	19.8
LB	16.6	4.1	42.4	14.6	22.4	26.6	5.6	39.6	6.5	21.8
PC	19.7	4.0	42.8	12.5	20.9	26.6	5.2	42.8	5.3	20.2
Mixed HLP										
mean	**19.1**	**4.8**	**43.3**	**11.5**	**21.4**	**24.9**	**7.0**	**42.7**	**4.9**	**20.5**
(SEM)	**(1.1)**	**(0.3)**	**(1.3)**	**(1.3)**	**(0.7)**	**(0.7)**	**(0.6)**	**(0.6)**	**(0.4)**	**(0.3)**
p value	**NS**	**0.01**	**NS**	**NS**	**0.003**	**NS**	**0.02**	**NS**	**NS**	**0.002**

Table 6.4 VLDL Composition Ratios - normal v mixed hyperlipidaemic subjects.

	-------	$VLDL_1$	----------	-------	-------	$VLDL_2$	----------	-------
	TG:PR	**FC:PR**	**EC:PR**	**FC:PL**	**TG:PR**	**FC:PR**	**EC:PR**	**FC:PL**
Normal								
mean	**5.8**	**0.14**	**1.00**	**0.16**	**2.5**	**0.21**	**1.61**	**0.29**
(SEM)	**(0.20)**	**(0.02)**	**(0.07)**	**(0.02)**	**(0.10)**	**(0.02)**	**(0.06)**	**(0.10)**
RA	10.1	0.3	1.6	0.15	3.9	0.3	1.5	0.19
PS	8.9	0.6	1.5	0.26	3.4	0.4	1.8	0.23
JC	7.1	0.3	1.8	0.19	1.7	0.2	1.6	0.19
RS	6.4	0.3	1.8	0.17	2.2	0.3	2.3	0.19
DM	9.6	0.7	2.6	0.29	2.5	0.4	2.8	0.26
LB	10.4	0.6	1.8	0.22	3.0	0.4	1.9	0.24
PC	9.7	0.6	1.7	0.27	2.7	0.2	1.8	0.16
Mixed HLP								
mean	**8.9**	**0.50**	**1.81**	**0.22**	**2.8**	**0.32**	**1.96**	**0.21**
(SEM)	**(0.60)**	**(0.07)**	**(0.13)**	**(0.02)**	**(0.30)**	**(0.04)**	**(0.17)**	**(0.01)**
p value	**0.002**	**0.002**	**0.0004**	**0.04**	**NS**	**0.03**	**NS**	**NS**

TG:PR = triglyceride:protein ratio, FC:PR = free cholesterol:protein ratio, EC:PR = esterified cholesterol:protein ratio, FC:PL = free cholesterol:phospholipid ratio.

Table 6.5 ApoB Pool Sizes - normal v mixed hyperlipidaemic subjects.

	Total ApoB (mg/dl)	**$VLDL_1$ Pool (mg)**	**$VLDL_2$ Pool (mg)**	**IDL Pool (mg)**	**LDL Pool (mg)**
Normal					
mean (SEM)	**60 (3.5)**	**42 (6.3)**	**93 (13)**	**269 (40)**	**1420 (99)**
range	**42-84**	**18-100**	**41-220**	**71-724**	**860-2248**
RA	93	145	141	224	2236
PS	106	196	166	180	2528
JC	156	132	325	618	3276
RS	121	345	267	411	3323
DM	234	328	477	651	3977
LB	128	206	338	405	2785
PC	142	368	249	441	4118
Mixed HLP					
mean (SEM)	**140 (18)**	**246 (37)**	**280 (43)**	**419 (67)**	**3178 (268)**
p value	**p=0.004**	**p=0.002**	**p=0.004**	**p=0.08**	**p=0.0005**

3. Kinetic Analysis (Table 6.6, a & b).

The kinetics of $VLDL_1$ were significantly different from normal, with an increased production rate and decreases in the fractional rates of direct catabolism and transfer. $VLDL_2$ kinetics were mostly normal with the exception of a reduced fractional transfer rate. IDL showed significant decreases in production and fractional transfer rates with a normal fractional rate of direct catabolism. The production rate of LDL was reduced as compared to normal although not significantly ($p=0.21$). Overall, the total apoB production was increased ($p=0.049$). Finally, the fractional catabolic rate of LDL was significantly reduced. Thus the pertinent findings were i). an increase in apoB production, specifically $VLDL_1$ production, ii). a decrease in apoB transfer rate down the delipidation chain, and iii). decreases in the catabolism of $VLDL_1$ and LDL. The complete set of rate constants and calculated leucine pool sizes is given in Appendix 5. Figure 6.1, (a & b), shows representative tracer mass curves from a normolipidaemic and a mixed hyperlipidaemic subject.

To characterise further the apoB metabolism in the two groups, Pearson correlation coefficients (r) were calculated between apoB pool sizes and production rates and the plasma lipid levels and kinetic parameters. A sample size of $n=7$ is perhaps too small for certain statistical analyses and this needs to be borne in mind when interpreting the results. Firstly, the data was assessed for normality using the Anderson-Darling normality test. The following needed transformation prior to further analysis - $VLDL_1$, $VLDL_2$ and IDL apoB pool sizes ($\log_e$ transformed), IDL and LDL apoB production rates (square root), total apoB production rate ($\log_e$), $VLDL_1$, $VLDL_2$ and IDL fractional rates of direct catabolism (square root), and $VLDL_1$ and IDL fractional rates of transfer ($\log_e$). The correlations were then calculated separately for the two groups. Tables 6.7 and 6.8 show the correlations for apoB pool sizes and apoB production rates with total cholesterol, total triglyceride and the kinetic parameters.

To test the significance of the correlations, regression analysis was performed on those correlations with r values >0.5 or <-0.5. Again here the small sample size of the mixed HLP group may be statistically limiting with many of the correlated parameters not retaining significance whilst those in the normal group largely did. This may also reflect the degree of scatter of the values in the mixed HLP group. Those correlations with p values of <0.05 and <0.005 are marked in Tables 6.7 and 6.8. No correction for multiple comparison has been performed but those correlations with a significance of $p<0.005$ are unlikely to have occurred by chance.

In the normolipidaemic group the $VLDL_1$ and $VLDL_2$ apoB pool sizes correlate positively with total triglyceride, whilst the IDL and LDL pool sizes correlate positively with total cholesterol. Total apoB correlates strongly with total plasma cholesterol. In the mixed hyperlipidaemic group, total apoB also correlates strongly with total plasma cholesterol but the individual subfraction apoB pools do not show significant correlations, perhaps due to the small sample size. However, there is a tendency for $VLDL_1$ to correlate with plasma triglyceride whilst $VLDL_2$, IDL and LDL correlate with total cholesterol.

In the normolipidaemic group the VLDL and IDL pool sizes showed negative correlations with $VLDL_2$ and IDL fractional transfer rates. Total apoB also showed a significant negative correlation with $VLDL_2$ fractional transfer rate. The apoB pool sizes appear to have little relation to the rates of production and direct catabolism of the lipoproteins in this group. In the mixed hyperlipidaemic group the negative correlation between VLDL and IDL pool sizes and $VLDL_2$ and IDL fractional transfer rates persists, and total apoB correlates negatively with $VLDL_2$ fractional transfer rate although not significantly. However, in this group the $VLDL_1$ and LDL apoB pools also correlate negatively with LDL fractional catabolic rate, a relationship that was not seen in the normolipidaemic group.

In both groups total apoB production correlated most strongly with $VLDL_1$ production. In the normolipidaemic group total apoB production also correlated positively with IDL production, the fractional rates of direct catabolism of $VLDL_2$ and IDL and the fractional catabolic rate of LDL, reflecting the homeostatic balance between production and catabolism of lipoproteins in the normal state. The mixed hyperlipidaemic group showed a reciprocal relationship between the production rates of $VLDL_1$, $VLDL_2$ and IDL such that $VLDL_1$ and $VLDL_2$ production were negatively correlated whilst $VLDL_2$ and IDL production were positively correlated. $VLDL_1$ production showed a non-significant positive correlation with $VLDL_1$ pool size in contrast to the negative correlation between $VLDL_2$ production and $VLDL_1$ pool size (again non-significant).

Therefore, in both groups total apoB and total cholesterol correlated positively, with the apoB pool sizes of each lipoprotein correlating with its predominant lipid. The apoB pool sizes were negatively correlated with the fractional transfer rates of apoB down the delipidation chain. Catabolism of the lipoproteins showed little relationship to the apoB pool sizes except for LDL catabolism and $VLDL_1$ and LDL pool sizes in the mixed hyperlipidaemic group. Total apoB production was largely determined by $VLDL_1$ apoB production in both groups, with the mixed hyperlipidaemic group showing a negative correlation between $VLDL_1$ and $VLDL_2$ production.

Table 6.6a Kinetic Results - $VLDL_1$ and $VLDL_2$, normal v mixed HLP subjects.

	$VLDL_1$ PROD (mg/d)	$VLDL_1$ FDC (pool/d)	$VLDL_1$ FTR (pool/d)	$VLDL_2$ PROD (mg/d)	$VLDL_2$ FDC (pool/d)	$VLDL_2$ FTR (pool/d)
Normal mean (SEM)	**686 (84)**	**6.17 (1.84)**	**13.5 (2.00)**	**176 (26)**	**1.26 (0.33)**	**6.60 (0.69)**
range	**264-1508**	**0.05-27.2**	**5.81-37.7**	**21-358**	**0.06-3.90**	**3.6-12.7**
RA	1078	2.8	4.5	176	0.8	5.2
PS	841	1.8	2.5	207	0.5	3.3
JC	993	0.06	7.6	279	0.8	3.2
RS	1349	2.8	1.1	190	0.003	2.2
DM	1098	0.8	2.6	192	0.4	1.7
LB	886	2.2	1.9	264	0.6	1.5
PC	1615	0.9	3.5	93	3.2	2.4
Mixed HLP mean (SEM)	**1123 (103)**	**1.62 (0.40)**	**3.39 (0.81)**	**200 (23)**	**0.90 (0.40)**	**2.79 (0.48)**
p value	**p=0.006**	**p=0.03**	**p=0.0002**	**NS**	**NS**	**p=0.0002**

PROD = production rate, FDC = fractional rate of direct catabolism, FTR = fractional transfer rate, NS = not significant, Mixed HLP = mixed hyperlipidaemic subjects.

Table 6.6b Kinetic Results - IDL and LDL, normal v mixed HLP subjects.

	IDL PROD (mg/d)	IDL FDC (pool/d)	IDL FTR (pool/d)	LDL PROD (mg/d)	LDL FCR (pool/d)	Total ApoB PROD (mg/d)
Normal mean (SEM)	**152 (31)**	**1.00 (0.24)**	**2.47 (0.36)**	**117 (28)**	**0.49 (0.04)**	**1131(109)**
range	**3-381**	**0.02-3.30**	**1.01-6.50**	**1-326**	**0.26-0.94**	**561-2209**
RA	27	1.1	1.9	80	0.28	1361
PS	11	0.6	3.0	43	0.22	1102
JC	56	0.7	1.0	70	0.21	1398
RS	10	0.2	1.2	47	0.17	1596
DM	28	0.4	0.9	55	0.16	1373
LB	79	0.6	0.8	93	0.16	1322
PC	9	0.5	0.9	149	0.13	1866
Mixed HLP mean (SEM)	**31 (10)**	**0.59 (0.11)**	**1.39 (0.30)**	**77 (28)**	**0.19 (0.02)**	**1431(91)**
p value	**p=0.002**	**NS**	**p=0.03**	**NS**	**p<0.0001**	**p=0.049**

FCR = fractional catabolic rate.

Table 6.7a ApoB Pool Size Correlations - Pearson correlation coefficients (r) for normal and mixed HLP subjects.

Pools - normal *mixed*	Total chol. (mmol/l)	Total trig. (mmol/l)	$VLDL_1$ PROD (mg/d)	$VLDL_1$ FDC (pool/d)	$VLDL_1$ FTR (pool/d)	$VLDL_2$ PROD (mg/d)	$VLDL_2$ FDC (pool/d)	$VLDL_2$ FTR (pool/d)
$VLDL_1$ apoB (mg)	0.023 *0.588*	0.798** *0.662*	0.475 *0.690*	-0.383 *0.169*	-0.513 *-0.643*	0.155 *-0.632*	0.349 *0.020*	-0.460 *-0.652*
$VLDL_2$ apoB (mg)	0.333 *0.695*	0.767** *0.282*	0.336 *0.047*	-0.276 *-0.499*	-0.398 *-0.126*	0.444 *0.293*	0.109 *-0.128*	-0.663* *-0.817**
IDL apoB (mg)	0.616* *0.567*	0.342 *0.235*	0.220 *0.295*	-0.215 *-0.644*	-0.176 *0.119*	0.174 *0.163*	-0.066 *0.041*	-0.656* *-0.609*
LDL apoB (mg)	0.734** *0.735*	0.346 *0.583*	-0.024 *0.672*	-0.103 *-0.523*	-0.430 *-0.041*	0.431 *-0.413*	-0.060 *0.310*	-0.487 *-0.628*
Total apoB (mg)	0.895** *0.907**	0.373 *0.481*	-0.103 *0.089*	-0.046 *-0.372*	-0.395 *0.078*	0.345 *0.036*	-0.264 *0.006*	-0.569* *-0.596*

Normal group - top line of each row, Mixed HLP - italics, bottom line of each row, PROD = production rate, FDC = fractional rate of direct catabolism, FTR = fractional transfer rate, NS = not significant, FCR = fractional catabolic rate, Mixed HLP = mixed hyperlipidaemic subjects.
** = $p<0.05$, ** = $p<0.005$ on regression analysis (NB Uncorrected for multiple comparison).*

Table 6.7b ApoB Pool Size Correlations (continued).

Pools - normal *mixed*	IDL PROD (mg/d)	IDL FDC (pool/d)	IDL FTR (pool/d)	LDL PROD (mg/d)	LDL FCR (pool/d)	Total PROD (mg/d)
$VLDL_1$ apoB (mg)	-0.338 *-0.552*	0.498 *-0.806**	-0.568* *-0.365*	-0.111 *0.151*	-0.381 *-0.793**	0.284 *0.579*
$VLDL_2$ apoB (mg)	-0.067 *0.419*	0.197 *-0.525*	-0.626* *-0.818**	0.101 *0.001*	-0.100 *-0.692*	0.442 *0.214*
IDL apoB (mg)	0.377 *0.350*	-0.091 *-0.422*	-0.691** *-0.885**	0.121 *0.188*	-0.054 *-0.610*	0.321 *0.489*
LDL apoB (mg)	0.033 *-0.235*	-0.243 *-0.606*	-0.463 *-0.673*	-0.128 *0.332*	-0.433 *-0.812**	0.106 *0.687*
Total apoB (mg)	-0.086 *0.156*	-0.400 *-0.372*	-0.425 *-0.581*	-0.076 *-0.062*	-0.373 *-0.508*	0.023 *0.140*

Table 6.8a Production Rate Correlations - Pearson correlation coefficients (r) for normal and mixed HLP subjects.

PROD - normal *mixed*	Total chol. (mmol/l)	Total trig. (mmol/l)	$VLDL_1$ PROD (mg/d)	$VLDL_1$ FDC (pool/d)	$VLDL_1$ FTR (pool/d)	$VLDL_2$ PROD (mg/d)	$VLDL_2$ FDC (pool/d)	$VLDL_2$ FTR (pool/d)
$VLDL_1$ PROD (mg/d)	-0.336 *0.202*	0.079 *0.507*	_____	0.405 *-0.016*	0.262 *-0.125*	0.078 *-0.805**	0.664** *0.401*	0.185 *-0.144*
$VLDL_2$ PROD (mg/d)	0.051 *-0.269*	0.107 *-0.689*	0.078 *-0.805**	0.352 *-0.226*	-0.364 *0.122*	_____	0.213 *-0.498*	-0.093 *-0.103*
IDL PROD (mg/d)	-0.133 *-0.052*	-0.544* *-0.233*	0.414 *-0.583*	0.427 *-0.259*	0.482 *0.290*	-0.105 *0.782**	0.315 *-0.053*	0.163 *-0.163*
LDL PROD (mg/d)	0.049 *0.084*	-0.056 *0.527*	-0.043 *0.547*	-0.219 *-0.162*	0.257 *0.314*	-0.355 *-0.464*	-0.117 *0.871**	-0.210 *-0.050*
Total PROD (mg/d)	-0.244 *0.169*	-0.040 *0.441*	0.842** *0.946***	0.509 *-0.127*	0.236 *-0.040*	0.326 *-0.609*	0.661* *-0.414*	0.084 *-0.229*

Normal group - top line of each row, Mixed HLP - italics, bottom line of each row, PROD = production rate, FDC = fractional rate of direct catabolism, FTR = fractional transfer rate, NS = not significant, FCR = fractional catabolic rate, Mixed HLP = mixed hyperlipidaemic subjects.
** = $p<0.05$, ** = $p<0.005$ on regression analysis (NB Uncorrected for multiple comparison).*

Table 6.8b Production Rate Correlations (continued).

PROD - normal *mixed*	IDL PROD (mg/d)	IDL FDC (pool/d)	IDL FTR (pool/d)	LDL PROD (mg/d)	LDL FCR (pool/d)	Total PROD (mg/d)
$VLDL_1$ PROD (mg/d)	0.414 *-0.583*	0.852 *-0.409*	-0.062 *-0.370*	-0.043 *0.547*	0.418 *-0.500*	0.842** *0.946***
$VLDL_2$ PROD (mg/d)	-0.105 *0.782**	0.051 *0.147*	-0.022 *-0.067*	-0.355 *-0.464*	-0.204 *0.247*	0.326 *-0.609*
IDL PROD (mg/d)	_____	0.199 *0.371*	-0.048 *-0.456*	0.291 *0.100*	0.631* *0.070*	0.598* *-0.296*
LDL PROD (mg/d)	0.291 *0.100*	0.075 *0.263*	-0.242 *-0.491*	_____	0.423 *-0.360*	0.226 *0.653*
Total PROD (mg/d)	0.598* *-0.296*	0.702** *-0.359*	-0.130 *-0.606*	0.226 *0.653*	0.528* *-0.552*	_____

One correlation that remained highly significant in both groups was $VLDL_1$ apoB production as a predictor of total apoB production. For this reason, the relationships of $VLDL_1$ production with total triglyceride, $VLDL_1$ apoB pool size, LDL apoB pool size, LDL fractional catabolic rate and total apoB production were explored further by drawing regression plots. A question that must be raised when comparing these two groups is whether they are in fact two different populations or merely a continuum of the same separated by an arbitrary cut-off (cholesterol 6.0mmol/l, triglyceride 2.0mmol/l)? Given the small sample size of the two groups this question is difficult to answer. However, regression plots were drawn for the parameters mentioned above, firstly on each group separately and then on the two groups joined. Finally, four extra subjects whose lipid levels overlapped both groups - cholesterol >6.0mmol/l but triglyceride <2.0mmol/l - were included in the total group. These 'intermediate' subjects (Table 6.9) were added to bridge the gap between the normolipidaemic and mixed HLP groups. Regression plots were then drawn for the complete group. The findings are summarised in Table 6.10. All the r values were stronger in the mixed hyperlipidaemic subjects than in the normal subjects. When the two groups were combined the r values for $VLDL_1$ production versus both triglyceride and $VLDL_1$ pool size increased whilst those for LDL pool size and LDL fractional catabolic rate decreased. This perhaps reflects the stronger relationship between $VLDL_1$ production and apoB metabolism in the mixed HLP group. The addition of the four 'intermediate' subjects did not improve the regression fits and in fact worsened them suggesting that these subjects had no influence on the slope of the regression. The regression plot for $VLDL_1$ production v total apoB production in the normal plus mixed HLP subjects is shown in Figure 6.2.

Table 6.9 'Intermediate' Subjects - Lipid Levels.

'Intermediate' Subject	Cholesterol (mmol/l)	Triglyceride (mmol/l)
1.	6.36	1.39
2.	6.28	1.95
3.	6.95	1.55
4.	6.95	1.60

Table 6.10 r (r^2) values for $VLDL_1$ Production v Variables - all groups.

$VLDL_1$ Production v... (mg/d)	Normal Subjects (n=15)	Mixed HLP Subjects (n=7)	Normal + Mixed HLP Subjects (n=22)	Normal + Mixed + Intermediate Subjects (n=26)
Triglyceride (mmol/l)	0.077 (0.006)	0.507 (0.257)	0.597 (0.357)	0.545 (0.297)
$VLDL_1$ apoB pool (mg)	0.475 (0.225)	0.690 (0.476)	0.704 (0.496)	0.654 (0.428)
LDL apoB pool (mg)	-0.024 (0.000)	0.672 (0.452)	0.587 (0.345)	0.532 (0.283)
LDL FCR (pool/d)	0.418 (0.175)	-0.500 (0.25)	-0.217 (0.047)	-0.105 (0.011)
Total apoB Production (mg/d)	0.842 (0.709)	0.946 (0.895)	0.866 (0.750)	0.863 (0.745)

4. LDL Subfraction Profiles (Table 6.11).

All seven subjects had a minority of LDL in the large, least dense fraction (LDLI). In six subjects the majority of LDL was divided equally between LDLII and LDLIII. Subject LB had mostly small, dense LDL (LDLIII). Of note this subject was the only one with the apoE 4/4 phenotype. Figure 6.3 shows the LDL subfraction profiles of subjects JC and LB, JC with both LDLII and LDLIII and LB with mostly LDLIII.

Table 6.11 LDL Subfraction Profiles (%) - mixed HLP subjects.

Subject	% LDLI (d=1.025-1.034g/ml)	% LDLII (d=1.034-1.044g/ml)	% LDLIII (d=1.044-1.060g/ml)
RA	16	44	40
PS	16	40	44
JC	12	47	41
RS	7	40	53
DM	13	41	46
LB	2	26	72
PC	20	30	50
Mean (SEM)	**12 (2.3)**	**38 (2.8)**	**49 (4.2)**

6.5 Discussion.

The seven subjects in this study consisted of six males and one post-menopausal female (LB). At first glance subject LB would appear out of keeping with the rest of the group on three accounts - her sex, her elevated body mass index (BMI) of greater than 30, and her apoE 4/4 phenotype. The remaining subjects had BMI's below 30 and apoE phenotypes of either 3/3 or 4/3. However, analysis of the results with this subject excluded caused no significant change to the findings. Her apoE 4/4 phenotype only appeared relevant in analysis of the LDL subfraction profiles.

In this study some findings were in keeping with previous work on FCHL but some were not. It is important to stress that the seven mixed hyperlipidaemic (HLP) subjects here were not classified as FCHL and may in fact have dyslipidaemia of a different aetiology. However, the majority of kinetic work on subjects with this type of lipid profile that is available for comparison has been performed in FCHL.

By virtue of selection, the mixed HLP and normal subjects did not overlap in their total cholesterol and triglyceride levels. In addition, the mixed HLP subjects had lower HDL cholesterol levels as is often seen with triglyceride elevations. The apoB levels were elevated in keeping with previous work on FCHL subjects. Compositional analysis revealed increased triglyceride:protein and cholesterol:protein ratios in $VLDL_1$. This is in contrast to previous work in FCHL subjects where the composition of VLDL has been reported as normal. However, these studies did not separate VLDL into the triglyceride-rich $VLDL_1$ and the less triglyceride-rich $VLDL_2$. An interesting finding was that of an increased free cholesterol:phospholipid ratio in $VLDL_1$ in the

mixed HLP subjects. The amount of free cholesterol in the phospholipid lattice of the lipoprotein surface is largely responsible for the surface fluidity. Increasing amounts of free cholesterol in the surface make the lattice more rigid and so reducing the amount of protein that is able to insert into the lattice, in particular apoCII. Since lipoprotein lipase requires apoCII as a cofactor for lipoplysis, an increase in $VLDL_1$ free cholesterol could lead to a reduction in particle catabolism. A reduced rate of direct catabolism of $VLDL_1$ was in fact seen in the mixed HLP group. Reduced lipoprotein lipase activity has been previously reported in a subset of individuals with FCHL (Babirak et al 1992).

Total apoB production, in particular that of $VLDL_1$, in the mixed HLP group was elevated. This concurs with the increased hepatic production of total VLDL apoB seen by other workers (Janus et al 1980, Kissebah et al 1981, Cortner et al 1991, Venkatesan et al 1993). In our subjects the increase was seen in the larger, triglyceride-rich $VLDL_1$ rather than in the smaller $VLDL_2$ as might have been expected by the moderately elevated plasma cholesterol (Gaw et al 1995). However, the mean plasma triglyceride level in the study of Gaw et al was lower than in our subjects. Contrary to the findings of Janus et al (1980) and Kissebah et al (1981), the transfer of apoB down the delipidation chain from $VLDL_1$ to LDL in this study was reduced. Kissebah suggested that in FCHL there was increased production of a small, dense VLDL which was rapidly 'metabolically channelled' to LDL, hence explaining the increased levels of apoB seen in these individuals. The reduced transfer of apoB from VLDL to LDL seen in our study is more in keeping with the large, triglyceride-rich nature of the VLDL that is produced in increased amounts as $VLDL_1$. This lipoprotein species is only slowly converted to LDL (Griffin and Packard 1994).

Comparison of the correlations in the normal and mixed HLP groups raises three points. Firstly, the predominant lipid within the plasma, i.e. cholesterol or triglyceride, is reflected in the apoB pool size of each lipoprotein - VLDL pool size is greater when triglyceride is relatively abundant whilst IDL and LDL pool sizes are larger when more cholesterol is present. Secondly, the apoB pool sizes and fractional transfer rates are negatively correlated in such a way to suggest that either enlarged pool sizes impede the passage of apoB down the delipidation chain, or that delayed passage of the apoB causes the enlarged pool sizes. Thirdly, although in both groups the major determinant of total apoB production is $VLDL_1$, the main difference between the groups appears to lie in the reciprocal relationship between $VLDL_1$, $VLDL_2$ and IDL production rates seen in the mixed HLP group with the resultant effect that this has on $VLDL_1$ apoB pool size, plus the negative relationship seen between LDL catabolism and the pool sizes of $VLDL_1$ and LDL. In the mixed hyperlipidaemic group the production of $VLDL_1$ appears to determine both its own pool size and that of LDL, whilst having a negative effect on the production of $VLDL_2$ and IDL. This, in keeping with the positive correlation between $VLDL_1$ and triglyceride, suggests that these individuals preferentially produce $VLDL_1$, a triglyceride-rich particle which is slowly metabolised and adds to the size of the LDL pool. The correlations between LDL catabolism and $VLDL_1$ and IDL apoB pool sizes could reflect both the delayed metabolism of particles originating as $VLDL_1$ and the type of LDL that results i.e. $VLDL_1$ is predominantly metabolised to small, dense LDL (LDLIII) which is only slowly cleared from the plasma.

The LDL subfraction profiles of the mixed HLP subjects showed both LDLII and LDLIII, as compared to normal males and post-menopausal females who mostly have LDLII alone (Caslake 1996). The smaller, denser LDL subfractions are cleared more slowly from the plasma and this in turn is reflected in the reduced LDL fractional catabolic rate seen here. The predominance of LDLIII in subject LB possibly reflects reduced clearance of LDL precursors on account of the apoE 4/4 phenotype.

The regression analyses were perhaps inconclusive when trying to determine whether the normal and mixed HLP groups were in fact different or merely a continuum of the same. However, the fact that the addition of a further group of subjects with elevated cholesterol levels but normal triglyceride levels did not improve the regression fits might suggest that it is triglyceride rather than cholesterol that has the main influence on apoB metabolism in this sort of individual. In the seven mixed HLP subjects the metabolism of apoB can be summarised as follows. An increased amount of apoB is being secreted by the liver, primarily in the form of $VLDL_1$. This $VLDL_1$ is only slowly cleared from the plasma and relatively slowly delipidated to $VLDL_2$, IDL and LDL. As a result of the increased production, reduced clearance and reduced transfer to LDL there is an increase in all of the lipoprotein pool sizes. This is seen clinically as an increase in plasma cholesterol and triglyceride levels. Due to metabolic channelling, $VLDL_1$ is delipidated to form mainly the small, dense subfractions of LDL. This LDL is in turn only slowly catabolised, further exacerbating the enlarged LDL pool size and adding to the elevated total plasma apoB seen in these subjects. That triglyceride can have such bearing on apoB metabolism has been reported before. Workers have found different levels of triglyceride at which the metabolism appears to change - 1.1mmol/l (Austin et al 1990), 1.3mmol/l (Tan et al 1995) and 1.6mmol/l (Superko and Krauss 1992).

Thus, the kinetics of apoB in these seven mixed hyperlipidaemic subjects showed some interesting differences from normolipaemic subjects and both some similarities and differences with FCHL. The apoB metabolism in these subjects appears to be more a consequence of triglyceride and the production of relatively triglyceride-enriched VLDL, with the resultant delayed delipidation and catabolism. The kinetic findings as compared to normal are summarised in Figure 6.4.

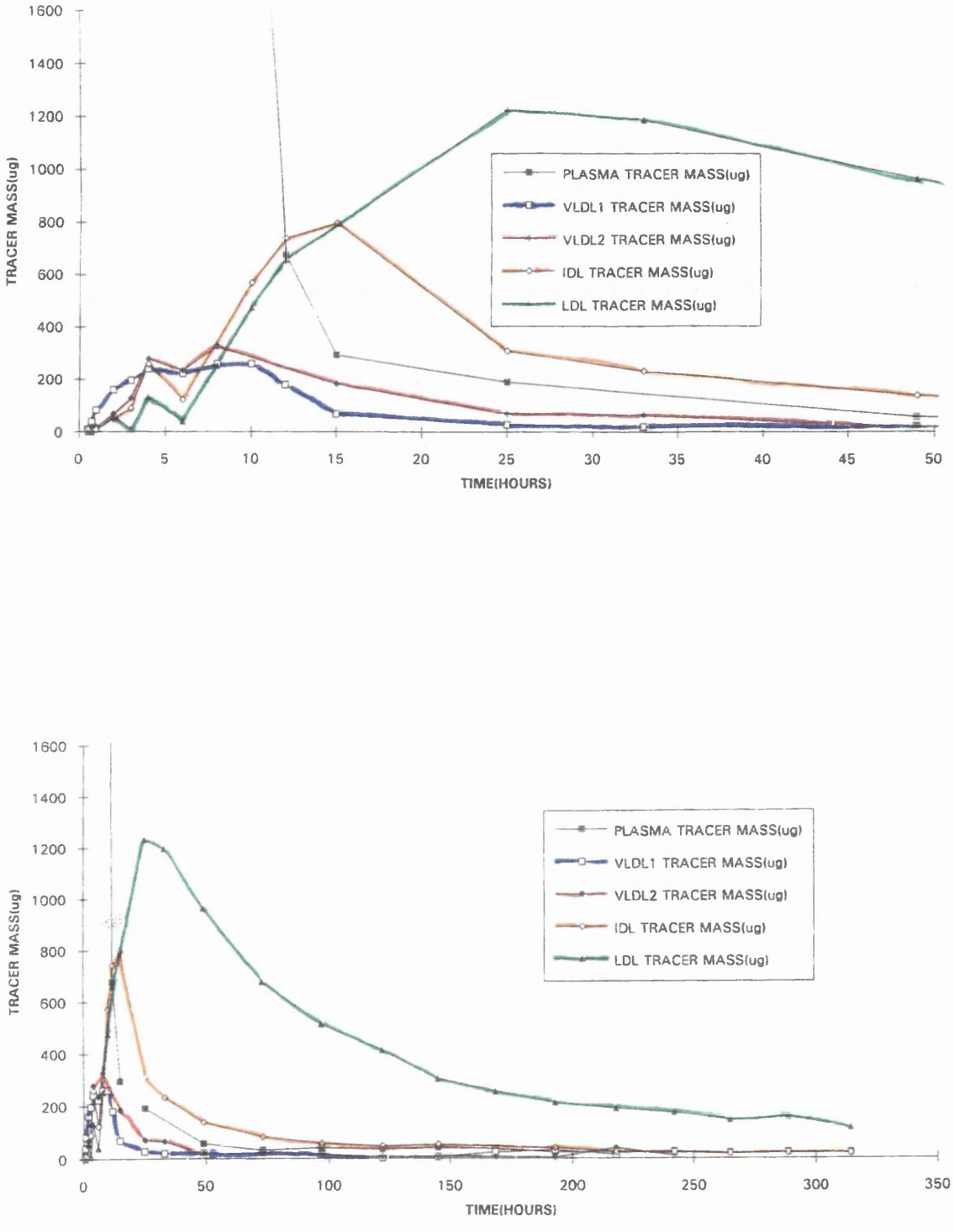


Figure 6.1a Tracer Mass Curves for a Normal Subject (0-50 & 0-350 hours).

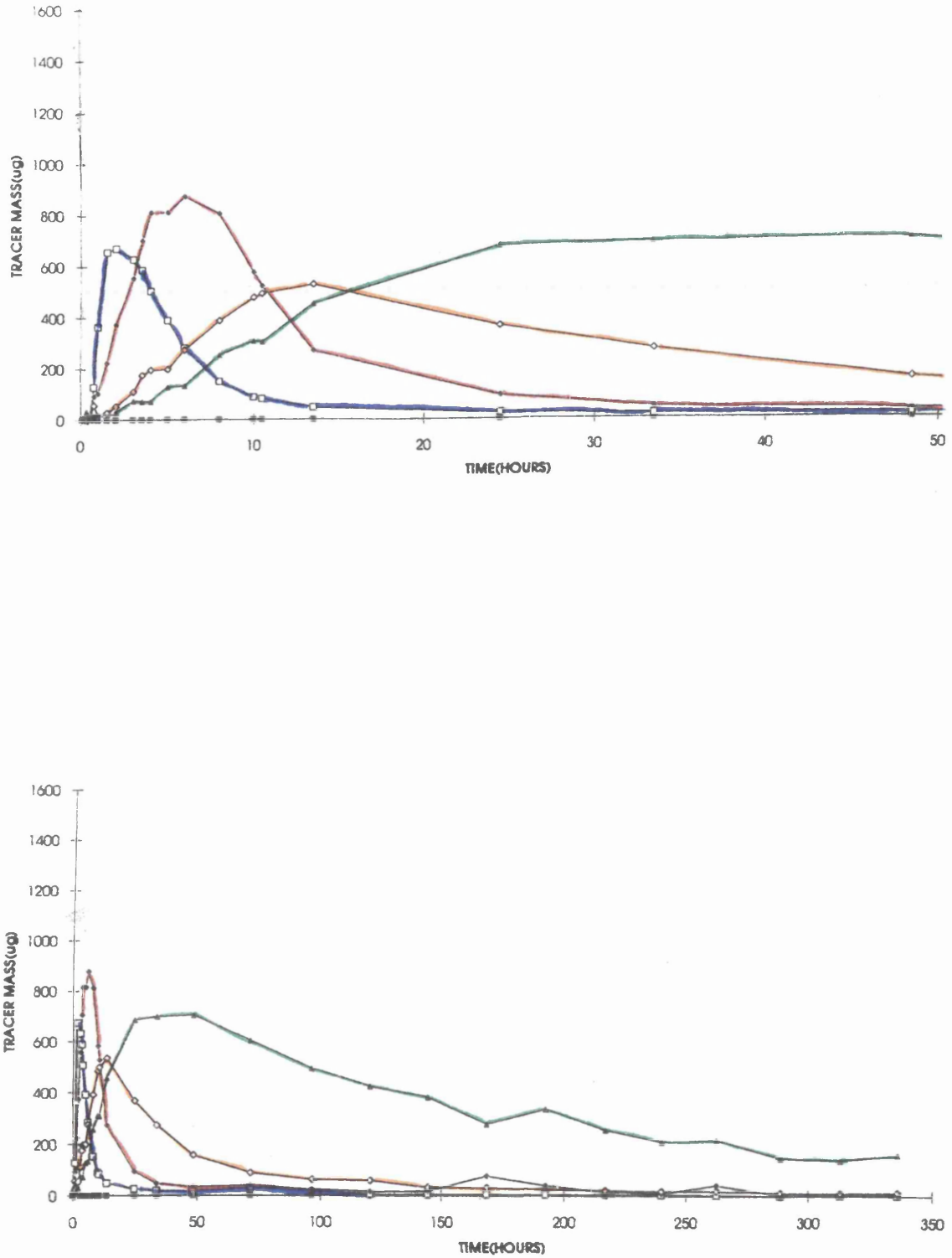
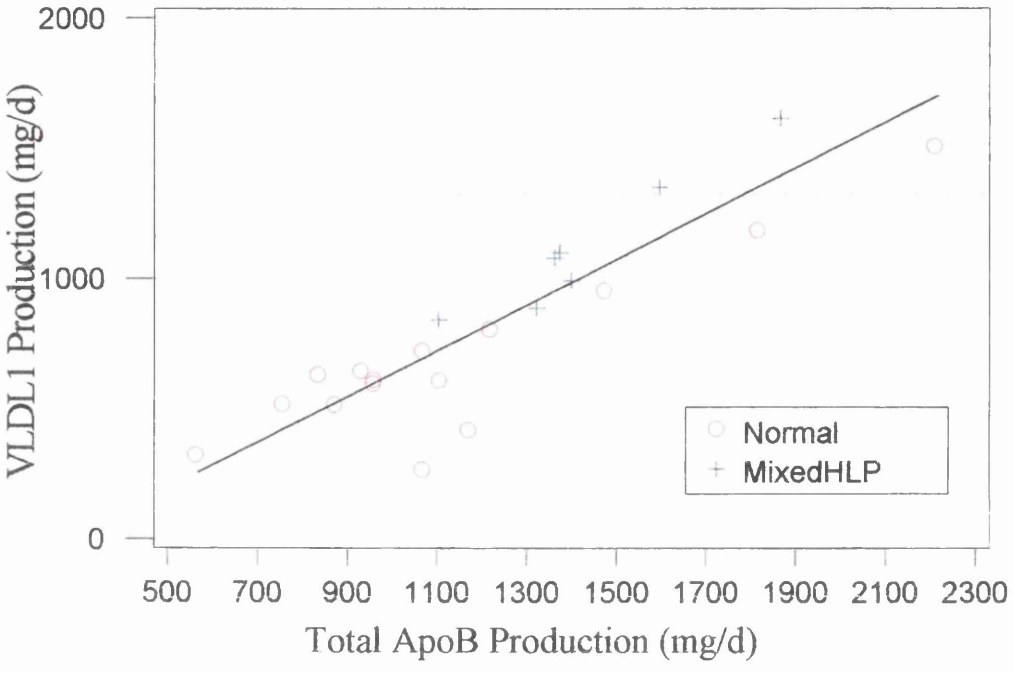


Figure 6.1b Tracer Mass Curves for a Mixed Hyperlipidaemic Subject (0-50 & 0-350 h).

Figure 6.2 VLDL1 Production v Total ApoB Production



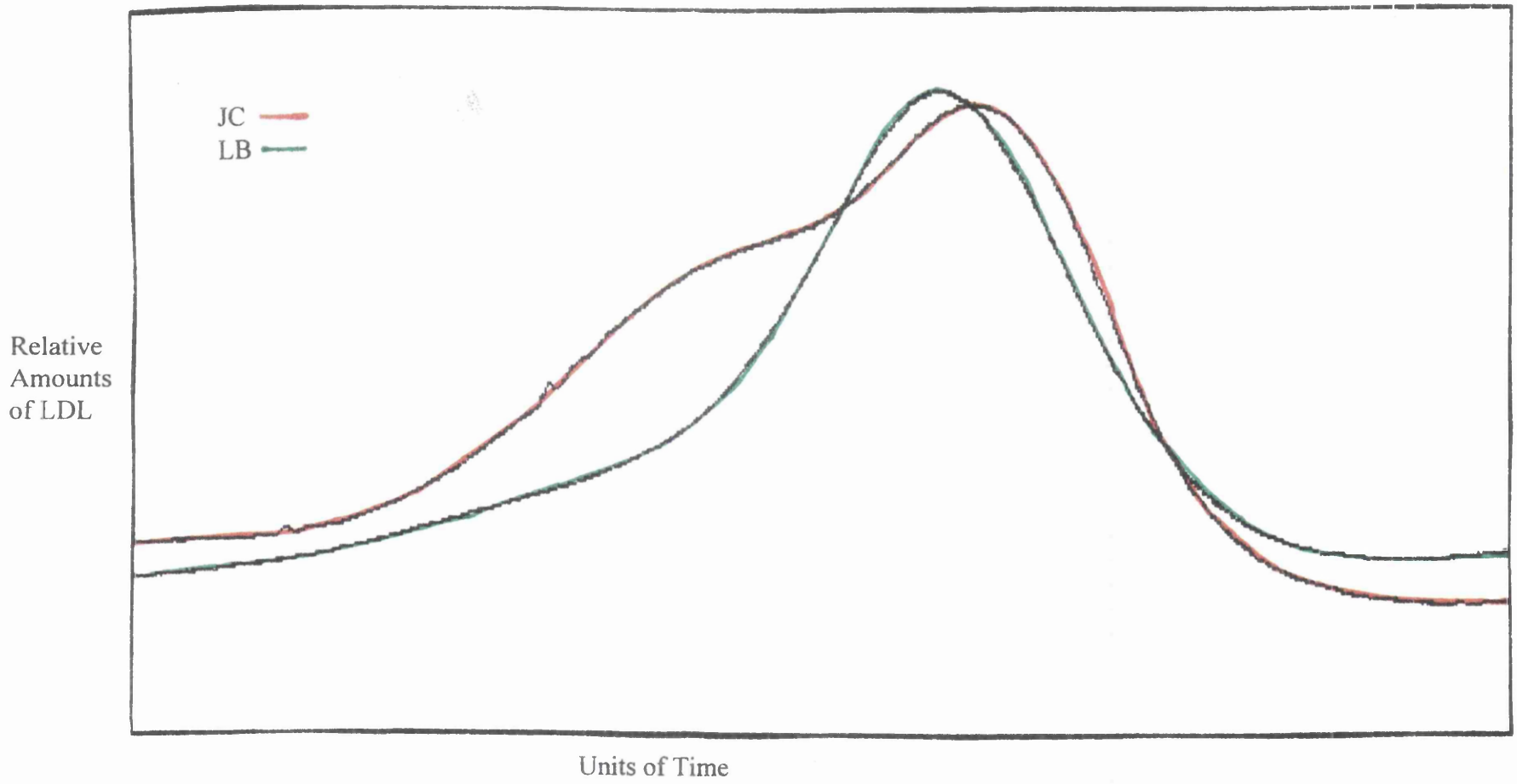


Figure 6.3 LDL Subfraction Profiles of Subjects JC and LB.

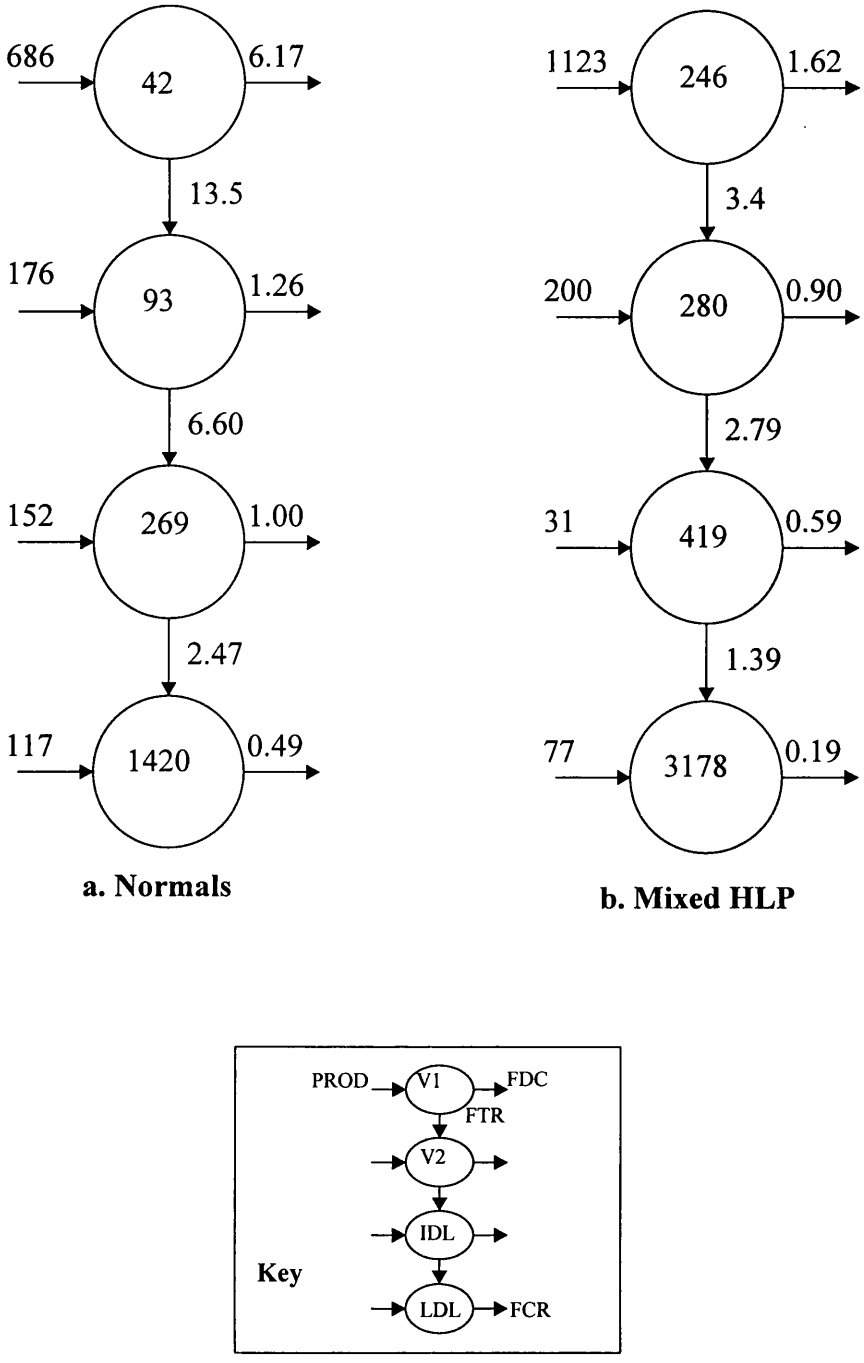


Figure 6.4 Pool Sizes and Kinetic Parameters - a. Normal and b. Mixed HLP Subjects (mean values).

Prod = production rate (mg/d), FDC = fractional rate of direct catabolism (pool/d), FTR = fractional transfer rate (pool/d), FCR = fractional catabolic rate (pool/d), V1 = $VLDL_1$, V2 = $VLDL_2$, IDL & LDL apoB pool sizes (mg).

Chapter 7 The Treatment of Mixed Hyperlipidaemia :- Mechanisms of Action of Simvastatin and Atorvastatin, a New HMG-CoA Reductase Inhibitor.

7.1 Introduction.

Mixed hyperlipidaemia can be relatively difficult to treat. Standard practice is to use one of the fibrate class of drugs or nicotinic acid, both of which are more effective at lowering triglyceride elevations alone rather than mixed elevations of cholesterol and triglyceride. More recently combination therapy with a statin plus a fibrate has been used for cases not responsive to a fibrate alone. Whilst this combination is effective there is an increased risk of side effects, in particular myopathy, and a likelihood of reduced patient compliance due to the increased number of drugs being taken. To date statins alone have proved less beneficial for mixed hyperlipidaemia. However, atorvastatin, a new member of the statin class, is reputed to be effective at lowering both cholesterol and triglyceride levels.

The 3-hydroxy-3-methylglutaryl Coenzyme A (HMG-CoA) reductase inhibitors, or statins, are a class of lipid-lowering drug derived originally from fungal metabolites (Endo et al 1976). Through their competitive inhibition of the rate limiting step in cholesterol synthesis (HMG-CoA conversion to mevalonate) they have proved efficacious in the lowering of plasma total cholesterol and LDL cholesterol. The mechanism of action responsible for this cholesterol lowering has been reported as an up-regulation of the LDL receptors, secondary to a reduced intrahepatic free cholesterol pool, thus leading to increased clearance of LDL from the plasma (Bilheimer et al 1983, Malmendier et al 1989). Other workers have found additional explanations for the cholesterol-lowering effect of statins, including reduced hepatic production of apoB-containing lipoproteins (Watts et al 1995), increased catabolism of LDL precursors (Gaw et al 1993), and reduced direct synthesis of LDL (Gaw et al 1996).

Atorvastatin is one of the more recently developed of the statins and has a good safety profile in keeping with others in this class. It has been shown to be at least as potent as the others, with dose related reductions in plasma LDL cholesterol of 25% to 60% at doses of 2.5mg to 80mg per day (Nawrocki et al 1995). It also has a significant triglyceride lowering effect, achieving reductions of up to 45% on maximum therapy of 80mg per day in patients with primary hypertriglyceridaemia (Bakker-Arkema et al 1996). It is, therefore, possible that this drug may be useful in the treatment of mixed hyperlipidaemia. The aim of this study was to characterise the effect of atorvastatin on the kinetics of apoB in a group of subjects with mixed hyperlipidaemia, and to compare this to the effect of simvastatin in the same group of subjects.

7.2 Subject Selection.

The characteristics of the seven subjects used in this study are described in Chapter 6. All subjects underwent a baseline turnover, as described in the previous chapter, but two subjects withdrew from the study after completing only one treatment phase, one following treatment with simvastatin and one following treatment with atorvastatin. There were no serious adverse reactions to either drug. Each subject gave signed informed consent and the study was approved by the Ethical Committee of Glasgow Royal Infirmary.

7.3 Methods.

The subjects underwent three stable isotope turnover studies of apoB kinetics using a bolus of tri-deuterated leucine at a dose of 7mg/kg body weight. The methodology for the turnovers was the same on each occasion, as previously described in Chapter 2. Following the baseline turnover, subjects were randomised to receive either simvastatin 40mg per day or atorvastatin 40mg per day, both taken last thing at night. Each treatment phase lasted eight weeks after which time subjects returned for a second turnover, continuing on their current therapy for the two week turnover period. They were then switched onto the alternative therapy without a washout period. The study was a randomised cross-over trial, but not blinded. Lipid and lipoprotein measurements were taken at the beginning and end of each turnover but at no point during the eight week treatment phases. Liver function tests and creatine kinase levels were monitored throughout but no subject showed elevations in these parameters. Twenty-four hour medical contact was available to each subject for advice regarding possible side effects, but in the main this was not required. Subjects were asked not to change any aspect of their lifestyles during the study, in particular not to lose or gain large amounts of weight. Three day diet diaries were completed by the subjects during the two weeks of each turnover and analysed, courtesy of the Department of Dietetics and Nutrition in this hospital, to ensure consistency in the dietary composition from one turnover to the next. Each subject's general practioner was informed of his patient's involvement in the study and given a summary of the patient's response to the two treatments at the end. Finally, the further management of each subject's hyperlipidaemia was arranged prior to his discharge from the study.

7.4 Results.

1. Lipids and Lipoproteins.

Following treatment with both simvastatin and atorvastatin there were significant reductions ($p<0.05$) in total cholesterol, total triglyceride, LDL cholesterol and VLDL cholesterol. In most cases, the percentage reduction by atorvastatin was greater than that by simvastatin (Table 7.1), although two subjects (PS and JC) showed a marginally greater reduction in triglyceride whilst on simvastatin rather than atorvastatin. For both drugs HDL cholesterol showed an increase although this did not reach significance in either case. There was a significant decrease in plasma apoB on both drugs but no change in apoAI, Lp(a), HDL_2 or HDL_3 levels.

Table 7.1 Lipids and Lipoproteins - baseline and treatment phases.

Subject	Turnover	Cholesterol (mmol/l)	Triglyceride (mmol/l)	VLDL-chol (mmol/l)	LDL-chol. (mmol/l)	HDL-chol. (mmol/l)
RA	Baseline	6.20	2.90	0.85	3.95	1.40
	Simvastatin	4.50	2.10	0.55	2.50	1.45
	Atorvastatin	4.05	1.80	0.55	2.10	1.40
PS	Baseline	6.45	2.60	0.80	4.60	1.05
	Simvastatin	3.95	1.70	0.65	2.25	1.05
	Atorvastatin	3.45	1.90	0.60	1.80	1.05
JC	Baseline	6.40	2.25	0.75	4.70	0.95
	Simvastatin	4.40	1.35	0.60	2.70	1.10
	Atorvastatin	3.80	1.55	0.60	2.20	1.05
RS	Baseline	6.35	2.60	1.15	4.55	0.70
	Simvastatin	3.20	1.70	0.60	1.90	0.70
	Atorvastatin	-	-	-	-	-
DM	Baseline	7.90	3.55	1.90	5.10	0.90
	Simvastatin	-	-	-	-	-
	Atorvastatin	4.05	1.95	0.70	2.20	1.10
LB	Baseline	6.60	2.95	1.25	4.45	0.90
	Simvastatin	4.60	2.40	1.00	2.60	1.00
	Atorvastatin	4.15	1.60	0.55	2.50	1.10
PC	Baseline	7.00	3.60	1.45	4.60	0.95
	Simvastatin	4.65	2.50	0.90	2.85	0.90
	Atorvastatin	4.15	2.45	1.00	2.25	0.95
Mean	Baseline	6.70	2.92	1.16	4.56	0.98
	Simvastatin	4.22**	1.96**	0.72*	2.47**	1.03
	Atorvastatin	3.95**	1.88**	0.67*	2.18**	1.11
% Change	Simvastatin	37%	33%	38%	46%	6%
	Atorvastatin	41%	36%	43%	52%	13%

** = $p < 0.05$, ** = $p < 0.005$, % change = % increase or decrease from baseline.*

Compositional analysis revealed some differences between the two treatment phases (Table 7.2). $VLDL_1$ showed an increase in the triglyceride content on both drugs, but atorvastatin also caused a reduction in $VLDL_1$ free cholesterol whilst simvastatin did not. $VLDL_2$ had a lower esterified cholesterol content on both drugs, whereas IDL only had lower esterified cholesterol on atorvastatin. LDL showed reduced free cholesterol on both drugs, with an increase in esterified cholesterol on atorvastatin. Thus, atorvastatin appears to have a more extreme effect than simvastatin in lowering the cholesterol content of the lipoproteins. Of note, there were no significant differences in the direct comparisons of simvastatin with atorvastatin.

Table 7.2 Subfraction Compositions - mean values (SEM), baseline and treatment phases.

Sub-fraction	Turnover	%protein	%free cholesterol	%esterified cholesterol	%tri-glyceride	%phos-pholipid
$VLDL_1$	Baseline	7.1 (0.4)	3.4 (0.3)	13 (0.9)	61 (1.1)	16 (0.2)
	Simvastatin	7.5 (0.3)	2.6 (0.4)	10 (0.9)	65 (0.7)*	15 (0.9)
	Atorvastatin	7.8 (0.2)	2.2 (0.3)*	11 (0.5)	65 (1.1)*	14 (0.7)
$VLDL_2$	Baseline	13 (0.80	4.2 (0.3)	26 (2.1)	36 (2.4)	20 (0.5)
	Simvastatin	14 (0.7)	3.9 (0.4)	20 (1.6)*	42 (1.4)	19 (1.6)
	Atorvastatin	15 (0.4)	3.7 (0.4)	21 (1.2)*	41 (1.8)	18 (0.4)
IDL	Baseline	19 (1.1)	4.8 (0.3)	43 (1.3)	11 (1.3)	21 (0.7)
	Simvastatin	20 (0.7)	4.4 (0.4)	38 (2.4)	16 (1.9)	22 (1.6)
	Atorvastatin	21 (0.6)	4.3 (0.3)	39 (0.9)*	16 (1.5)	20 (0.4)
LDL	Baseline	25 (0.7)	7.0 (0.6)	43 (0.6)	4.9 (0.4)	20 (0.3)
	Simvastatin	24 (0.5)	4.4 (0.4)*	46 (1.3)	5.3 (0.6)	21 (0.6)
	Atorvastatin	25 (0.4)	4.6 (0.2)*	45 (0.7)*	5.3 (0.3)	20 (0.4)

** = $p < 0.05$, ** = $p < 0.005$, SEM = standard error of the mean.*

2. ApoB Pool Sizes.

Neither drug caused a significant change to the $VLDL_1$ apoB pool size. $VLDL_2$ and IDL pool sizes were significantly ($p<0.05$) reduced by both drugs, whilst the LDL pool sizes showed highly significant reductions on both drugs ($p<0.005$). Both drugs also significantly reduced the total apoB pool (Table 7.3). Again there were no significant differences in the direct comparisons of simvastatin with atorvastatin.

Table 7.3 ApoB Pool Sizes - baseline and treatment phases.

Subject	Turnover	$VLDL_1$-B (mg)	$VLDL_2$-B (mg)	IDL-B (mg)	LDL-B (mg)	Total-B (mg/dl)
RA	Baseline	145	141	224	2236	93
	Simvastatin	123	149	224	2020	85
	Atorvastatin	137	77	160	1066	49
PS	Baseline	196	166	180	2528	106
	Simvastatin	131	141	353	1746	82
	Atorvastatin	151	148	243	1278	63
JC	Baseline	132	325	618	3276	156
	Simvastatin	84	146	251	1373	64
	Atorvastatin	159	163	227	1522	72
RS	Baseline	345	267	411	3323	121
	Simvastatin	101	138	181	1700	65
	Atorvastatin	-	-	-	-	-
DM	Baseline	328	477	651	3977	234
	Simvastatin	-	-	-	-	-
	Atorvastatin	147	189	281	1985	77
LB	Baseline	206	338	405	2785	128
	Simvastatin	99	226	193	1164	58
	Atorvastatin	69	139	254	1330	62
PC	Baseline	368	249	441	4118	142
	Simvastatin	322	212	258	2218	83
	Atorvastatin	233	229	166	1756	65
Mean	Baseline	246	280	419	3178	140
	Simvastatin	143	169*	243*	1703**	73*
	Atorvastatin	149	158*	222*	1490**	65*
%Change	Simvastatin	42%	40%	42%	46%	48%
	Atorvastatin	39%	41%	47%	53%	54%

** = $p < 0.05$, ** = $p < 0.005$, % change = % increase or decrease from baseline.*

3. Kinetic Analysis (Table 7.4).

Analysis of the kinetic results gave some unexpected findings, particularly in $VLDL_1$. The original hypothesis was that the reductions in plasma triglyceride levels would be due to reduced hepatic $VLDL_1$ apoB production. In fact, the converse appeared to happen. Initial inspection of the tracer mass curves before and after treatment (Figure 7.1) showed a change in the shape of the $VLDL_1$ curve with a higher peak value on treatment suggestive of an increase in $VLDL_1$ production and a steeper decline in the slope which would fit with an increased clearance rate. These observations were in fact borne out by the kinetic analysis. On atorvastatin there was a highly significant ($p=0.0032$) increase in $VLDL_1$ production. On simvastatin there was a tendency for $VLDL_1$ production to increase but this did not reach significance ($p=0.24$). All subjects showed increased $VLDL_1$ production on atorvastatin, whilst on simvastatin two showed a reduction in $VLDL_1$ production (RS and LB). The fractional rate of direct catabolism (FDC) of $VLDL_1$ increased significantly on atorvastatin ($p=0.042$) with all subjects showing an increase, whilst on simvastatin there was a trend towards an increased $VLDL_1$ FDC which did not quite reach significance ($p=0.08$), with one subject (RS) showing a reduced $VLDL_1$ FDC. (This subject was one of the two who also did not show increased $VLDL_1$ production on simvastatin). There was no significant effect of either drug on the fractional transfer rates of $VLDL_1$ apoB to $VLDL_2$ apoB. Neither simvastatin nor atorvastatin caused any significant difference to the kinetics of $VLDL_2$ or IDL apoB. On atorvastatin there was a highly significant increase in the fractional catabolic rate (FCR) of LDL ($p=0.0015$) with all patients showing this increase. This can be seen in the increased steepness of the LDL curve decline on treatment (Figure 7.2). On simvastatin there was a tendency for increased LDL FCR but this narrowly missed significance ($p=0.052$), with one patient (LB) showing no change in the FCR. These kinetic findings are illustrated in Figures 7.1 and 7.2. The complete sets of rate constants and calculated leucine pool sizes on each treatment regime are given in appendices 6 and 7.

Table 7.4a Kinetic Analysis - $VLDL_1$ and $VLDL_2$, baseline and treatment phases.

Subject	Turnover	$VLDL_1$ PROD (mg/d)	$VLDL_1$ FDC (pools/d)	$VLDL_1$ FTR (pools/d)	$VLDL_2$ PROD (mg/d)	$VLDL_2$ FDC (pools/d)	$VLDL_2$ FTR (pools/d)
RA	Baseline	1078	2.8	4.5	176	0.8	5.2
	Simvastatin	2736	18.0	4.0	568	1.3	5.8
	Atorvastatin	1760	9.0	3.5	98	0.5	6.9
PS	Baseline	841	1.8	2.5	207	0.5	3.3
	Simvastatin	1262	2.8	6.7	200	2.6	5.0
	Atorvastatin	1468	3.9	5.7	312	2.8	5.0
JC	Baseline	993	0.06	7.6	279	0.8	3.2
	Simvastatin	1774	12.0	9.1	259	1.2	5.8
	Atorvastatin	1556	6.0	3.8	383	0.02	5.6
RS	Baseline	1349	2.8	1.1	190	0.003	2.2
	Simvastatin	855	1.4	7.0	136	3.7	3.0
	Atorvastatin	-	-	-	-	-	-
DM	Baseline	1098	0.8	2.6	192	0.4	1.7
	Simvastatin	-	-	-	-	-	-
	Atorvastatin	1533	7.5	2.9	253	0.7	3.0
LB	Baseline	886	2.2	1.9	264	0.6	1.5
	Simvastatin	562	3.8	2.1	86	0.8	0.5
	Atorvastatin	1449	20.0	1.3	434	2.9	0.9
PC	Baseline	1615	0.9	3.5	93	3.2	2.4
	Simvastatin	2651	6.3	2.0	437	2.2	2.7
	Atorvastatin	1893	4.0	4.5	388	1.3	4.9
Mean (SEM)	Baseline	1123 (103)	1.62 (0.4)	3.39 (0.8)	200 (23)	0.9 (0.4)	2.8 (0.5)
	Simvastatin	1640 (372)	7.38 (2.6)	5.15 (1.2)	281 (76)	2.0 (0.4)	3.8 (0.9)
	Atorvastatin	1610 (72)**	8.40 (2.5)*	3.62 (0.6)	311 (50)	1.4 (0.5)	4.4 (0.9)

** = $p < 0.05$, ** = $p < 0.005$, SEM = standard error of the mean, PROD = production rate, FDC = fractional rate of direct catabolism, FTR = fractional transfer rate.*

Table 7.4b Kinetic Analysis - IDL and LDL, baseline and treatment phases.

Subject	Turnover	---------- PROD (mg/d)	----IDL----- FDC (pools/d)	---------- FTR (pools/d)	LDL PROD (mg/d)	LDL FCR (pools/d)
RA	Baseline	27	1.1	1.9	80	0.28
	Simvastatin	142	1.0	3.3	107	0.45
	Atorvastatin	59	1.0	3.0	39	0.45
PS	Baseline	11	0.6	3.0	43	0.22
	Simvastatin	46	0.2	1.9	31	0.41
	Atorvastatin	37	0.5	2.5	37	0.59
JC	Baseline	56	0.7	1.0	70	0.21
	Simvastatin	108	1.2	2.6	43	0.50
	Atorvastatin	60	1.9	3.0	20	0.44
RS	Baseline	10	0.2	1.2	47	0.17
	Simvastatin	34	0.5	1.7	101	0.25
	Atorvastatin	-	-	-	-	-
DM	Baseline	28	0.4	0.9	55	0.16
	Simvastatin	-	-	-	-	-
	Atorvastatin	147	0.4	2.1	147	0.37
LB	Baseline	79	0.6	0.8	93	0.16
	Simvastatin	86	0.8	0.2	119	0.14
	Atorvastatin	266	0.9	0.5	266	0.31
PC	Baseline	9	0.5	0.9	149	0.13
	Simvastatin	0	0.2	2.1	44	0.26
	Atorvastatin	0	2.6	4.4	0	0.35
Mean (SEM)	Baseline	31 (10)	0.6 (0.1)	1.4 (0.3)	77 (14)	0.19 (0.02)
	Simvastatin	69 (21)	0.6 (0.2)	2.0 (0.4)	74 (16)	0.34 (0.06)
	Atorvastatin	95 (40)	1.2 (0.4)	2.6 (0.5)	85 (42)	0.42 (0.04)**

** = $p < 0.05$, ** = $p < 0.005$, SEM = standard error of the mean, PROD = production rate, FDC = fractional rate of direct catabolism, FTR = fractional transfer rate, FCR = fractional catabolic rate.*

4. LDL Subfraction Profiles (Table 7.5).

On simvastatin there was a significant reduction in the concentrations of all three LDL subfraction species. On atorvastatin there was a significant reduction in LDLII and LDLIII concentrations, with a non-significant reduction in LDLI concentration. Analysis of the changes in percentage distribution of each subfraction in response to treatment gave some unexpected findings. Simvastatin caused no significant change to the percentage amounts of LDLI, LDLII and LDLIII, presumably due to its effect of equally lowering all three subfractions (the reductions of each subfraction were 60% LDLI, 49% LDLII and 56% LDLIII). Atorvastatin, on the other hand, showed a highly significant increase in the percentage of LDLII, with an associated but non-significant increase in LDLI, and a significant decrease in the percentage of LDLIII. Thus, on atorvastatin there appears to be a shift in the distribution of the LDL subfractions from a predominance of small, dense species (LDLIII) to an increased proportion of larger, less dense lipoproteins (LDLI). In conjunction with this, there is an overall reduction in the total amount of LDL, with the largest reduction being in LDLIII concentration (69%) and a smaller, non-significant reduction in LDLI concentration (27%). Figure 7.3 gives a representative illustration of the changes in the LDL subfraction profiles in one subject.

Table 7.5 LDL Subfraction Profiles - concentrations with percentages given in brackets, baseline and treatment phases.

Subject	Turnover	LDLI conc^n mg/dl (percentage)	LDLII conc^n mg/dl (percentage)	LDLIII conc^n mg/dl (percentage)
RA	Baseline	53 (16)	150 (44)	133 (40)
	Simvastatin	12 (37)	47 (37)	67 (53)
	Atorvastatin	20 (23)	50 (57)	18 (20)
PS	Baseline	56 (16)	136 (40)	147 (44)
	Simvastatin	30 (16)	108 (58)	49 (26)
	Atorvastatin	23 (13)	108 (61)	46 (26)
JC	Baseline	50 (12)	204 (47)	180 (41)
	Simvastatin	22 (9)	151 (63)	67 (28)
	Atorvastatin	27 (12)	100 (47)	87 (41)
RS	Baseline	24 (7)	132 (40)	178 (53)
	Simvastatin	14 (8)	70 (40)	92 (52)
	Atorvastatin	-	-	-
DM	Baseline	40 (13)	130 (41)	147 (46)
	Simvastatin	-	-	-
	Atorvastatin	35 (21)	71 (44)	58 (35)
LB	Baseline	6 (2)	99 (26)	278 (72)
	Simvastatin	7 (9)	19 (23)	56 (68)
	Atorvastatin	8 (4)	98 (50)	92 (46)
PC	Baseline	45 (20)	65 (30)	114 (50)
	Simvastatin	10 (7)	7 (5)	108 (78)
	Atorvastatin	58 (40)	67 (47)	16 (11)
Mean	Baseline	40 (12)	131 (38)	168 (49)
	Simvastatin	16* (10)	67* (39)	73** (51)
	Atorvastatin	28 (19)	82* (51)**	53** (30)*
% change	Simvastatin	60%	49%	56%
	Atorvastatin	27%	37%	69%

** = $p < 0.05$, ** = $p < 0.005$, % change = % increase or decrease from baseline.*

7.5 Discussion.

The reductions seen here in lipids and lipoproteins due to simvastatin and atorvastatin are in keeping with those seen in a multitude of other studies on the statin class of drugs, including the recent large 4S and WOSCOPS trials. In general, atorvastatin caused greater mean percentage reductions than simvastatin, with both drugs at a daily dose of 40mg, but the difference was not statistically significant - total cholesterol 41% v 37%, triglyceride 36% v 33%, VLDL cholesterol 43% v 38%, and LDL cholesterol 52% v 46%. The increase in HDL cholesterol has also been seen previously with the statins. In this study some subjects showed an increase in HDL cholesterol on treatment whilst some showed no change and thus the result was not statistically significant. In keeping with this was the lack of change in the apoAI levels and HDL subfractions. Compositional analysis showed a decrease in the cholesterol content of the lipoproteins in response to the statins, a finding which has been reported previously (Gaw et al 1993). Here atorvastatin appears to have a more significant effect than simvastatin which may reflect a slightly greater inhibition of HMG-CoA reductase such that the lipoproteins are being secreted by the liver with a lower cholesterol content. The increase in $VLDL_1$ triglyceride content was an unexpected finding but is in keeping with the observed kinetic changes, as will be discussed below.

Total apoB concentration was reduced by 48% on simvastatin and 54% on atorvastatin. The greatest reductions in each case were in LDL apoB pool sizes (46% and 53% respectively), but there were also substantial reductions in the apoB pools of $VLDL_2$ (40%, 41%) and IDL (42%, 47%). Again atorvastatin caused the greater reductions but the difference from simvastatin was not significant. Of note, neither drug caused a significant reduction in $VLDL_1$ apoB pool size but again this is in keeping with the kinetic observations.

A highly unexpected finding in this study was the significant increase in $VLDL_1$ production rate of 43% that was seen on atorvastatin. This was contrary to the original hypothesis that atorvastatin would lower plasma triglyceride levels by a reduction in VLDL production. This reduced VLDL production has been previously reported in a kinetic study of simvastatin (Watts et al 1995). However, the study of Watts et al did not distinguish between the two subfractions of VLDL and used a monoexponential function for the analysis which is perhaps not entirely appropriate when modelling the kinetics of a heterogeneous class of lipoproteins such as VLDL. Of note was the fact that in the current study the majority of the subjects also showed an increase in $VLDL_1$ production on simvastatin but two showed a decrease and so there was no significant change overall. The fractional rate of direct clearance of $VLDL_1$ whilst on atorvastatin was also significantly increased with the resultant effect of no change in the $VLDL_1$ apoB pool size. Simvastatin largely caused a similar effect on $VLDL_1$ direct catabolism but again the increase was not significant. However, there was also no significant change in the $VLDL_1$ apoB pool size on this drug. Increased catabolism of VLDL has been noted before in subjects with mixed hyperlipidaemia, in this instance following treatment with pravastatin (Parhofer et al 1993).

The unexpected increase in $VLDL_1$ production could possibly be explained if there existed a hepatic VLDL receptor. If this receptor, in addition to the LDL receptor, is up-regulated in response to reduced intrahepatic cholesterol there will be increased clearance of $VLDL_1$, as was reflected in the increased $VLDL_1$ fractional rate of direct catabolism. (Atorvastatin has proved effective in homozygous familial hypercholesterolaemia, i.e. subjects with practically no LDL receptors available for up-regulation, (Marais, unpublished observation) and so the LDL receptor is not necessarily responsible for increased $VLDL_1$ clearance). Increased clearance of these triglyceride-rich lipoproteins will result in an increased triglyceride load being returned to the liver. Here the apoB is degraded and the triglyceride becomes available for re-secretion with newly synthesised apoB. The neutral lipid content of the hepatocyte has been suggested to determine the amount of post-translational apoB that is secreted relative to the amount that is degraded. This in turn could stimulate the production of triglyceride-rich lipoproteins i.e. $VLDL_1$, hence the observed increased production rate of $VLDL_1$. The finding of increased $VLDL_1$ triglyceride in the compositional analysis adds weight to this concept of 'futile cycling' of triglyceride. Triglyceride turnover studies on these same subjects using deuterated glycerol have indicated that this futile cycling is occurring (Millar, unpublished observation).

That the kinetics of $VLDL_2$ and IDL in this study were unaffected by either drug is in contrast to the work of Gaw et al (1993) who observed an increase in the catabolism of LDL precursors. The subjects in this study did show a tendency to increased rates of direct catabolism of $VLDL_2$ and IDL but these did not reach significance. However, the pool sizes of these lipoproteins were significantly reduced. The other significant kinetic finding of this study was the increased fractional catabolic rate of LDL on both drugs. This is in keeping with the widely accepted mechanism of action of the statins, whereby a reduced intrahepatic cholesterol pool, secondary to HMG-CoA reductase inhibition, stimulates the up-regulation of LDL receptors and so increases LDL clearance from the plasma. The increased LDL catabolism, especially on atorvastatin, could also be a reflection of the nature of the LDL that is binding to the receptor. The larger, less dense LDL species (LDLI) are known to bind with greater affinity to the LDL receptor than the small, dense species (LDLIII). Atorvastatin appears to change the nature of the LDL subfraction profile such that LDLI predominates whereas this is not the case with simvastatin (see below). This may be one reason for the increased LDL fractional catabolic rate on simvastatin not quite reaching statistical significance. The kinetic findings are summarised in Figure 7.4.

The atorvastatin-induced change in the LDL subfraction profiles was a second unexpected finding in this study. Both simvastatin and atorvastatin reduced the concentrations of all three LDL subfractions (although the reduction in LDLI was not significant for atorvastatin). However, the relative proportions by which each subfraction is reduced differ between the two drugs. Simvastatin reduces each subfraction by roughly the same amount - 60% reduction in LDLI, 50% reduction in LDLII and a 55% reduction in LDLIII. If anything, the largest reduction is in LDLI, which is the subfraction with the highest affinity for the LDL receptor and so more likely to show increased clearance when the LDL receptors are up-regulated. In contrast, on atorvastatin the majority of the LDL reduction is in LDLIII. LDLI is 25% reduced, LDLII is 35% reduced and LDLIII is 70% reduced. Thus the LDL

subfraction profile on atorvastatin is changed from a predominance of LDLIII to a predominance of the less dense species LDLI and LDLII. This shift in the LDL subfraction profile has been noted before with the fibrate class of drugs (Caslake 1996) but never consistently with a statin. However, in a study of the effect of pravastatin in primary moderate hypercholesterolaemia Vega et al (1990) did note that three out of the ten patients studied converted their LDL subfraction pattern from pattern B to pattern A in response to treatment. The mechanism behind this was given as a reduction in triglyceride in these patients which is also the suggested explanation for the effect that fibrates have on altering the LDL subfraction profile. In this study, it could be that the marginally greater effect of atorvastatin than simvastatin on lowering triglyceride is enough to cause the observed change in the LDL subfraction profile.

Direct comparison of simvastatin and atorvastatin gave no significant differences between the two. However, on comparing each to the baseline results the general impression to emerge is that atorvastatin is slightly more efficacious at lowering lipid and lipoprotein levels than simvastatin when both drugs are used at the same dosage (40mg daily). Atorvastatin certainly shows more extensive effects on the apoB kinetics than simvastatin, in particular on $VLDL_1$ production and direct catabolism, and also on LDL catabolism. However, given the fact that simvastatin showed a tendency to similar effects, although not reaching significance, it is possible that this difference is merely a result of subject selection and a small sample size. Perhaps if larger numbers were studied simvastatin would also show significant changes in these parameters. The striking difference between the two lies in the effects on the LDL subfraction profiles. Direct comparison of the percentage of LDLIII on simvastatin as compared to atorvastatin is the only value that approaches a significant difference ($p=0.07$). Although both drugs lower plasma LDL concentration, atorvastatin causes a shift in the LDL subfraction profile to one of a less atherogenic nature whereas simvastatin does not. That this effect has only previously been seen with the fibrates points to the triglyceride-lowering action of atorvastatin as responsible. Perhaps the marginally greater efficacy of atorvastatin at lowering triglyceride is just enough to change the nature of the lipoproteins being transferred down the delipidation chain, such that the resultant LDL is predominantly of a large, less dense nature (LDLI and LDLII). It could be suggested that each subject has an individual 'threshold' triglyceride level at which hepatic apoB production will change such that LDLI and LDLII predominate in the plasma, and that the subjects selected for this study happen only to reach this threshold whilst on atorvastatin. This would require a much larger sample size to resolve.

To summarise, the results of this study showed an unexpected increase in $VLDL_1$ production and an unexpected change in the nature of the LDL subfraction profiles in this group of subjects when treated with atorvastatin 40mg daily, two effects not previously reported with a statin.

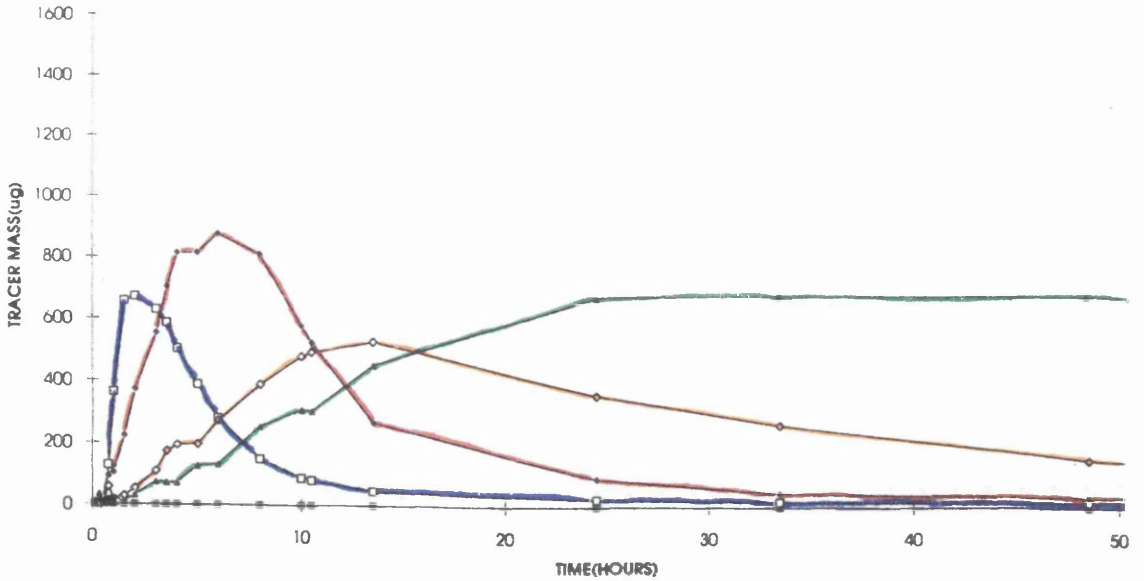


Figure 7.1a Tracer Mass Curves, 0-50 hours - baseline.

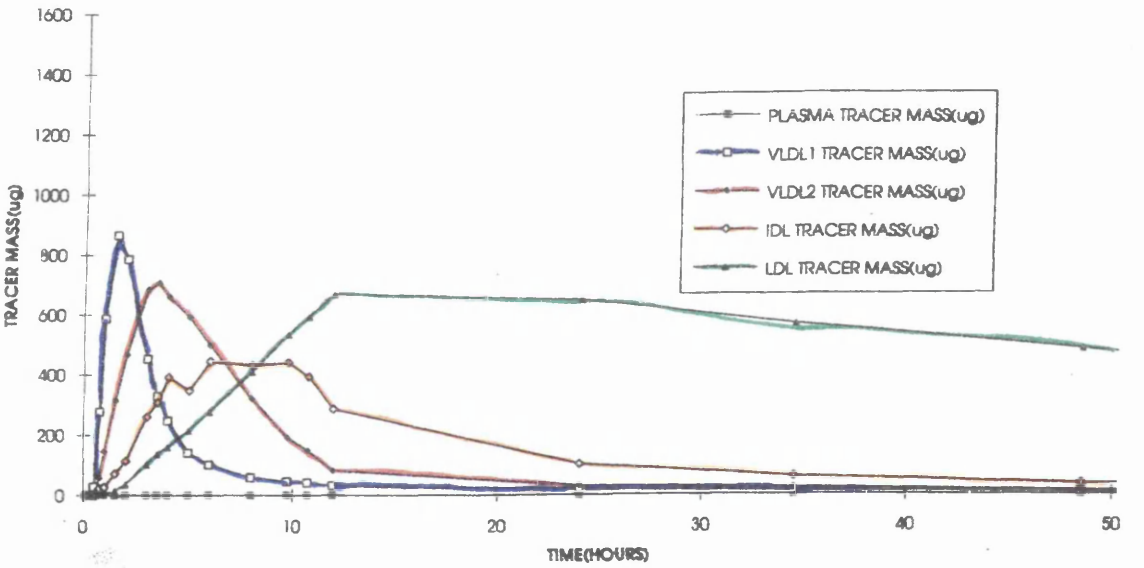


Figure 7.1b Tracer Mass Curves, 0-50 hours - simvastatin.

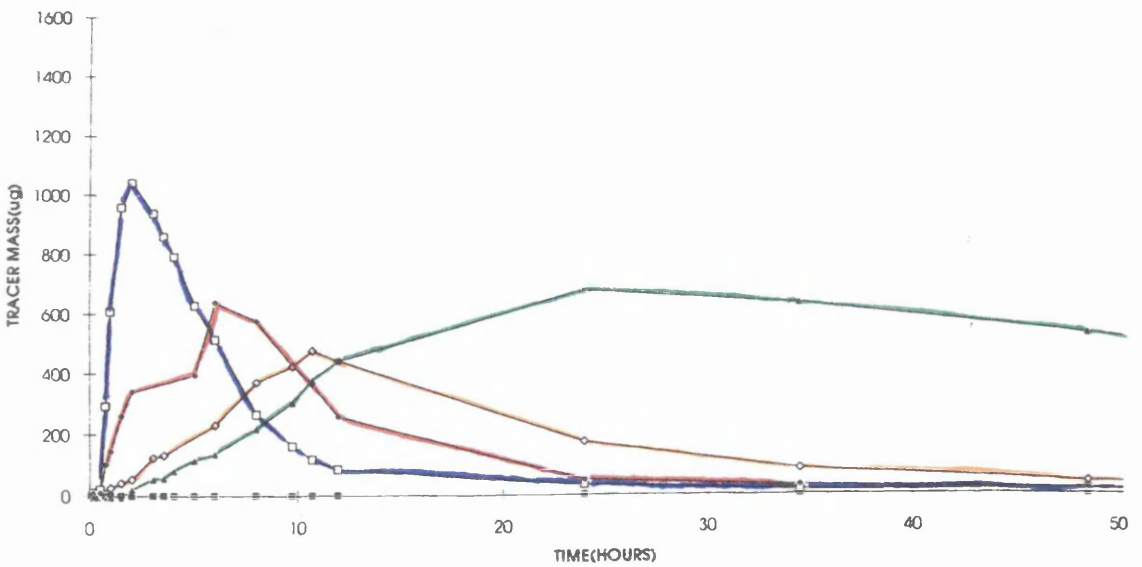


Figure 7.1c Tracer Mass Curves, 0-50 hours - atorvastatin.

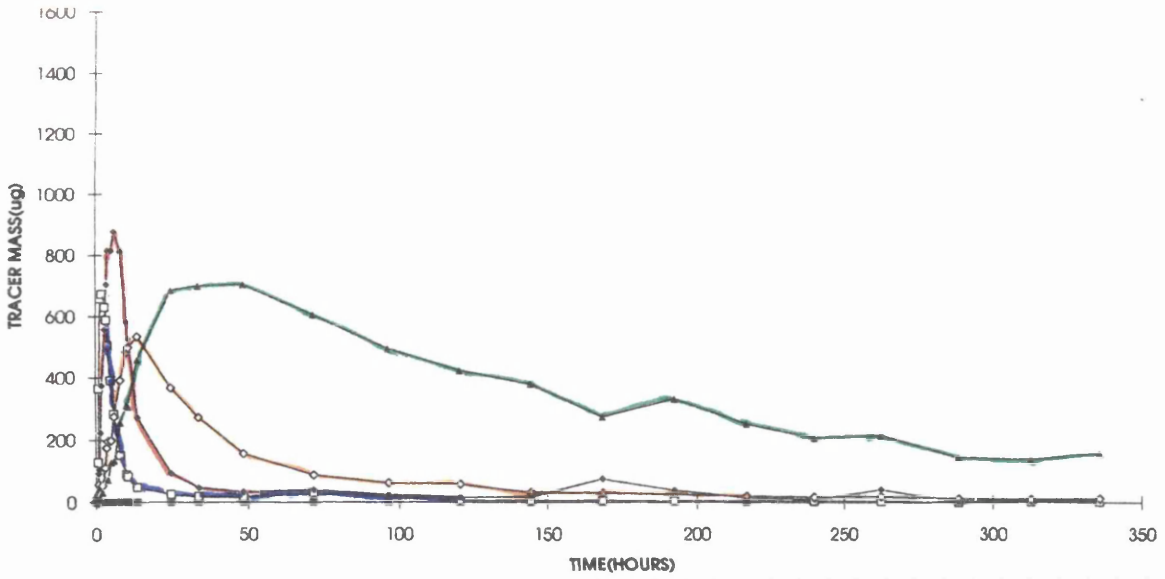


Figure 7.2a Tracer Mass Curves, 0-350 hours - baseline.

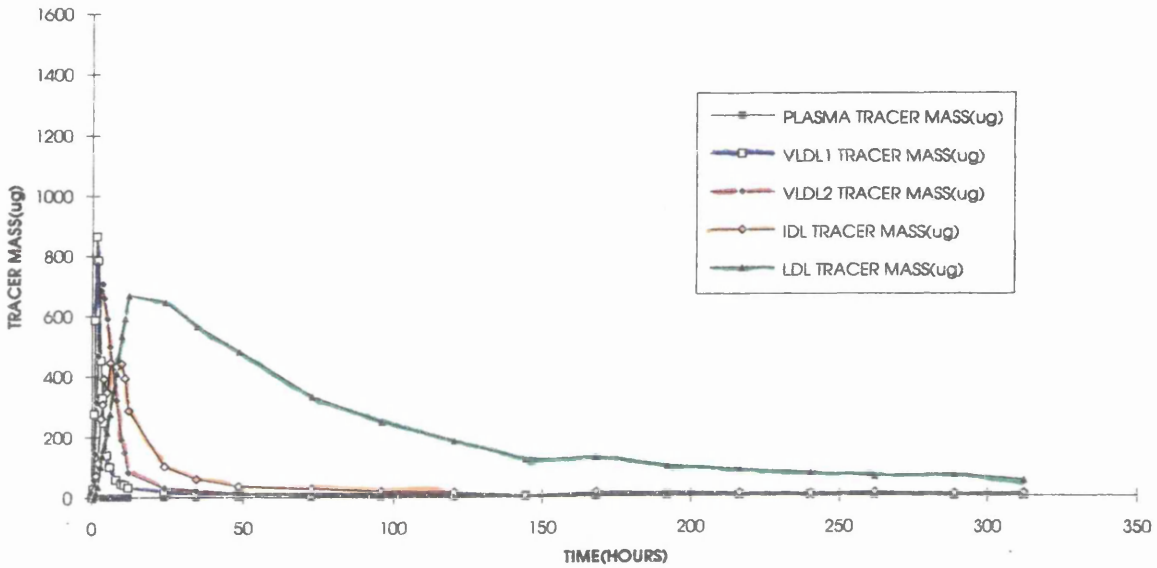


Figure 7.2b Tracer Mass Curves, 0-350 hours - simvastatin.

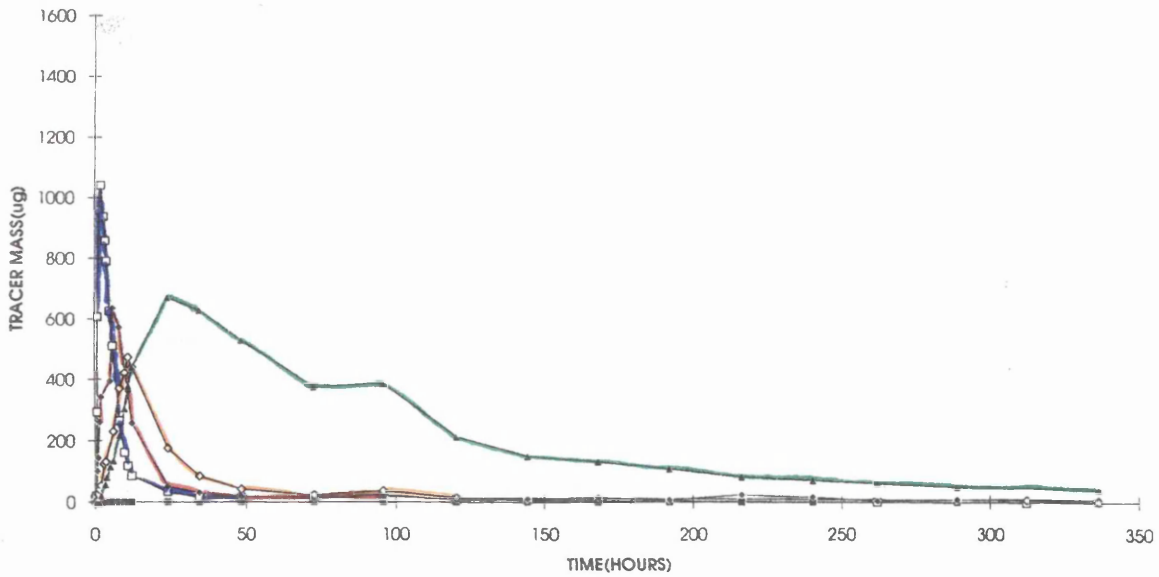


Figure 7.2c Tracer Mass Curves, 0-350 hours - atorvastatin.

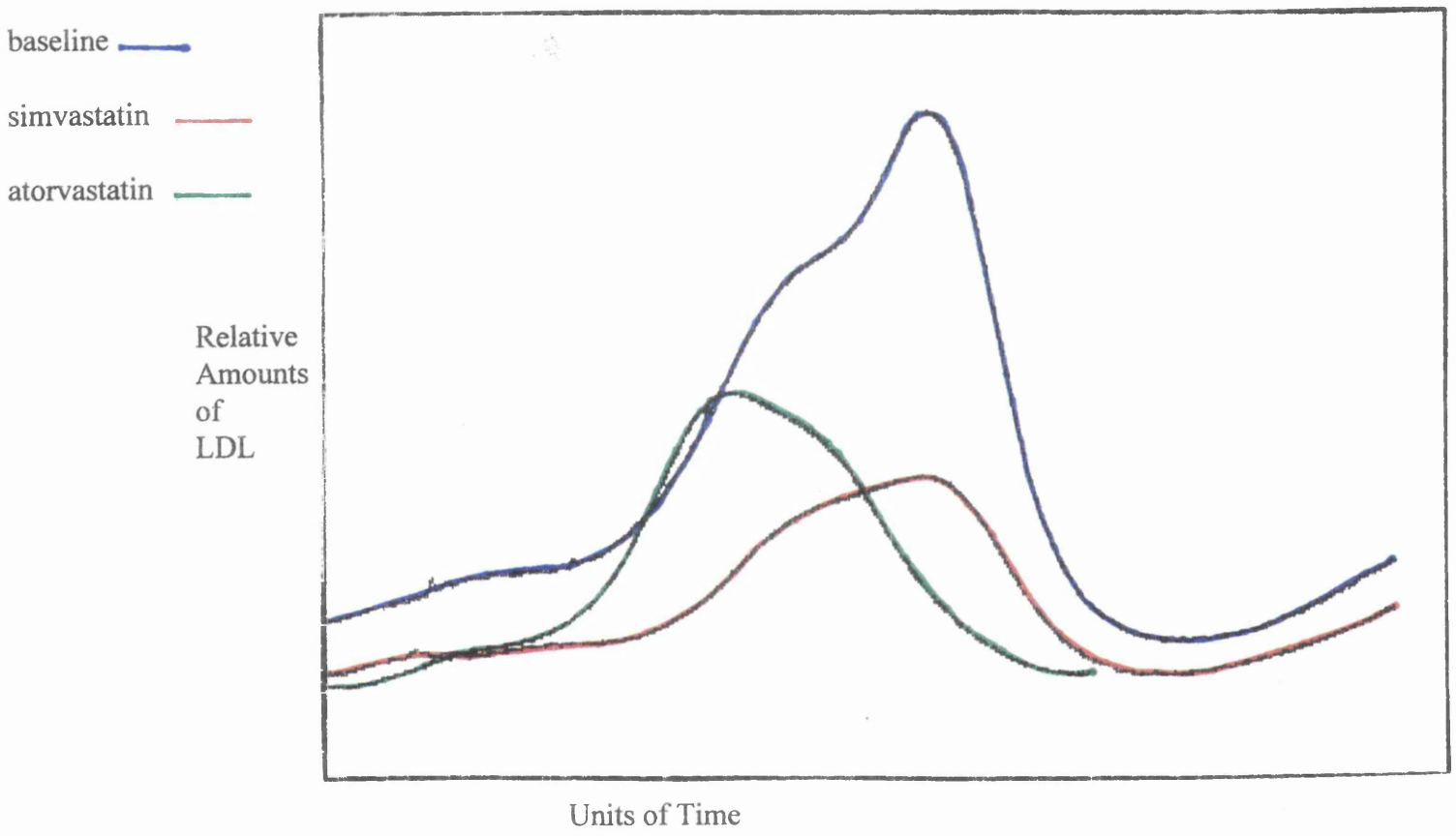


Figure 7.3 LDL Subfraction Profiles - effects of treatment.

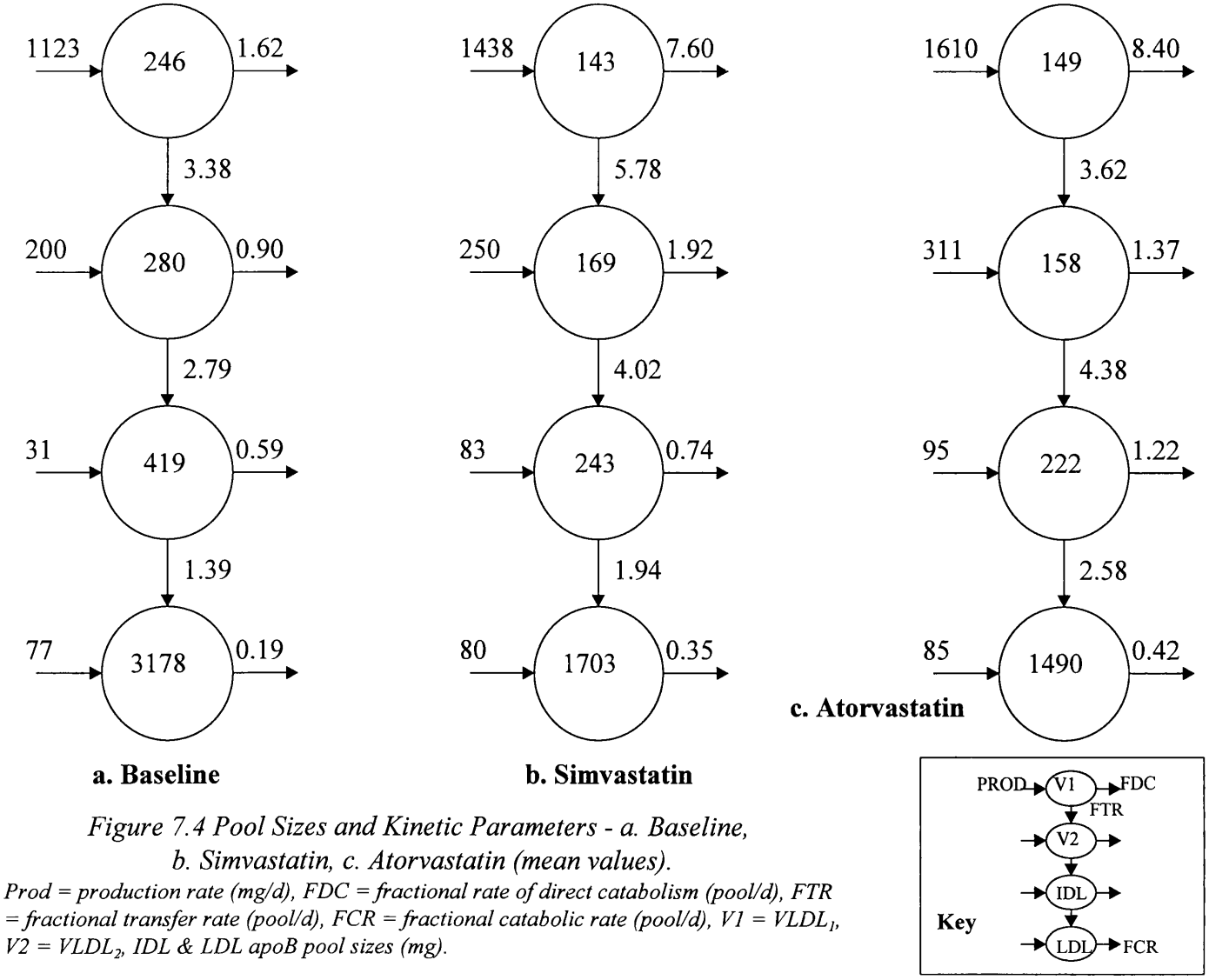


Figure 7.4 Pool Sizes and Kinetic Parameters - a. Baseline, b. Simvastatin, c. Atorvastatin (mean values).

Prod = production rate (mg/d), FDC = fractional rate of direct catabolism (pool/d), FTR = fractional transfer rate (pool/d), FCR = fractional catabolic rate (pool/d), V1 = $VLDL_1$, V2 = $VLDL_2$, IDL & LDL apoB pool sizes (mg).

Chapter 8 General Discussion.

By the beginning of the century the association between elevated plasma lipid levels, in particular plasma cholesterol, and atherosclerosis had become widely accepted. Since then research has moved forward from focusing primarily on the lipids themselves to concentrating on the lipoproteins and more recently on the apolipoproteins. As this century now closes, the research seems to have swung full circle with the realisation that intracellular lipid concentrations can influence the synthesis of apolipoproteins, the secretion of lipoproteins and hence the plasma lipid levels. To a certain extent this thesis has followed a similar pattern by first selecting subjects by virtue of their plasma lipid levels, studying their lipoprotein subfractions and then characterising their apolipoprotein B metabolism. The results were then discussed in the context of the effect that intrahepatic lipid levels have on the nature and amounts of apoB containing lipoproteins that are secreted by the liver.

The primary aim of this thesis was to characterise the kinetics of apoB containing lipoproteins in certain pathological states. In the main this aim was achieved. The secondary aim was to test the applicability of stable isotope methodology and multicompartmental modelling techniques, established in normolipidaemia, to non-physiological conditions. In reaching these aims three objectives were achieved. First, a non-kinetic study of a large group of normolipidaemic subjects highlighted various inter-relationships between lipoproteins and indices of obesity, insulin resistance and lipase activity that can have a bearing on apoB kinetics (chapter 3). Secondly, the kinetics of apoB containing lipoproteins were characterised in five dyslipidaemic states, namely familial hypercholesterolaemia and familial defective apoB-100 (chapter 4), hypobetalipoproteinaemia and analbuminaemia (chapter 5), and mixed hyperlipidaemia (chapter 6). Finally, the mechanisms of action of two HMG-CoA reductase inhibitors were determined in the group of mixed hyperlipidaemic subjects, with particular reference to the effects on apoB kinetics (chapter 7).

Throughout, the findings were compared to those reported in the current literature, with both agreement and disagreement with other work. Where results appeared to conflict with other work explanations were sought. The nature of stable isotope work means that there are several limitations to its use. It is labour intensive and time consuming, therefore, often restricting sample populations to a small size. In a small sample, inter-individual variation has much more of an influence on the results than would be the case in a larger sample. This in turn has an effect on the significance or otherwise of results. In addition, there is less stable isotope kinetic work available for comparison than is the case for radio-isotopes. Although the two often give similar conclusions, differences can arise, in particular with regard to synthetic rates and tracer recycling. The different methods of data analysis used in the literature i.e. monoexponential functions, linear regression analysis and multicompartmental modelling, add further complications when results are being compared. Furthermore, a method used in this thesis that is not widely used in the literature is that of separating two classes of VLDL on the basis of density. These two VLDL subfractions behave in a metabolically distinct manner. While it is accepted in the literature that VLDL is a heterogeneous class of proteins, much of the kinetic work

studies only total VLDL. This again leads to limitations when putting the current work into the context of other reports. With the studies of the familial defective apoB-100, hypobetalipoproteinaemic and analbuminaemic subjects there was very little other work available for comparison, both due to the rarity of the conditions and to the fact that stable isotope work in general is still limited. Certainly, the study of apoB kinetics in analbuminaemia is the first of its kind.

8.1 Methodological Considerations.

Throughout this thesis stable isotope methodology and multicompartmental modelling techniques have been used that were originally developed for use in normolipidaemic subjects (Demant et al 1996). These techniques were chosen as they are believed to be the 'gold standard' for kinetic analysis. Despite the limitations mentioned above, stable isotopes have great benefits over radio-isotopes - they are safe to handle, are administered endogenously and can be used repeatedly. Other workers have shown no difference in the particular isotope used (Lichtenstein et al 1990) and this study used tri-deuterated leucine. It is an essential amino acid, is ubiquitous in body protein and is minimally recycled. In addition, leucine is plentiful in apoB. The use of multiple labelling improves the sensitivity of mass spectrometry measurements. ApoB is an ideal marker for studying the metabolism of VLDL, IDL and LDL as it stays with the lipoproteins from their synthesis to their catabolism. The methods of lipoprotein and apoB separation are well established and in general required no modification for GC-MS analysis, but with two exceptions. In the case of the analbuminaemic subject, the very high apoB pool sizes possibly caused some error in the ultracentrifugal separation of IDL and LDL due to overflow from LDL into IDL. This in turn may have caused an overestimation of the IDL pool size (Marais, unpublished observation). This error could possibly have been avoided by using a smaller amount of plasma than the standard 2ml from which to separate the lipoproteins. However, since the main kinetic finding in this subject was one of increased $VLDL_2$ production it is unlikely that the discrepancy in the IDL pool size had any adverse bearing on the results. In the subject with hypobetalipoproteinaemia, the very low levels of apoB necessitated the separation of total VLDL rather than its subfractions in order to obtain measurable amounts of apoB. As a consequence, the multicompartmental model had to modified in such a way as to model VLDL as a whole rather than as $VLDL_1$ and $VLDL_2$. This resulted in a model with fewer compartments than the original but still provided interpretable results.

Multicompartmental modelling was used as it is probably the best method for analysing the complexities of the lipoprotein system, in particular accommodating the heterogeneous nature of the lipoproteins (Parhofer et al 1991). The model used here is that of Demant et al (1996) and has been developed from a combination of radio-isotope and stable isotope studies that have identified the key elements of the lipoprotein system, notably the intrahepatic synthesis of apoB, the delipidation cascade and the direct input into and loss from each lipoprotein fraction. This model is particularly good at defining the production rates of the lipoproteins, especially the differential production of $VLDL_1$ and $VLDL_2$. In addition, the kinetics of plasma free leucine are taken into account, with particular reference to its recycling and subsequent effect on the 'tails' of the lipoprotein enrichment curves. What is perhaps

lacking in this model is the dual delipidation pathway for apoB entering the plasma either as $VLDL_1$ or as $VLDL_2$, as was illustrated in the model of Packard et al (1995). However, use of this model requires the independent labelling of $VLDL_1$ and $VLDL_2$ which is not possible with endogenous stable isotope labelling techniques. Demant's model could perhaps also be improved by the expansion of the LDL compartments to allow for a more polydisperse LDL, especially for the study of hypertriglyceridaemic subjects. This would necessitate the isolation of apoB in the subfractions of LDL rather than just LDL as a whole. One further concern with using this model was that the parameter constraints that were applied to make the model uniquely identifiable were based on known normal physiology and might therefore not be applicable in the non-physiological state. However, this proved not to be the case. Thus, this model was successfully applied for use in dyslipidaemic states.

As mentioned above, one major difference between this work and much of the kinetic work in the current literature is the separate modelling of two VLDL subfractions. VLDL has been shown in other studies to consist of a number of metabolically heterogeneous subfractions that are grouped together by virtue of their centrifugal density. That $VLDL_1$ and $VLDL_2$ are metabolically distinct has been illustrated in this work. The massive overproduction of $VLDL_2$ in the analbuminaemic subject and the increased $VLDL_1$ production in the mixed hyperlipidaemic subjects may not have been identified had VLDL been modelled as a whole. In addition, the effect of atorvastatin on increasing both $VLDL_1$ production and catabolism (the 'futile cycling' effect) would not have been seen. Thus, it is justifiable to determine the separate kinetics of $VLDL_1$ and $VLDL_2$.

8.2 The Regulation of ApoB Metabolism in Man.

Observations from the above studies and from other work suggest that the lipid content of the hepatocyte is central to determining the amount and nature of the apoB containing lipoproteins that are secreted. ApoB-100 is synthesised in its final form within the hepatocyte. To this lipid is added prior to its secretion. The availabilty of lipid within the hepatocyte determines the relative proportion of apoB that is secreted as lipoprotein, as compared to the amount that is degraded. The type of lipid predominating within the hepatocyte determines the nature of the lipoprotein secreted i.e. if the hepatocyte is relatively enriched in triglyceride the main lipoprotein produced is $VLDL_1$, whilst if cholesterol is predominant $VLDL_2$ is preferentially produced. What determines the relative amounts of lipid in the hepatocyte was to a certain extent illustrated in the study of normolipidaemia. This highlighted the effect of insulin resistance and obesity on increasing the intrahepatic triglyceride content. The study of the analbuminaemic subject showed how a deficiency of albumin can also have an effect on intrahepatic lipid levels, in this case probably cholesterol ester, and so affect lipoprotein production. The hypobetalipoproteinaemic subject acted as an illustration of a genetic defect resulting in reduced apoB synthesis and hence reduced lipoprotein secretion.

$VLDL_1$ passes relatively slowly down the delipidation chain and is thus exposed to neutral lipid exchange. Catalysed by CETP, HDL_2 donates cholesterol ester to VLDL and in return receives triglyceride, resulting in the formation of relatively triglyceride-

enriched HDL_2 and cholesterol ester-enriched VLDL. The action of hepatic lipase on this HDL_2 results in the formation of HDL_3, and a lowering of plasma HDL cholesterol levels. HDL_2 is then reformed from HDL_3 by the cholesterol accumulating action of LCAT. Continued delipidation of the VLDL by the action of hepatic lipase produces LDL that is predominantly of a small, dense, protein-rich nature (LDLIII). This type of LDL is only slowly cleared from the plasma by the LDL receptor (perhaps due to the conformation of the apoB on the lipoprotein surface) and so accumulates in the plasma. It is cleared by receptor-independent mechanisms such as the scavenger receptors on macrophages with the resultant foam cell formation, inflammatory reaction and atherosclerosis development. Due to its small size, this dense LDL is able to directly penetrate the arterial endothelium so leading to subendothelial cholesterol deposition and hence plaque formation.

In contrast, $VLDL_2$ is rapidly delipidated and cleared from the plasma, with only a small proportion of its apoB reaching the LDL density range. That which does preferentially forms the larger, less dense species of LDL which in turn are rapidly cleared from the plasma by the LDL receptor. Exposure to neutral lipid exchange is minimised by the rapid clearance of the lipoproteins. This differential production of $VLDL_1$ or $VLDL_2$ is illustrated in the studies of the normolipidaemic, CHD sufferers and the mixed hyperlipidaemic individuals who were preferentially producing increased amounts of $VLDL_1$ with the resultant consequences described above and the development of the atherogenic lipoprotein phenotype. The FH and FDB subjects on the other hand showed how a deficiency in the catabolism of LDL, either due to receptor deficiency or ligand abnormality, can affect apoB metabolism. Of note, was the ability of the FDB subjects to normally catabolise their LDL precursors so reducing the potential for neutral lipid exchange and the formation of small, dense LDL.

This overview of apoB metabolism, with the mentioned points of regulation, is illustrated in Figure 8.1.

8.3 Pharmacological Manipulation of ApoB Metabolism.

The study of the mechanisms of action of simvastatin and atorvastatin, two HMG-CoA reductase inhibitors, illustrated one method of iatrogenic modification of apoB metabolism, in addition to allowing a direct comparison of the two drugs. The subjects used were those from the mixed hyperlipidaemia study. This group of individuals have traditionally been treated with the fibrate class of drug but newer claims for the HMG-CoA reductase inhibitors suggest that they are equally as effective in treating such individuals, in particular at lowering moderate elevations in plasma triglyceride. In addition, the recent 4S and WOSCOPS have done much to promote the safety and efficacy of this class of drug. The inhibition of HMG-CoA reductase results in the decreased intracellular production of cholesterol. By way of a homeostatic response, there is an up-regulation of LDL receptors in an attempt to return the intracellular cholesterol content to normal. This is seen as an increase in the catabolic rate of LDL, an effect that was noted with both drugs (although not significantly with simvastatin).

One unexpected finding of this study was the increase in $VLDL_1$ production that atorvastatin in particular caused (this effect was inconsistant with simvastatin and did not reach statistical significance). In addition there was an increase in $VLDL_1$ direct catabolism with no resultant change in the $VLDL_1$ apoB pool size. These findings were contrary to the original hypothesis that these drugs would cause a reduction in $VLDL_1$ production. The results suggest that there is a degree of 'futile cycling' of $VLDL_1$ occurring, possibly involving a hepatic VLDL receptor, a finding that is in keeping with results of triglyceride turnover studies in these same patients (Millar, unpublished observation) and also with the current thinking described above that the lipid content of the hepatocytes can determine the nature of the lipoproteins secreted.

A second unexpected finding was the ability of atorvastatin to change the nature of the LDL subfraction profile such that there was a shift in the profile from a predominance of small, dense LDL species to a predominance of the larger, less dense species. This effect has previously been reported for the fibrate class of drug (Caslake 1996), but not for the HMG-CoA reductase inhibitors, and so lends weight to the possibility that it is the triglyceride-lowering action of atorvastatin that causes this shift. Atorvastatin must therefore have an effect on the lipoproteins such that less are channelled into the small, dense species of LDL. Perhaps by virtue of its greater effect on $VLDL_1$ catabolism (and hence its triglyceride lowering) than simvastatin a smaller proportion of apoB comes down the delipidation chain from $VLDL_1$ as opposed to $VLDL_2$. The relationships between plasma triglyceride levels and LDL subfraction profiles have been reported by other workers (Tan et al 1995) with the suggestion that for any one individual once the plasma triglyceride falls below a certain 'threshold' level the LDL subfraction will change.

The fibrates lower plasma triglycerides by activation of lipoprotein lipase and so increasing the delipidation of triglyceride-rich lipoproteins. By reducing the residence time of these lipoproteins in the plasma they minimise their exposure to neutral lipid exchange and hence the development of small, dense LDL and low HDL cholesterol levels. Thus, they are able to cause a shift in the LDL subfraction profile by a reduction in intrahepatic triglyceride availability. This is an effect not classically characteristic of the HMG-CoA reductase inhibitors but it now appears that atorvastatin (and perhaps other drugs of this class) is capable of such an effect. If this proves to be the case, it will serve to further popularise these drugs for a broader use in dyslipidaemia in particular in the light of the 4S and WOSCOPS.

8.4 Future Studies.

The studies in this thesis have highlighted the synthesis and catabolism of apoB containing lipoproteins as points of regulation of apoB metabolism. In so doing, further questions have been raised. A prime area for further study is the futile cycling of triglyceride that appears to happen in mixed hyperlipidaemic subjects treated with atorvastatin. That this occurs perhaps indicates the existance of a hepatic receptor for $VLDL_1$ that is up-regulated in response to the inhibition of HMG-CoA reductase and subsequent reduction in intrahepatic cholesterol. Identification of this receptor would require cell culture studies, plus, for example, kinetic studies of FH homozygotes who were receptor negative for the LDL receptor. The recycling of triglyceride can be

identified by lipid turnover studies and this is already being undertaken in this laboratory. Triglyceride is endogenously labelled with penta-deuterated glycerol and its subsequent metabolism is analysed (Millar, unpublished).

The effects of atorvastatin on apoB metabolism can be further expanded both by using increased doses of the drug and by studying subjects with different forms of dyslipidaemia such as FH homozygotes (Marais, unpublished) or subjects with hypertriglyceridaemia secondary to, for example, lipoprotein lipase deficiency. In addition, the ability or otherwise of simvastatin to have similar effects on apoB metabolism can be determined by using an increased sample size of mixed hyperlipidaemic subjects and by increasing the dose of simvastatin used. Kinetic studies of some of the newer lipid-lowering agents currently being developed, for example microsomal transfer protein inhibitors, would also lead to further understanding of the regulation of apoB metabolism.

The further characterisation of apoB kinetics in analbuminaemia, an example of secondary hyperlipidaemia, would be interesting should more affected individuals be identified. Although itself a relatively benign condition, its clarification may help in the understanding of the nature of the dyslipidaemia of nephrotic syndrome, a condition characterised by heavy proteinuria, hypoproteinaemia and generalised oedema. Here studies have suggested that the increased apoB production is secondary to increased amounts of free fatty acids being diverted to the liver (Joven et al 1990). It is from 'experiments of nature' such as analbuminaemia that an understanding of more serious pathologies can be developed, with the ultimate aim of improved treatment or, ideally, prevention.

Thus, in meeting the aims and objectives of this thesis some questions regarding the metabolism of apoB containing lipoproteins have been answered but, as is always the case with research, more have been raised. What is most important is that the research should ultimately be applicable in the clinical context, in this case for the reduction in coronary heart disease.

"In expanding the field of knowledge we but increase the horizon of ignorance."
(Henry Miller, 'The Wisdom of the Heart', 1941)

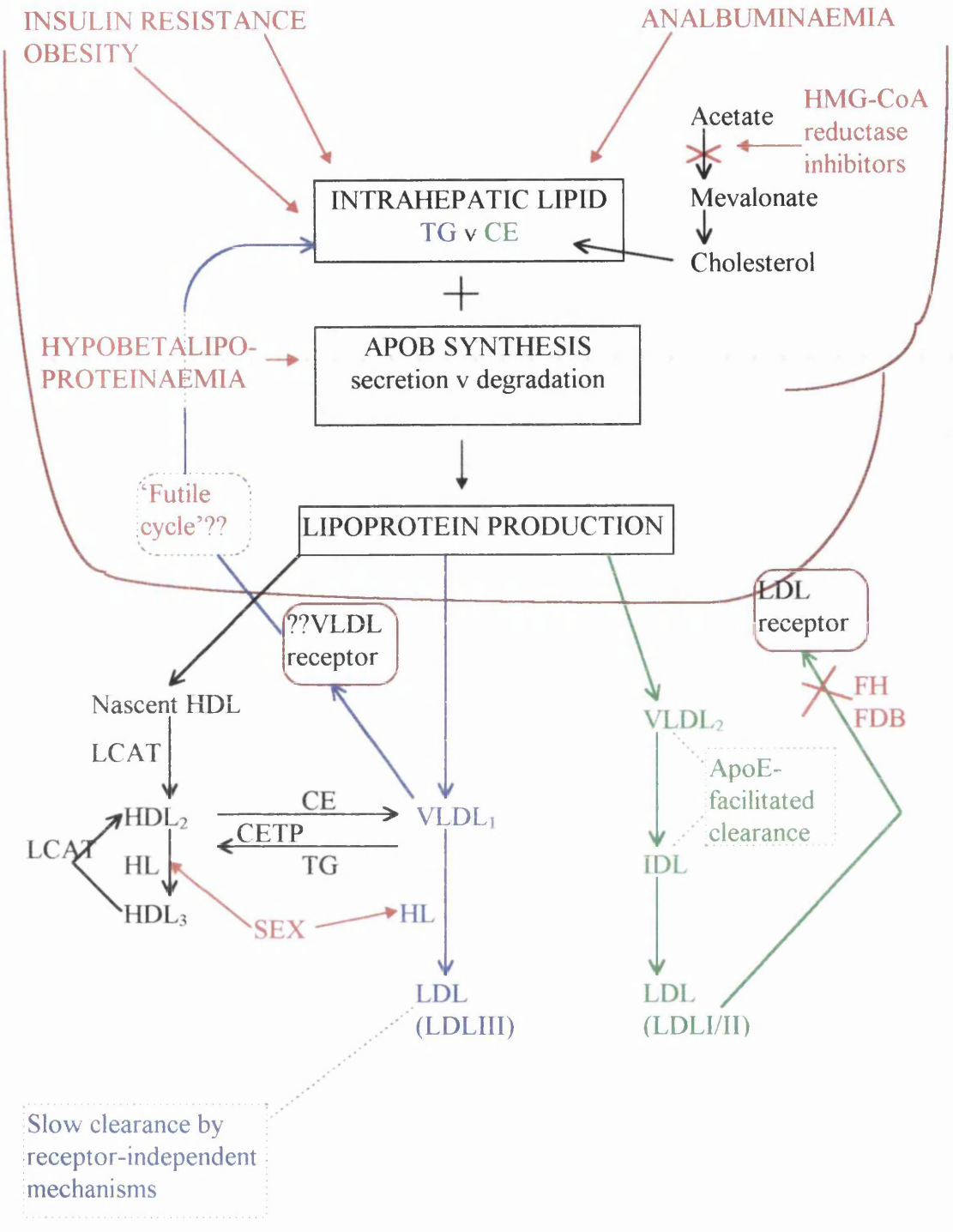


Figure 8.1 The Regulation of ApoB Metabolism in Man.

Key -	TG	triglyceride
	CE	cholesterol ester
	HL	hepatic lipase
	LCAT	lecithin:cholesterol acyl transferase
	CETP	cholesterol ester transfer protein
	FH	Familial hypercholesterolaemia
	FDB	Familial defective apoB-100

Glossary.

ALP	Atherogenic lipoprotein phenotype
Apo	Apolipoprotein
BHA	British Hyperlipidaemia Association
BMI	Body mass index
CETP	Cholesterol ester transfer protein
CHD	Coronary heart disease
DBP	Diastolic blood pressure
DGUC	Density gradient ultracentifugation
ECG	Electrocardiograph
FCHL	Familial combined hyperlipidaemia
FCR	Fractional catabolic rate
FDB	Familial defective apoB-100
FDC	Fractional rate of direct catabolism
FH	Familial hypercholesterolaemia
FTR	Fractional transfer rate
GC-MS	Gas chromatography-mass spectrometry
HDL	High density lipoprotein
HL	Hepatic lipase
HLP	Hyperlipidaemia
HMG-CoA	3-Hydroxy-3-methylglutaryl Coenzyme A
HyperapoB	Hyperapobetalipoproteinaemia
IDL	Intermediate density lipoprotein
IRS	Insulin resistance syndrome
LCAT	Lecithin:cholesterol acyl transferase
LDL	Low density lipoprotein
Lp(a)	Lipoprotein (a)
LPL	Lipoprotein lipase
MRFIT	Multiple Risk Factor Intervention Trial
NEFA	Non-esterified fatty acids
NIDDM	Non-insulin dependent diabetes mellitus
SBP	Systolic blood pressure
4S	Scandinavian Simvastatin Survival Study
WOSCOPS	West of Scotland Coronary Prevention Study
VLDL	Very low density lipoprotein

Appendix 1 - Manufacturers and Suppliers of Reagents, Hardware and Software.

Baker Instruments Ltd
Rusham Park, Whitehall Lane
Egham, Surrey
TW20 9NW, UK

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

BDH Laboratory Supplies
McQuilkin & Co
21 Polmadie Avenue, Glasgow
G5 0BB, UK

Bio-Rad Laboratories
2000 Alfred Nobel Drive
Hercules
CA 94547, USA

Boehringer Mannheim UK (Diagnostics and Biochemicals) Ltd
Bell Lane
Lewes, East Sussex
BN7 1LG, UK

Chromacol Ltd
3 Little Mundells, WGC
Herts,
AL7 1EW, UK

Fisons Instruments
Crewe Road
Wythenshaw, Manchester
M23 9BE, UK

Genzyme Biochemicals
50 Gibson Drive, Kings Hill
West Malling, Kent
ME19 6HG, UK

VA Howe & Company Ltd
Beaumont Close
Banbury
OX16 7RG, UK

Imed
San Diego
CA 92131-1192, USA

Innogenetics NV
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Appendix 2 - Turnover Bleeding Schedule.

Time Point	Blood Vol.(ml EDTA)	Plasma Use & Rotor Nos.	
Day 0 0'	30	2*1ml plasmas	
	+ 6.0 (yellow) for safety	2*2ml SW40	1+2
		3.5ml BQ & HDL subs, 3.5ml LDL subs	
		1ml Apo's A1,B & Lp(a)	
1'	2	plasma glycerol	
2'	4.5	2*1ml plasmas	
5'	4.5	"	
10'	10	" + 2ml SW40	3
15'	4.5	2*1ml plasmas	
20'	10	" + 2ml SW40	4
30'	14.5	2*1ml plasmas + 2ml SW40	5
45'	14.5	" spare plasma for pools	6 = 1
1hr	14.5	"	1
1.5hr	10	"	2
2hr	10	"	3
3hr	10	"	4
3.5hr	10	"	5
4hr	10	"	6 = 2
5hr	10	"	1
6hr	10	"	2
8hr	10	"	3
10hr	10	"	4
*patient given fat-free meal			
11hr	10	"	5
14hr	10	"	6 = 3
Day 1 24hr	10	"	1
36hr	10	"	2 + 3pools
Day 2 48hr	10	"	3 = 4
Day 3 72hr	10	"	1
Day 4 96hr	10	"	2 + 3pools
Day 5 120	10	"	3 = 5
Day 6 144	10	"	1
Day 7 168	10	" + 1ml chol, trig, HDL	2
Day 8 192	10	"	3
Day 9 216	10	"	4
Day 10 240	10	"	5
Day 11 264	10	"	6 = 6
Day 12 288	10	"	1
Day 13 312	10	"	2
Day 14 336	20	" + 3.5ml BQ	3 = 7
	+6.0 (yellow),4.5 (purple), &2.0 (grey) for clinical		
	+urinalysis		
	Total = 397.5ml		

NB All samples fasted except 11hr , 14hr , 36hr . 2*24hr urine samples .

Appendix 3 - Rate Constants and Calculated Leucine Pool Sizes for FH and FDB Subjects.

	AC (FH)	WT (FH)	RM (FH)	MM (FH)	RH (FH)	JR (FDB)	NS (FDB)
k (15,4)	0.04	0.08	0.06	2.47	0.02	0.02	0.02
d(5,15)	0.79	0.87	0.56	0.66	0.25	0.45	0.47
d(8,15)	0.11	0.06	0.20	0.20	0.59	0.18	0.33
d(11,15)	0.08	0.00	0.20	0.14	0.04	0.16	0.12
d(13,15)	0.03	0.07	0.04	0.00	0.11	0.21	0.08
k(0,6)	1.65	0.05	4.6	1.24	0.05	0.33	1.74
k(0,7)	0.01	0.07	0.02	0.30	0.13	0.10	0.84
k(8,6)	0.56	0.59	1.36	0.40	0.29	1.55	2.35
k(0,9)	0.03	0.29	0.06	0.005	0.22	0.00	0.00
k(0,10)	0.01	0.00	0.005	0.02	0.04	0.10	0.84
k(11,9)	0.26	0.96	0.20	0.14	1.00	0.03	0.42
k(12,9)	0.12	0.35	0.07	0.06	0.13	0.06	0.05
k(0,11)	0.00	0.08	0.05	0.00	0.00	0.00	0.00
k(0,12)	0.01	0.04	0.01	0.01	0.01	0.06	0.01
k(13,11)	0.07	0.20	0.17	0.20	0.06	0.03	0.13
k(0,13)	0.01	0.01	0.01	0.01	0.01	0.01	0.01
$VLDL_1$ leucine (mg)	11.6	18.9	5.0	12.8	5.2	9.1	1.6
$VLDL_2$ leucine (mg)	29.0	32.9	22.2	21.4	86.0	24.4	6.3
IDL leucine (mg)	55.5	49.7	73.8	53.1	118.2	57.3	28.1
LDL leucine (mg)	269.8	350.7	313.0	354.0	808.8	386.4	203.9

Appendix 4 - Rate Constants and Calculated Leucine Pool Sizes for Hypobetalipoproteinaemic and Analbuminaemic Subjects.

	Hypobetalipoprotein-aemic Subject (LR)	Analbuminaemic Subject (AK)
k (15,4)	0.02	0.08
d(5,15)	------	0.15
d(8,15)	0.81 - total VLDL	0.85
d(11,15)	0.00	0.00
d(13,15)	0.19	0.00
k(0,6)	------	3.78
k(0,7)	------	0.002
k(8,6)	------	0.22
k(0,9)	0.00 - total VLDL	0.52
k(0,10)	0.001 - total VLDL	0.01
k(11,9)	0.44 - total VLDL	0.51
k(12,9)	0.02 - total VLDL	0.03
k(0,11)	0.00	0.52
k(0,12)	0.004	0.003
k(13,11)	0.38	0.16
k(0,13)	0.02	0.008
$VLDL_1$ leucine (mg)	------	7.23
$VLDL_2$ leucine (mg)	1.94 = total VLDL	74.8
IDL leucine (mg)	6.1	411.8
LDL leucine (mg)	26.1	576.5

Appendix 5 - Rate Constants and Calculated Leucine Pool Sizes for Mixed Hyperlipidaemic Subjects.

	RA	PS	JC	RS	DM	LB	PC
k(15,4)	0.01	0.01	0.01	0.02	0.01	0.01	0.01
d(5,15)	0.79	0.77	0.71	0.85	0.80	0.67	0.87
d(8,15)	0.13	0.19	0.20	0.12	0.14	0.20	0.05
d(11,15)	0.02	0.01	0.04	0.01	0.02	0.06	0.01
d(13,15)	0.06	0.04	0.05	0.03	0.04	0.07	0.08
k(0,6)	0.22	0.13	0.00	0.57	0.06	0.19	0.05
k(0,7)	0.01	0.01	0.02	0.01	0.00	0.00	0.00
k(8,6)	0.37	0.19	0.51	0.23	0.18	0.17	0.23
k(0,9)	0.05	0.03	0.04	0.00	0.02	0.03	0.20
k(0,10)	0.01	0.01	0.02	0.01	0.00	0.00	0.01
k(11,9)	0.32	0.20	0.15	0.08	0.08	0.07	0.10
k(12,9)	0.04	0.02	0.01	0.01	0.02	0.02	0.05
k(0,11)	0.05	0.03	0.04	0.00	0.02	0.03	0.01
k(0,12)	0.04	0.02	0.01	0.01	0.02	0.02	0.02
k(13,11)	0.12	0.37	0.07	0.12	0.09	0.08	0.14
k(0,13)	0.01	0.01	0.01	0.01	0.01	0.01	0.01
$VLDL_1$ leucine (mg)	14.4	18.9	11.6	30.1	25.3	18.4	33.1
$VLDL_2$ leucine (mg)	13.8	17.9	28.1	23.2	38.5	27.8	22.3
IDL leucine (mg)	25.1	16.6	55.5	36.4	51.1	35.0	39.5
LDL leucine (mg)	199.9	238.3	279.6	282.8	310.8	223.5	365.2

Appendix 6 - Rate Constants and Calculated Leucine Pool Sizes for Mixed Hyperlipidaemic Subjects - on Simvastatin.

	RA	PS	JC	RS	DM	LB	PC
k(15,4)	0.02	0.01	0.02	0.01	-	0.01	0.02
d(5,15)	0.77	0.82	0.82	0.76	-	0.66	0.85
d(8,15)	0.16	0.13	0.12	0.12	-	0.10	0.14
d(11,15)	0.04	0.03	0.05	0.03	-	0.09	0.00
d(13,15)	0.03	0.02	0.02	0.09	-	0.14	0.01
k(0,6)	2.56	0.19	1.22	0.10	-	0.50	0.57
k(0,7)	0.07	0.03	0.10	0.001	-	0.001	0.005
k(8,6)	0.58	0.49	0.95	0.53	-	0.28	0.18
k(0,9)	0.06	0.16	0.05	0.24	-	0.10	0.13
k(0,10)	0.07	0.03	0.10	0.001	-	0.001	0.005
k(11,9)	0.30	0.28	0.22	0.19	-	0.06	0.15
k(12,9)	0.02	0.04	0.04	0.002	-	0.002	0.01
k(0,11)	0.06	0.00	0.05	0.03	-	0.10	0.001
k(0,12)	0.02	0.01	0.04	0.002	-	0.001	0.01
k(13,11)	0.27	0.25	0.22	0.12	-	0.03	0.27
k(0,13)	0.02	0.02	0.02	0.01	-	0.01	0.01
$VLDL_1$ leucine (mg)	10.9	11.9	7.50	9.00	-	10.6	27.5
$VLDL_2$ leucine (mg)	12.9	12.8	13.0	11.1	-	24.9	18.6
IDL leucine (mg)	20.4	31.9	22.8	16.7	-	20.5	21.9
LDL leucine (mg)	172.4	155.5	124.8	152.6	-	129.5	190.5

Appendix 7 - Rate Constants and Calculated Leucine Pool Sizes for Mixed Hyperlipidaemic Subjects - on Atorvastatin.

	RA	PS	JC	RS	DM	LB	PC
k(15,4)	0.01	0.01	0.02	-	0.01	0.01	0.01
d(5,15)	0.90	0.80	0.77	-	0.73	0.60	0.83
d(8,15)	0.05	0.16	0.19	-	0.13	0.18	0.17
d(11,15)	0.03	0.02	0.03	-	0.07	0.11	0.00
d(13,15)	0.02	0.02	0.01	-	0.07	0.11	0.00
k(0,6)	0.48	0.20	1.12	-	1.71	1.60	0.26
k(0,7)	0.01	0.01	0.19	-	0.19	0.004	0.02
k(8,6)	0.18	0.29	0.70	-	0.87	0.10	0.30
k(0,9)	0.03	0.14	0.00	-	0.01	0.26	0.07
k(0,10)	0.01	0.01	0.19	-	0.06	0.004	0.02
k(11,9)	0.27	0.23	0.34	-	0.27	0.08	0.28
k(12,9)	0.06	0.01	0.08	-	0.06	0.002	0.001
k(0,11)	0.03	0.05	0.08	-	0.01	0.08	0.26
k(0,12)	0.06	0.01	0.08	-	0.02	0.001	0.001
k(13,11)	0.24	0.36	0.23	-	0.28	0.05	0.45
k(0,13)	0.02	0.02	0.02	-	0.02	0.01	0.01
$VLDL_1$ leucine (mg)	14.0	12.6	15.0	-	13.0	6.80	19.5
$VLDL_2$ leucine (mg)	7.92	12.3	16.4	-	16.4	14.0	20.1
IDL leucine (mg)	14.7	21.5	19.8	-	24.3	25.2	13.9
LDL leucine (mg)	108.8	95.8	143.9	-	171.5	132.1	176.0

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