The Regulation of Hepatic Lipoprotein Metabolism in the St Thomas' Mixed Hyperlipidaemic Rabbit

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List of abbreviations

ACAT	acyl coenzyme A: cholesterol acyltransferase
acetyl CoA	acetyl coenzyme A
ALP	atherogenic lipoprotein phenotype
apo	apolipoprotein
APOBEC-1	apoB mRNA editing enzyme catalytic polypeptide-1
ASP	acylation stimulatory protein
AST	aspartate aminotransferase
bp	base pairs
CE	cholesteryl ester
CETP	cholesteryl ester transfer protein
CHD	coronary heart disease
C_t	threshold cycle
d	density
d_3 leucine	L-[5,5,5 $^2H_3$]-leucine
DEPC	diethyl pyrocarbonate
DNase	deoxyribonuclease
dNTP	deoxynucleoside triphosphate
F value	Svedberg flotation unit (d = 1.21)
FA	fatty acids
FC	free cholesterol
FCH	familial combined hyperlipidaemia
FCR	fractional catabolic rate
FDB	familial defective apoB
FDC	fractional rate of direct catabolism
FFA	free fatty acids
FH	familial hypercholesterolaemia
FHTG	familial hypertriglyceridaemia
FTR	fractional transfer rate
GAPDH	glyceraldehyde - 3 - phosphate dehydrogenase
GC-MS	gas chromatography - mass spectrometry

GGT	gamma glutamic acid
HDL	high density lipoprotein
HL	hepatic lipase
HMG CoA reductase	3-hydroxy, 3-methylglutaryl-coenzyme A reductase
HPRT	hypoxanthine-guanine phosphoribosyltransferase
HPV	hepatic portal vein
HSL	hormone sensitive lipase
IDL	intermediate density lipoprotein
IVC	inferior vena cava
LCAT	lecithin-cholesterol acyl transferase
LDL	low density lipoprotein
LDL-C	LDL cholesterol
Lp(a)	lipoprotein(a)
LPL	lipoprotein lipase
LRP	LDL receptor related protein
MI	myocardial infarction
MTP	microsomal triglyceride transfer protein
NIDDM	non insulin dependent diabetes mellitus
non $VLDL_1$	$VLDL_2$ + IDL + LDL
NS	non significant
NZW	New Zealand white rabbit
PCR	polymerase chain reaction
PDI	protein disulphide isomerase
PL	phospholipid
PR	production rate
rbc	red blood cells
RER	rough endoplasmic reticulum
RNase	ribonuclease
RT	reverse transcriptase
SDS	sodium dodecyl sulphate
SEM	standard error of the mean
Sf value	Svedberg flotation units (d = 1.063)
SMHL	St Thomas' mixed hyperlipidaemic rabbit

Taq	*Thermus Aquaticus*
TC	total cholesterol
TCA	trichloroacetic acid
TEMED	tetramethylethylenediamine
TG	triglyceride
T_m	melting temperature
VLDL	very low density lipoprotein
WHHL	Watanabe heritable hyperlipidaemic rabbit

Summary

The aim of this thesis was to characterise further the lipoprotein abnormalities present in the St Thomas' Mixed Hyperlipidaemic (SMHL) rabbit, assess the suitability of this strain as an animal model for the human disorder familial combined hyperlipidaemia, and to investigate the genetic defect giving rise to the disorder.

The SMHL rabbit colony was derived from the original St Thomas' Hospital rabbit colony, first described in 1987 as exhibiting elevated plasma cholesterol, plasma triglyceride or both. Indirect evidence for overproduction of VLDL and LDL apoB as the underlying metabolic defect was obtained. To investigate lipoprotein metabolism in these rabbits further, we made use of techniques already in place in our laboratory, developed a liver perfusion method and took advantage of new molecular biological techniques for mRNA quantitation.

All studies were performed on rabbits fed a 0.08% cholesterol diet, which was found to exaggerate an already dyslipidaemic phenotype in the SMHL rabbits. Plasma cholesterol levels were found to be elevated in both young and mature male SMHL rabbits in comparison to NZW controls ($p < 0.001$, $p = 0.04$ respectively). Plasma triglyceride levels were elevated in young SMHL rabbits compared to young NZW ($p < 0.001$), but there was no difference between the strains in mature rabbits. These changes were found to be as a result of an increased mass of each of the lipoproteins studied: very low density lipoprotein $(VLDL)_1$, $VLDL_2$, intermediate density lipoprotein (IDL) and low density lipoprotein (LDL), (Sf 60 - 400, Sf 20 - 60, Sf 12 - 20 and Sf 0 - 12 respectively) but there was no alteration in lipoprotein composition or apolipoprotein content. The SMHL rabbits exhibited insulin resistance with exaggerated free fatty acid (FFA) and insulin responses to an oral glucose dose. Cholesteryl ester transfer protein (CETP) activities were increased 3 fold in the SMHL rabbits ($p < 0.01$), but there were no differences in hepatic lipase (HL) or lipoprotein lipase (LPL) activities.

Investigation of the kinetics of lipoprotein metabolism using stable isotope labelling of apoB, yielded poor results due to the assays being at the limit of detection. There were no significant differences in production rates or catabolic rates of any of the lipoprotein species, however, trends towards increased $VLDL_1$ and IDL production, and decreased IDL catabolism were seen.

To investigate directly the output of apoB containing lipoproteins from the liver of the SMHL rabbits, we developed a method to perfuse the rabbit livers. This involved the perfusion of the organ for 3 hours with a recirculating, oxygenated Krebs Henseleit buffer at 37°C, and the isolation of lipoproteins from the perfusate at regular intervals throughout. We performed these studies in both young and mature NZW and SMHL rabbits. In the mature rabbits, total cholesterol output was increased in the SMHL rabbits, but not significantly so, and there was no difference in total triglyceride output. $VLDL_1$, $VLDL_2$, IDL and LDL were separated by density gradient ultracentrifugation, and cholesterol, triglyceride and apoB measured in each fraction. For the analysis of results, $VLDL_2$, IDL and LDL were grouped together to form a 'cholesterol rich' non $VLDL_1$ fraction. In the mature rabbits $VLDL_1$ and non $VLDL_1$ cholesterol output rates were increased but not significantly so. There was no difference in $VLDL_1$ triglyceride output rate, but non $VLDL_1$ triglyceride output rate was significantly increased in SMHL rabbits ($p < 0.05$). Output rates of apoB showed much greater differences, with non $VLDL_1$ apoB output rate exhibiting a 6 fold elevation in the SMHL rabbits compared to the NZW rabbits ($p = 0.01$). In the young rabbits, cholesterol output rate was significantly increased 2 fold ($p < 0.01$) in the SMHL rabbits, and triglyceride output rate was increased 3 fold (NS). $VLDL_1$ and non $VLDL_1$ cholesterol output rates were significantly increased (4 fold, $p < 0.05$ and 5 fold, $p < 0.05$ respectively). Non $VLDL_1$ triglyceride output rate was significantly increased 6.5 fold ($p < 0.05$) in SMHL rabbits. Again, output rates of apoB showed large differences, $VLDL_1$ apoB output rate was significantly increased 3.5 fold ($p < 0.05$) in SMHL rabbits, and non $VLDL_1$ apoB output rate was significantly increased 13 fold in SMHL rabbits ($p < 0.005$).

To investigate the genetics of the disorder, based on the information we had collected, we identified a group of candidate genes that had the potential to contribute to the

phenotype. Using reverse transcriptase polymerase chain reaction, we obtained a measure of the relative expression of the candidate genes in hepatic mRNA. We examined transcription of apoB, VLDL receptor, LDL receptor, CETP, microsomal triglyceride transport protein (MTP), protein disulphide isomerase (PDI) and cholesterol 7 alpha-hydroxylase, and found that, in relation to a housekeeping gene, glyceraldehyde 3 phosphate dehydrogenase (GAPDH), apoB, CETP and VLDL receptor mRNAs were present at significantly higher levels in SMHL rabbits ($p = 0.005$, $p = 0.002$, $p = 0.02$ respectively) than NZW rabbits. There were no significant differences in any of the other genes between the rabbit strains.

Therefore, we concluded that the SMHL rabbits exhibit a number of characteristics in common with the disorder familial combined hyperlipidaemia, namely increased plasma cholesterol, plasma triglyceride or both, increased apoB production across the lipoprotein spectrum, a possible decreased catabolism of apoB containing lipoproteins, insulin resistance and increased CETP activity. The driving force is likely to be an increased rate of apoB synthesis due to an upregulation of translation of this gene, with ancillary effects on the VLDL receptor and CETP activity. Consequently, SMHL rabbits are, it appears, an excellent model for the human disorder familial combined hyperlipidaemia.

Author's Declaration

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

Hazel Alison Ardern
April 1999

Dedication

To my parents, to Moira and to John, with thanks for your understanding, support and encouragement.

Chapter 1. Introduction

'I know what *we've got to do,' said Hazel, 'but I still can't see* how.' *'We're going to need some new ideas,' agreed Blackberry. They knew that it was on these and on nothing else that their lives, and the lives of their children, depended.*

Richard Adams, Watership Down

1.1. The prevalence of Coronary Heart Disease

Coronary heart disease (CHD) is the most common cause of death in the Western world, with rates in the United Kingdom, and particularly Scotland, being amongst the highest (Marmot, 1988). Keys (1957,1970) first suggested that this could be due to differences in diet, and showed that although the Japanese have one of the lowest cholesterol levels and CHD prevalence at home, when they emigrated to California they took on the lifestyle and subsequently the CHD risk of an American (Keys, Minnesota, Kimura, *et al*, 1957).

Indeed, countries such as the USA and Finland have undertaken successful education programmes, recommending dietary and lifestyle changes that have more than halved their CHD deaths (Stamler and Stamler, 1984, Puska, Vartiainen, Tuomilehto, *et al*, 1998). In 1992 the Secretary of State for Health in the United Kingdom proposed that by the year 2000, death rates from CHD should be reduced by 40% in the under 65s, and by 30% in those aged 65 - 74, by making lifestyle changes such as a reduction in smoking, obesity, plasma cholesterol and blood pressure.

However, not all lipid related CHD risk is due to diet. There is a strong genetic component which for affected individuals increases risk. A number of common genetic hyperlipidaemias have been described which affect up to 0.5% of the population but are found in 10% of subjects who have had a myocardial infarction (MI). The characterisation of the metabolic basis of genetic hyperlipidaemia will assist not only in understanding the specific problem but will offer general insight into the regulation of lipoprotein metabolism and new approaches to lipid lowering treatments.

1.2. Lipids

The term lipid defines a heterogeneous group of substances which contain fatty acids (FA) or are of steroid derivation. They require organic solvents to solubilise them as they are immiscible in water. Cholesterol (and cholesteryl ester), triglyceride and phospholipid are the major lipids in the human body.

1.2.1 Cholesterol

Cholesterol is an essential component of cell membranes and plays a major role in controlling their fluidity. It is the main sterol in the human body and is the precursor of bile acids, oestrogens, androgens, vitamin D and other steroid hormones. It can exist as free cholesterol, or as cholesteryl ester when complexed with a fatty acid. Cholesterol is synthesised *de novo* in the body from acetyl Coenzyme A (acetyl-CoA), in a series of steps regulated by the enzyme 3-hydroxy, 3-methylglutaryl-Coenzyme A reductase (HMG CoA reductase). The other source of cholesterol is the diet.

The liver is of central importance in cholesterol transport in the body. It regulates lipoprotein production and much of the catabolism of these lipid: protein complexes. Hepatic cholesterol homeostasis is regulated via the activation of key enzymes such as HMG CoA reductase (cholesterol synthesis), acyl coenzyme A:cholesterol acyltransferase (ACAT) (cholesterol esterification) and cholesterol 7 alpha-hydroxylase (conversion of cholesterol to bile acids for excretion). The function of cholesterol of greatest interest for the present discussion is as a component of plasma lipoproteins.

1.2.2 Triglyceride

Triglycerides, also called triacylglycerols, are formed by esterifying the hydroxyl groups of glycerol with FA. Fatty acids are hydrocarbon chains of varying length, and can be saturated (no double bonds) or unsaturated (one or more double bonds). Long chain FA circulate bound to albumin in the plasma (Spector, 1975), while short chain FA are soluble. Triglycerides are the major energy store in mammals, providing 9 kcal/g, and are the form in which most FA are stored, with 95% of the body's

triglyceride being found in the adipose tissue. Triglycerides are synthesised by many tissues, with the liver being the main site, using circulating free fatty acids (FFA). Ingested fat provides the FA for triglyceride synthesis in the gut. Circulating triglycerides in lipoproteins are hydrolysed to provide FA supplies for energy production by the muscle or for storage in the adipose tissue (reviewed in Coppack, Jensen and Miles, 1994). *De novo* synthesis from acetyl-CoA derived from glucose and other dietary carbohydrates, is also a source of FA for triglyceride synthesis.

1.2.3 Phospholipid

Phospholipids, like triglycerides, are derived from glycerol. Fatty acid chains are esterified to the glycerol backbone at positions 1 and/or 2, and a phosphoric acid is found at position 3, attached to another small molecule, that gives the phospholipid specific features and contributes to its amphipathic nature. The majority are synthesised by the liver, but all tissues can make phospholipids. These lipids act as emulsifiers, enabling hydrophobic molecules such as cholesteryl ester and triglyceride to exist in aqueous solutions. They form a protective barrier by lining up with their polar head groups towards the aqueous environment, and their non polar hydrocarbon chains towards the hydrophobic inside. Because of these properties, phospholipids are an essential component (40-50%) of biological membranes.

1.3. Lipoproteins

1.3.1 Human lipoproteins

Mammals and many other higher species transport neutral lipids (cholesteryl ester and triglyceride) in aqueous solutions such as plasma in the form of lipoprotein particles. These consist of a hydrophobic core of neutral lipid, surrounded by a hydrophilic monolayer of phospholipid, free cholesterol and protein. Plasma lipoproteins vary between species, with the most detailed investigations having been performed on human lipoproteins. There are five discrete populations of human lipoproteins that can be distinguished by composition, size and density (Table 1.1.). These are chylomicrons, very low density lipoproteins (VLDL), intermediate density lipoproteins (IDL), low density lipoproteins (LDL) and high density lipoproteins

(HDL). These can be grouped into two main families on the basis of their apolipoprotein (apo) content - apoA containing lipoproteins (HDL), and apoB containing lipoproteins (VLDL, IDL and LDL containing apoB-100 and chylomicrons containing apoB-48). Each lipoprotein class can be further subdivided into discrete subpopulations with different functions.

Table 1.1. % composition by weight, density and size of human plasma lipoproteins

	Chylo	VLDL	IDL	LDL	HDL
CE	2	7	8	10	4
Triglyceride	84	55	32	9	4
FC	5	12	23	38	14
Phospholipid	7	18	21	22	28
Protein	2	8	16	21	50
Density (g/ml)	<0.95	<1.006	1.006 - 1.019	1.019 - 1.063	1.063 - 1.021
Flotation Rate (Sf value, F value for HDL)	>400	20 - 400	12 - 20	0 - 12	0 - 9
Diameter (Å)	> 800	300 - 800	250 - 300	200 - 250	75 - 200

Chylomicrons (chylo), cholesteryl ester (CE), free cholesterol (FC)
Sf values (Svedberg Flotation Units measured against a density of 1.063 g/ml), and F values (Svedberg Flotation Units measured against a density of 1.21 g/ml).
(Warwick, 1991)

Lipoprotein fractions can be separated by centrifugation on the basis of their density and flotation value (Alaupovic, Gustafson, Sanbar, *et al*, 1964), by electrophoresis (paper or gel) on the basis of charge (Fredrickson, Levy and Lees, 1967a) or by size (Mills, Lane and Weech, 1984). Each class has a specific role in the transport of lipid, but all act coordinately to control lipid flux throughout the body.

1.3.2 Chylomicrons

Chylomicrons are very large triglyceride rich lipoprotein particles, formed exclusively in the mucosal cells of the small intestine from absorbed dietary fat (Figure 1.1.). Chylomicrons are distinguishable from the other lipoproteins as they are the only class to contain apoB48 (see section 1.4.3). ApoAI, AII and AIV, apoCs and apoE are also associated with chylomicrons (Table 1.3.). In humans, chylomicron levels peak 2-4 hours after a meal and then return to fasting levels within 8 hours and so the particles are usually absent in the fasting state. In the rabbit the study of chylomicrons is complicated by the fact that they take longer to fast due to their coprophagic behaviour.

1.3.3 Very low density lipoprotein

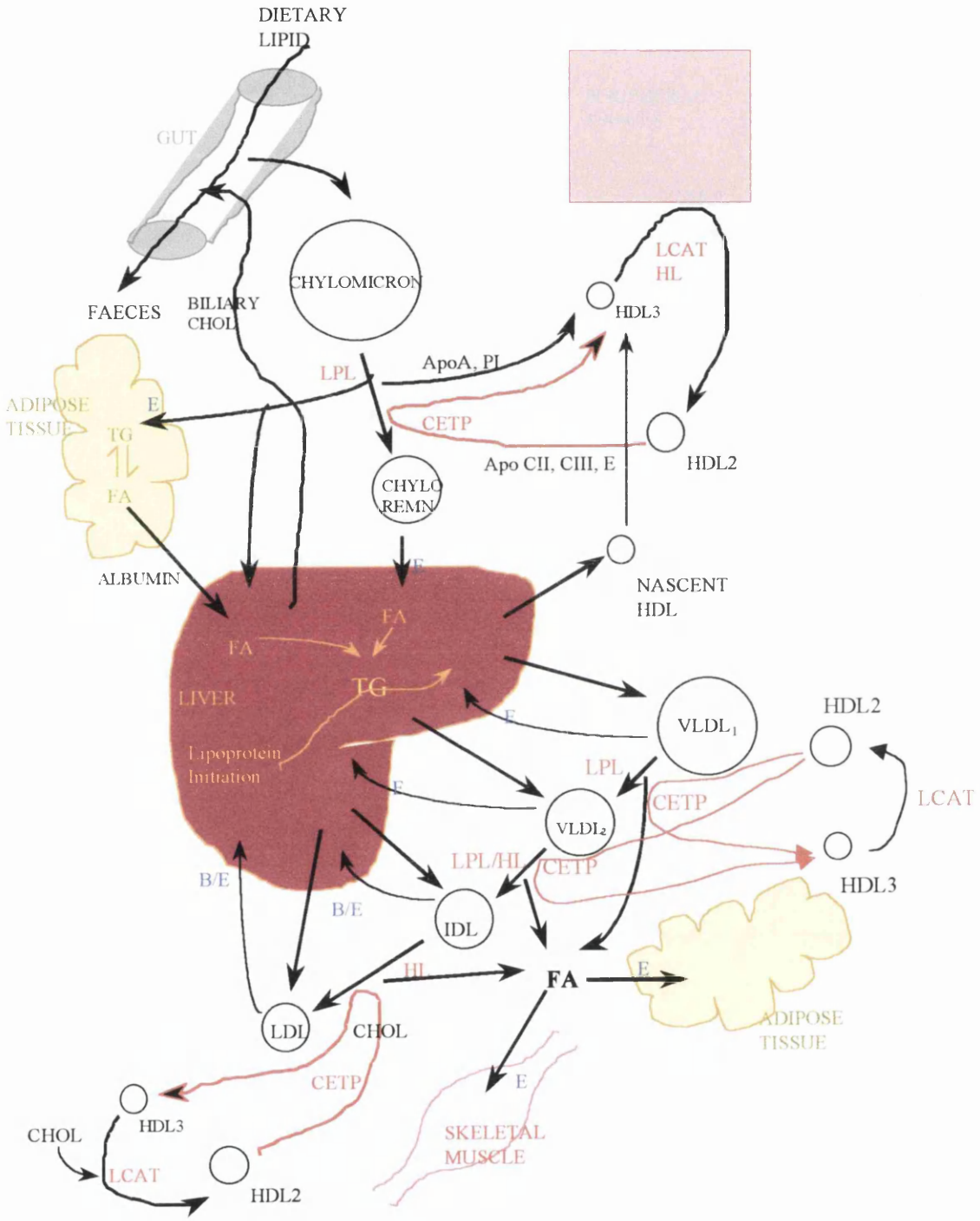
VLDL are triglyceride rich lipoproteins, similar to chylomicrons but with two major differences - the apoB is the full length apoB100, and the triglycerides are from an endogenous source. VLDL are synthesised in the liver, with their lipid content reflecting that of the hepatocyte. VLDL contain apoB100, apoCs and apoE on their surface (Table 1.3.).

VLDL particles are commonly subfractionated into two groups - large triglyceride rich $VLDL_1$ (Sf 60 - 400), and small, relatively cholesteryl ester rich $VLDL_2$ (Sf 20 - 60). These subfractions have distinct metabolic properties; both can be synthesised by the liver, but $VLDL_2$ can also be formed as a result of catabolism of $VLDL_1$. Kinetic studies have shown that $VLDL_1$ and $VLDL_2$ are metabolised down parallel lipolytic pathways and generate LDL products of differing properties (Packard, Munro, Lorimer, *et al,* 1984).

1.3.4 Intermediate density lipoprotein

IDL are also known as VLDL remnants, however they are a distinct subspecies which are important in their own right and are believed to be atherogenic. They are thought to be directly synthesised by the liver, as well as being formed as a result of catabolism

Figure 1.1. Overview of lipoprotein metabolism



Lipid is absorbed into the body from the diet in the form of chylomicrons. Triglyceride (TG) from these particles is hydrolysed by LPL and stored in adipose tissue, and apolipoproteins and phospholipid from chylomicrons are transferred to newly forming HDL particles. These newly forming HDL obtain cholesterol from peripheral tissues, and CETP exchanges cholesterol in the HDL particles with triglyceride in the chylomicron particles, forming chylomicron remnants (chylo remn). In this way, cholesterol is transferred from peripheral tissues to the liver, with chylomicron remnants being taken up by the liver via the apoE recognising LRP. Triglyceride in the adipose tissue is transported to the liver as FA bound to albumin, and is secreted from the liver as $VLDL_1$, $VLDL_2$, IDL or LDL. These particles can be taken back up into the liver by the LRP or the LDL receptor, or can be delipidated by the action of LPL and HL. FA liberated by these enzymes is delivered to adipose tissue or skeletal muscle. Additionally, VLDL or IDL particles can be taken up by adipose tissue of skeletal muscle via the apoE recognising VLDL receptor. Cholesterol is also transported to VLDL, IDL and LDL particles from HDL in exchange for triglyceride, a process mediated by CETP. Cholesterol from peripheral tissues can be delivered to these apoB100 containing particles and hence to the liver, via HDL, in processes that involve LCAT and CETP.

of VLDL. IDL can be cleared from the circulation by the liver, or can be further catabolised by hepatic lipase (HL) to LDL. Humans have relatively low levels of IDL compared to other subfractions, however rabbits have much more substantial IDL concentrations. IDL contains apoB100, apoC and apoE (Table 1.3.), and has been shown to consist of two subpopulations on gradient gel electrophoresis (Musliner, Giotas and Krauss, 1986, Meyer, Caslake and Packard, 1997)

1.3.5 Low density lipoprotein

The majority of cholesterol in the plasma is carried in LDL. LDL are formed from the delipidation of IDL, and may also be directly synthesised by the liver (Huff, Telford, Woodcroft, *et al*, 1985, Ginsberg, 1995, Packard and Shepherd, 1997). ApoB100 constitutes 95% of the protein on LDL, and there is only one apoB per particle. LDL are cleared from the plasma by the hepatic LDL receptor, and also by receptor-independent mechanisms that are currently poorly characterised, resulting in the delivery of cholesterol to peripheral tissues. Subclasses of LDL exist with differing atherogenic potential. In normolipidaemic subjects, 3 subclasses can be readily identified (LDL I, LDL II and LDL III) (Griffin, Caslake, Yip, *et al*, 1990). Small, dense LDL III is most abundant in subjects with raised plasma triglyceride levels and is held to be the most atherogenic. It has a longer residence time in the circulation, is more susceptible to oxidation, has been shown to bind more readily to arterial wall proteoglycans (Anber, Millar, McConnell, *et al*, 1997) and due to its small size is easily taken up into the arterial wall.

1.3.6 High density lipoprotein

In humans, HDL is synthesised both by the liver and the small intestine. The main protein is apoAI, but HDL also contains apoAII, apoC and apoE (Table 1.3.). HDL is commonly subdivided into HDL_2 (d = 1.063 - 1.125 g/ml) and HDL_3 (d = 1.125 - 1.21 g/ml).

HDL is involved in reverse cholesterol transport. In this process free cholesterol from cells and the surface layer of triglyceride rich lipoproteins is captured in HDL and

esterified by the action of LCAT. Cholesteryl esters from HDL are then either taken up directly by the liver (Sherrill, Innerarity and Mahley, 1982, Franceschini, Maderna and Sirtori, 1991) or passed to VLDL and LDL by the action of CETP. The acceptor lipoprotein is then subject to receptor mediated degradation in the liver. This mechanism of transport of cholesterol from the peripheral tissues back to the liver for excretion gives the HDL particle its anti-atherogenic properties.

1.3.7 Lipoprotein(a)

Lipoprotein(a) (Lp(a)) is an LDL sized cholesterol rich lipoprotein particle containing apoB and apo(a). However, unlike LDL, Lp(a) is not derived from a triglyceride rich precursor, it is probably secreted directly from the liver. Increased levels of Lp(a) have been associated with CHD (Dahlen, Guyton, Attar, *et al,* 1986). Lp(a) has been found to accumulate in atherosclerotic plaques (Smith and Cochran, 1990) and to have a role in the atherothrombotic process (Chapman, Huby, Nigon, *et al,* 1994).

1.3.8 Rabbit lipoproteins

The lipoprotein profile of the rabbit is distinguishable from that of the human mainly by the fact that there is a significant IDL fraction while generally VLDL and LDL fractions are substantially lower than in humans (Chapman, 1980). There is evidence (Ghiselli, 1982) that LDL can be directly synthesised in rabbits in addition to being a delipidation product. HDL is the dominant lipoprotein class, being present in higher concentrations than in humans.

The composition of rabbit lipoproteins (Table 1.2.) is variable as reported in the literature (reviewed in Chapman, 1980), due to the variety of strains and the effects of diet on lipoprotein concentrations and compositions. VLDL and IDL have an intermediate composition between human VLDL and LDL. Rabbit LDL is triglyceride enriched and cholesterol poor in relation to human LDL. However, rabbit VLDL, LDL and HDL are of similar size range, structure and surface charge as human species.

Table 1.2. Percentage composition (by weight) and size of plasma lipoproteins in New Zealand White (NZW) rabbits

	VLDL	IDL	LDL
CE	6.6 ± 1.1	23.5	19.3 ± 3.5
Triglyceride	62.2 ± 0.4	30.6	26.4 ± 3.0
FC	3.7 ± 0.4	6.3	5.1 ± 0.5
Phospholipid	15.6 ± 2.1	19.7	20.0 ± 2.7
Protein	11.9 ± 1.3	19.9	29.3 ± 2.8
Diameter (Å)	440	249	222

(Havel, Kita, Kotite, *et al*, 1982)

Rabbit apolipoproteins are similar to those in humans, although not entirely homologous (Shore and Shore, 1976). One aspect of lipoprotein metabolism in the rabbit that makes it a much more suitable model for the human situation than for example the rat, is that apoB48 is synthesised solely in the intestine, and apoB100 in the liver. Additionally, hepatic cholesterol synthesis occurs at similar rates in the rabbit and the human (20 and 16% of the rat level respectively). Furthermore the rabbit has similar properties of LCAT and cholesteryl ester transfer activity in plasma to humans (De Parscau and Fielding, 1984).

1.4. Apolipoproteins

Apolipoproteins are the protein 'couriers' on lipoprotein particles that help solubilise cholesterol and triglyceride for transport (Table 1.3.). They regulate the activities of enzymes important in lipoprotein metabolism, and act as ligands for targeting lipoproteins to specific cell receptors (Mahley, Innerarity, Rall, *et al,* 1984). Apolipoproteins share many common features and are believed to have evolved from a common origin, as their amino acid sequences show striking homologies.

Table 1.3. Properties of apolipoproteins

Apo	Molecular Weight	Origin	Lipoprotein Association	Functions
AI	28 500	Liver, intestine	HDL, chylomicrons	Activates LCAT
AII	17 400	Liver, intestine	HDL, chylomicrons	Activates HL, inhibits LCAT
AIV	46 000	Intestine	Chylomicrons	Activates LCAT, activates LPL via apoCII
B100	512 000	Liver	VLDL, IDL, LDL	Triglyceride transport (VLDL), receptor-mediated LDL catabolism
B48	241 000	Intestine	Chylomicrons	Triglyceride transport
CI	6 300	Liver, intestine	HDL, VLDL, chylomicrons	Activates LCAT, lipase
CII	8 800	Liver, intestine	HDL, VLDL, chylomicrons	Activates LPL
CIII	8 800	Liver, intestine	HDL, VLDL, chylomicrons	Inhibits LPL
E	35 000	Liver, brain, macrophages	VLDL, IDL, HDL	Receptor mediated catabolism
(a)	200 000 - 700 000	Liver	Lp(a)	Not known

Lipoprotein lipase (LPL)

Adapted from Schaefer, Eisenberg and Levy (1978) and Laker and Evans (1996).

1.4.1 Apolipoprotein (a)

Apo(a) is highly glycosylated and exists in more than 30 different isoforms which vary in size from 200 to 700 kDa. This is due to the presence of a variable number of

kringle 4 repeats. The plasma concentration of Lp(a) in general appears to be inversely related to the size of the apo(a) (Gaw, Boerwinkle, Cohen, *et al,* 1994). Apo(a) is synthesised by the liver, and attaches to apoB by a disulphide bond, which causes the Lp(a) particle to be cleared less efficiently by the LDL (B/E) receptor. However, Lp(a) in familial defective apoB (FDB) and familial hypercholesterolaemia (FH) patients is cleared at the same rate as normals, therefore there must be a clearance mechanism other than the LDL receptor (Perombelon, Gallagher, Myant, *et al,* 1992). Apo(a) has a high degree of homology with plasminogen and an interference with the thrombolysis pathway has been suggested as a potential mechanism for the atherogenicity of Lp(a) (Maher and Brown, 1995).

1.4.2 Apolipoprotein A

There are three main forms of apoA - apoAI, apoAII and apoAIV. The intestine is capable of synthesising all apoA proteins, but apoAI and apoAII are also synthesised in the liver. The genes for apoAI and apoAIV are found on chromosome 11, while that for apoAII is found on chromosome 1. ApoAI, the main apolipoprotein on HDL, is considered important in reverse cholesterol transport and is also found on chylomicrons. ApoAII is found mostly on HDL. Both apoAI and apoAII act as cofactors for LCAT. ApoAIV is found on chylomicrons and in small amounts on HDL, but the majority is free in plasma. It may also act as an activator of LCAT.

1.4.3 Apolipoprotein B

The apoB gene was the last of the major human apolipoprotein genes to be cloned. (Chen, Yang, Chen, *et al,* 1986). The apoB gene is approximately 43 kb long, containing 29 exons. ApoB is heterogeneous, but exists in two main forms: hepatic apoB100, a 512 kDa protein and intestinal apoB48, a 241 kDa protein. These proteins are synthesised from the same gene situated on chromosome 2, with apoB48 consisting of the N-terminal 48% of apoB100 (Marcel, Hogue, Theolis, *et al* 1982). A novel post-transcriptional modification of the apoB100 mRNA sequence occurs in intestinal cells only, which produces a stop codon at position 2153 so that the message when translated produces apoB48 (Powell, Wallis, Pease, *et al,* 1987). A cDNA was cloned and identified (Teng, Burant and Davidson, 1993) as apoB mRNA

editing enzyme catalytic polypeptide-1 (APOBEC-1), which in the presence of other components of the apoB mRNA editing enzyme, caused the site-specific deamination of a cytosine to a uracil (Davidson, Anant and MacGinnitie, 1995).

ApoB48 is found only on chylomicrons, and does not bind to the LDL (B/E) receptor as the receptor binding site of apoB is found in the C-terminal half of the protein. ApoB100 is the main protein associated with VLDL, IDL and LDL, accounting for more than 95% of the protein on LDL, and is necessary for their secretion and uptake by the LDL (B/E) receptor. Unlike the other apolipoproteins, apoB is not exchanged between lipoproteins.

1.4.4 Apolipoprotein C

There are 3 different apoCs - apoCI, apoCII and apoCIII. They are derived from the liver and gut and are found on HDL from where they are transferred to VLDL and chylomicrons. The apoCI and apoCII genes are found on chromosome 19, while the apoCIII gene is on chromosome 11, in a cluster with apoAI and apoAIV.

ApoCI is an activator of LCAT, and also binds phospholipids. ApoCII is the activator of LPL and people deficient in apoCII are severely hypertriglyceridaemic. ApoCIII appears to have an inhibitory effect on the hydrolysis and clearance of chylomicrons and VLDL via LPL (Thompson, 1994) and is the most abundant of the C apolipoproteins.

1.4.5 Apolipoprotein E

The apoE gene is found on chromosome 19, adjacent to apoCI. The gene has similarities to apoAI and apoAIV, suggesting a common ancestral origin. ApoE, a 34 kDa protein, exists in three different isoforms, $apoE_2$, $apoE_3$ and $apoE_4$, which have different avidity for the lipoprotein receptors. ApoE is synthesised in the liver and is found on chylomicron remnants, VLDL and large HDL particles. ApoE is involved in the hepatic uptake of chylomicron remnants and VLDL via the LDL receptor and the LDL receptor related protein (LRP, section 1.5.3).

1.5. Lipoprotein metabolism

Every day, grams of lipid are both absorbed by the intestine and synthesised by the liver and intestine, and require to be transported to peripheral tissues where the lipid is stored, or used (Figure 1.1.). The liver is responsible for the packaging of cholesterol and triglyceride into apoB containing lipoproteins, preferring to use FA from the diet or from adipose tissue stores rather than newly synthesised lipid. ApoA containing lipoproteins recycle excess cholesterol from the tissues to apoB containing lipoproteins (a process known as reverse cholesterol transport), and these cholesterol rich lipoproteins are subsequently cleared by the liver.

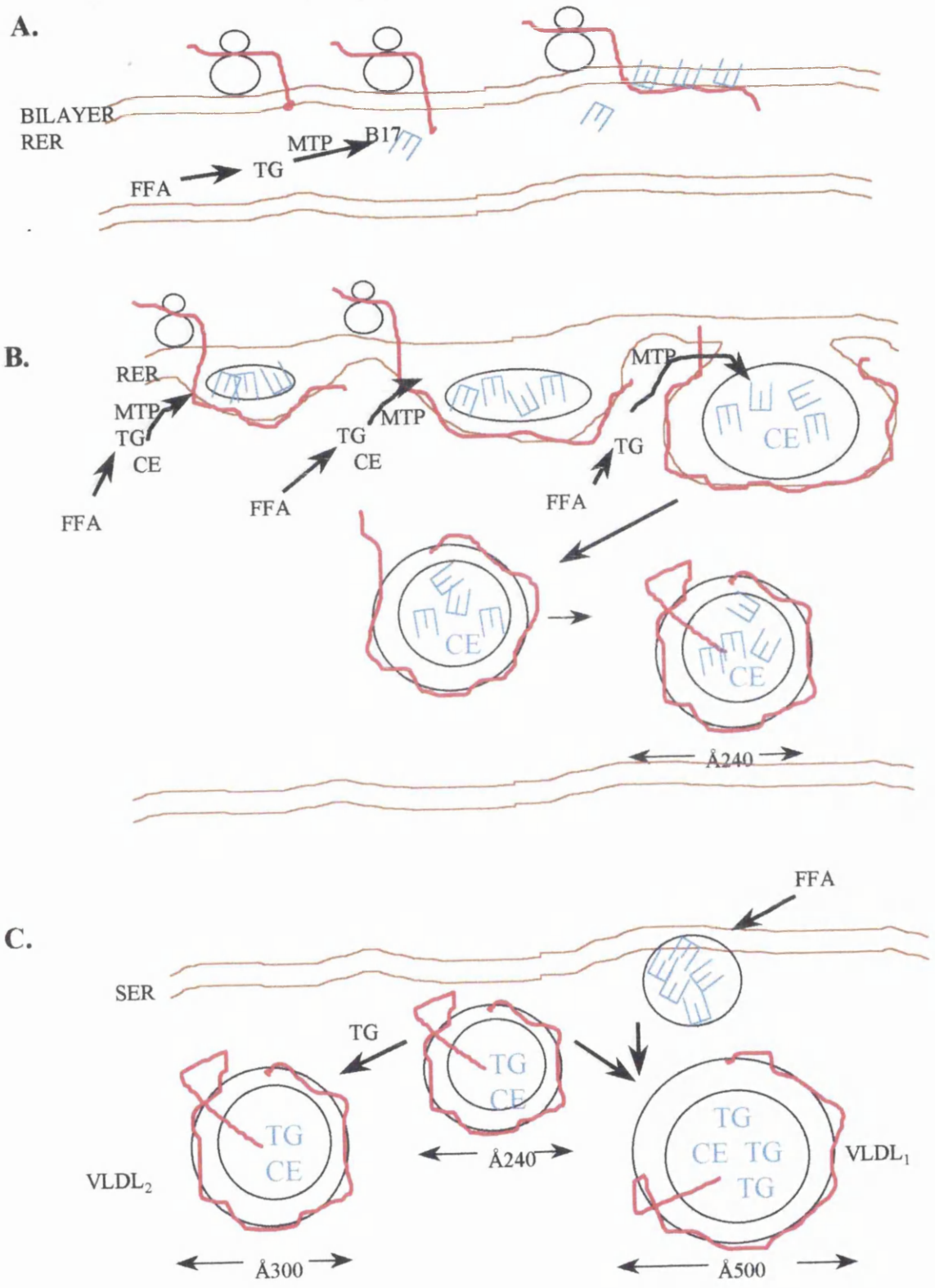
1.5.1 Lipoprotein production

Lipoproteins are synthesised in two sites - chylomicrons are synthesised by the intestine, and VLDL, IDL and LDL by the liver. The components of chylomicrons are synthesised, assembled and secreted by cells of the intestinal mucosa. Absorption of fat stimulates intestinal synthesis of apolipoproteins and lipids. Cholesterol from bile, the diet or from mucosal cells shed during renewal of the lining of the intestine, is also present. Chylomicrons are large, apoB containing, triglyceride rich lipoproteins, consisting of 95% triglyceride and 2% cholesterol by weight. They are secreted into intestinal lymph and thence into the circulation where they acquire apoE and apoCII. ApoCII is the co-factor for LPL which hydrolyses the triglyceride within the chylomicron into FA which are taken up by adipose tissue and muscle for storage or for use as energy. Cholesteryl ester is transferred from HDL to chylomicrons in return for phospholipid and apolipoproteins, and within only a few minutes of the chylomicron entering the circulation, it has been converted to a chylomicron remnant which is taken up by the LRP in the liver with apoE as the ligand, thereby delivering cholesteryl ester to the liver for storage or for the synthesis of VLDL and bile acids (Figure 1.1.).

Lipoprotein particles produced by the parenchymal cells of the liver are mainly VLDL, the main transporter of triglyceride in the post absorptive state, but there is evidence that smaller particles, IDL and LDL are directly secreted from the liver too (Packard, *et al*, 1984, Huff *et al*, 1985, Packard and Shepherd, 1997). The lipid content of the

lipoprotein particles reflects that of the hepatocyte. VLDL are synthesised in a two-step process (Figure 1.2.) (Schumaker, Phillips and Chatterton, 1994) and secreted after approximately 30 minutes. ApoB is co-translationally translocated into the rough endoplasmic reticulum (RER) and in the process it is glycosylated and associated with lipid to allow the apoB to take on the correct conformation. The lipid forms a core around which the apoB can fold. This may possibly involve pauses in translocation. The rate of secretion of apoB in lipoproteins is thought to depend on the availability of lipid as apoB protein is apparently produced at a constant rate. Proper association of the lipid with the apoB is critical for efficient secretion of VLDL as lipid and molecular chaperones promote the correct folding of apoB. Factors that decrease the availability of lipid can block VLDL secretion and result in degradation of the apoB. Microsomal triglyceride transfer protein (MTP), a heterodimer of protein disulphide isomerase (PDI) and a 98kDa specific subunit, is important in delivering triglyceride and cholesteryl ester to the RER for this purpose, and PDI catalyses the formation of disulphide bonds in apoB. An absence of MTP can cause abetalipoproteinaemia (Wetterau, Aggerbeck, Bouma, *et al*, 1992) and an MTP inhibitor has been shown to normalise atherogenic lipoprotein levels in the Watanabe rabbit (Section 1.7.1) (Wetterau, Gregg, Harrity, *et al*, 1998). The second step of the assembly process occurs in the smooth endoplasmic reticulum where a large triglyceride droplet combines with the LDL sized apoB particle to produce a VLDL particle. This is then secreted via the golgi and the space of Disse into the circulation. If the apoB fails to associate with sufficient lipid during particle initiation, it is internally degraded. Degradation can occur throughout lipoprotein assembly (Dixon and Ginsberg, 1993).

Figure 1.2. Synthesis of VLDL



A. ApoB (shown in red) is cotranslationally translocated into the lumen of the RER. The N terminus forms a globule, and as MTP adds lipid to the membrane nearby, the globule unfolds. Some apoB associates with the membrane. ApoB17 is the largest apoB found unassociated with lipid. If lipid is not available, as the apoB grows larger it becomes unstable and is degraded by molecular chaperones.

B. MTP adds more lipid (mainly TG (E), but also cholesteryl ester) to the developing apoB. At apoB90, enough lipid is added and the particle buds off. The remaining 10% of apoB forms a bow structure across the lipoprotein particle.

C. Small amounts of triglyceride are added to this lipoprotein to make $VLDL_2$, or it associates with a triglyceride droplet in the smooth endoplasmic reticulum (SER) to form $VLDL_1$. These VLDL particles are secreted from the cell via the golgi apparatus and the space of Disse. If at any stage there is an insufficiency of lipid, the apoB can become unstable and be internally degraded.

1.5.2 Lipolysis and interconversion of lipoproteins

Circulating VLDL obtains apoCs and apoE from HDL. More than 90% of the triglycerides are hydrolysed by LPL, and much of the apoC and apoE recycled to HDL. The particle becomes smaller in a stepwise manner and forms IDL and subsequently through the action of HL (an enzyme found in liver sinusoids), LDL (Figure 1.1.). CETP transfers cholesteryl ester from HDL to the VLDL, and by the time LDL is formed, all apolipoproteins except apoB are lost. Both IDL and LDL can be cleared from the circulation by the liver. Some VLDL may also be cleared by the liver. The metabolism of VLDL, IDL and LDL particles can be traced readily as apoB stays with each particle throughout its lifetime in the circulation.

HDL particles are important in removing cholesterol from extrahepatic tissues. This reverse cholesterol transport mechanism is vital in the return of cholesterol to the liver for excretion (Figure 1.1.). Cholesterol from peripheral tissues and from cell membranes is transferred to small HDL_3. LCAT acts to esterify the cholesterol within the HDL, which maintains a gradient for free cholesterol. As the particles enlarge, they become HDL_2 and some cholesteryl ester is transferred to apoB containing lipoproteins by CETP in exchange for triglyceride. Cholesterol can also be returned to the liver directly from HDL (Sherrill, *et al*, 1982, Franceschini, *et al*, 1991). Triglyceride in HDL is hydrolysed by HL and the HDL becomes an HDL_3 particle again.

1.5.3 Lipoprotein catabolism

Catabolism of lipoproteins is by receptor mediated or receptor independent mechanisms. The LDL receptor (Goldstein and Brown, 1977), a transmembrane glycoprotein found on chromosome 19, is the main receptor involved in the clearance of apoB containing lipoproteins. This receptor recognises apoB or apoE containing lipoproteins via their arginine and lysine rich regions which bind to the receptor and facilitate the catabolism of VLDL, IDL and LDL. That the LDL receptor is the main method of uptake of LDL is demonstrated by accumulation of the lipoprotein when the function of the LDL receptor is lost in FH. The LDL receptor is found mainly in the liver and the adrenal cortex, and also to some extent in the intestine and skin

fibroblasts. In fact the LDL receptor enables uptake of cholesterol by all cells of the body when faced with cholesterol depletion.

In rabbits, 90% of LDL is catabolised by the LDL receptor, with the remainder catabolised by receptor independent mechanisms. The liver is responsible for the catabolism of 50-70% of the total LDL (Pittman, Carew, Attie, *et al,* 1982).

The LRP binds apoE containing lipoproteins but not LDL. Its activity is promoted by LPL and HL, which mediate lipoprotein binding if present on the particle's surface (Beisiegel, 1995). LRP is found on hepatocytes, fibroblasts, neurons, and monocyte-derived macrophages. It is thought that this receptor may be involved in atherogenesis by causing the accumulation of lipoproteins in macrophages and smooth muscle cells (Lupu, Heim, Backman, *et al*, 1994). Additionally, the abundance of LRP in neurons and the presence of apoE in the brain has led to the proposal that the LRP may play a role in apoE metabolism in the brain, and hence have a link to Alzheimers disease (Ji, Pitas and Mahley, 1998). Through apoE and lipase present as a ligand on the particle surfaces, the LRP binds chylomicron remnants, and is the main method of clearance of chylomicrons. The LRP also binds VLDL, acting as a back-up for clearing these particles.

The VLDL receptor (chromosome 9) which is structurally related to the LDL receptor, was first described in rabbits and binds apoE containing lipoproteins (VLDL and IDL) clearing them from the circulation (Takahashi, Kawarabayasi, Nakai, *et al*, 1992). It is expressed mainly in the heart, skeletal muscle, adipose tissue, brain and ovary. In rabbit aortic intima-media, expression of the VLDL receptor is upregulated by cholesterol feeding. Its basic function is likely to be to provide FA to muscle and adipose tissue in a mechanism complementary to that facilitated by LPL. The VLDL receptor is only present in very small amounts in the liver.

Receptor mediated clearance by the above mechanisms is in general saturable, due to there being a limited number of receptors. Therefore at times of lipoprotein excess in the circulation, receptor independent clearance becomes important. Such mechanisms include pinocytosis (engulfing of extracellular fluid by invagination of the plasma membrane) or adsorptive endocytosis by cell surface receptors with low affinity for

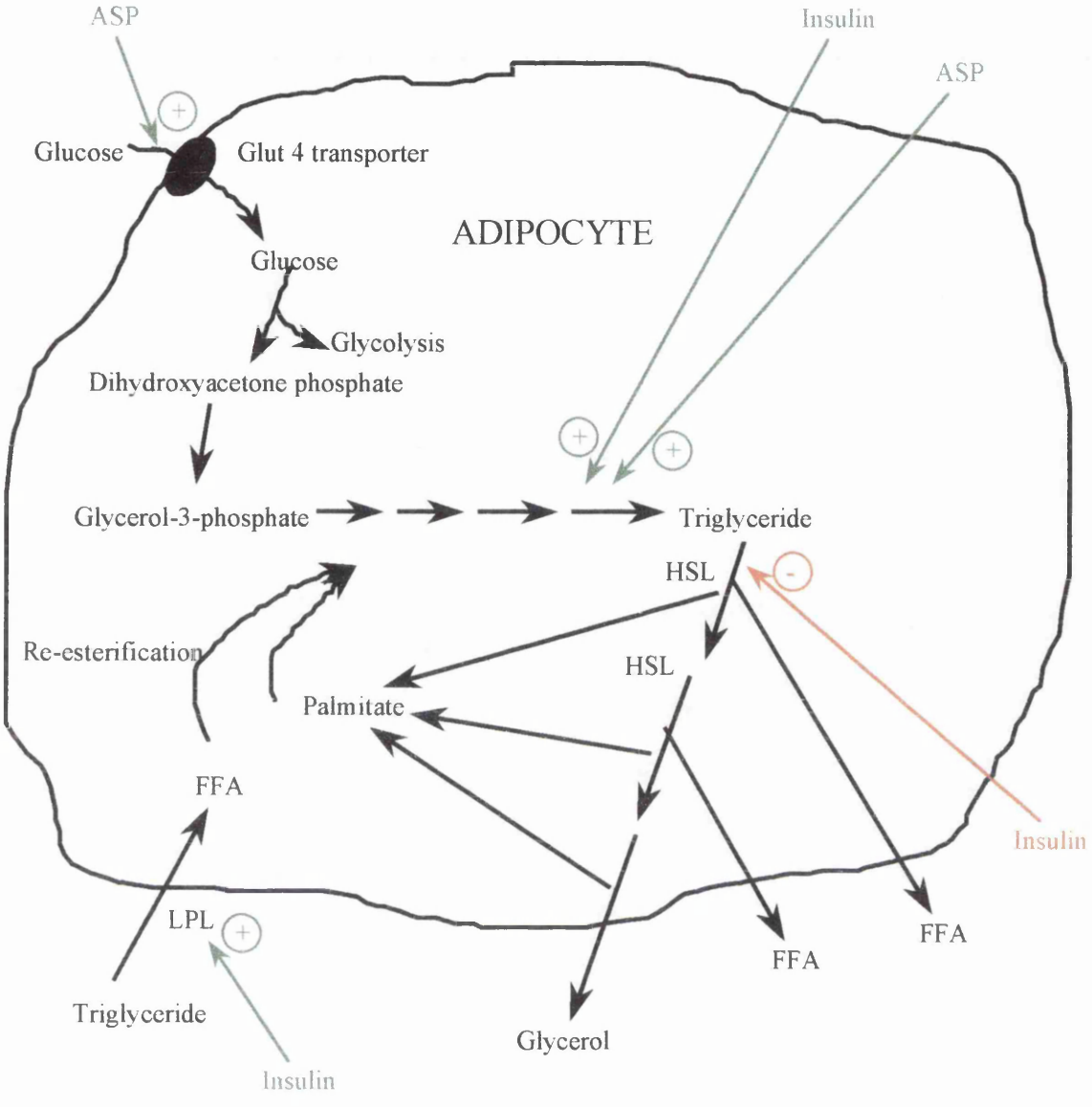
LDL. Receptor uptake of modified LDL may also occur by high affinity receptors on cells of the reticulo-endothelial system eg the acetyl-LDL receptor. Shepherd, Packard and Gibson (1985) demonstrated that the reticulo-endothelial system contributes significantly to catabolism of total human LDL *in vivo* in rabbits. However others (Pittman *et al*, 1982) using different techniques have found no major contribution of the reticulo-endothelial system to receptor independent clearance.

1.5.4 The effects of insulin

Insulin is the major anti-lipolytic hormone (Coppack *et al* 1994), acting mainly in the post-prandial state. In times of FFA and/or glucose plenty it causes adipose tissue or muscle to store triglyceride or glycogen, by inhibiting hormone sensitive lipase (HSL), thus suppressing FA release from the adipose tissue, or by stimulating glucose uptake and glycogen synthesis (Figure 1.3.). Insulin has been shown to decrease VLDL (and apoB) production, and to increase LPL activity on the adipose tissue surface (Durrington, Newton, Weinstein, *et al*, 1982, Patsch, Franz and Schonfeld, 1983, Sparks, Sparks, Bolognin, *et al*, 1986).

The role of insulin resistance in a number of metabolic disturbances was first described by Reaven (1988). Insulin resistance results in an elevated plasma triglyceride and FFA concentration, and a decreased HDL cholesterol concentration. There is also a preponderance of small dense LDL. Together these changes are known as the atherogenic lipoprotein phenotype (ALP). This loss of ability to control glucose and FFA levels results in hyperglycaemia and hypertriglyceridaemia, and is a common feature of disorders such as familial combined hyperlipidaemia (FCH), familial hypertriglyceridaemia (FHTG), non-insulin dependent diabetes mellitus (NIDDM) and obesity (Frayn 1993).

Figure 1.3. Adipocyte lipolysis



Glucose is taken up into the adipocyte by the Glut4 transporter. Triglyceride is hydrolysed to FFA by LPL and FFA are taken up into the adipocyte. Gucose and FFA are both stored as triglyceride in the adipocyte, and in times of energy surplus, insulin promotes the storage of triglyceride and inhibits the action of HSL which hydrolyses triglyceride to glycerol and FFA. Acylation stimulatory protein (ASP) also promotes the uptake of glucose, and its storage as triglyeride. Irregularities in the action of insulin and ASP have both been suggested as contributory factors to the development of the FCH phenotype.

1.6. Common Hyperlipidaemias

Several attempts have been made to form a definitive list of lipoprotein abnormalities. Initially, classification was by phenotype as the underlying cause of the abnormality was unknown. However the realisation that the same phenotype could have a different underlying cause, meant classification by genotype was necessary. As information on the genotypic causes of the various hyperlipidaemias is still incomplete it is easier to observe the phenotype, therefore phenotypic classification is still used widely. Originally Gofman, Rubin, McGinley, *et al* (1954) separated lipoproteins by analytical ultracentrifugation and described three patterns of lipoprotein distribution. Later Fredrickson, Levy and Lees (1967b) described 5 classes of hyperlipoproteinaemia which were subsequently modified by the World Health Organisation to include a 6th class (Table 1.4.).

Table 1.4. World Health Organisation classification of hyperlipoproteinaemia

Type	TC	LDL-C	TG	Lipoprotein abnormality	Underlying genetic disorder
I	↑	↓→	↑	Excess chylomicrons	LPL deficiency, ApoCII deficiency
IIa	↑→	↑	→	Excess LDL	FH, FCH and others
IIb	↑	↑	↑	Excess LDL and VLDL	FH, FCH and others
III	↑	↓→	↑	Excess chylomicron remnants and IDL	$ApoE_2E_2$ and other defects
IV	↑→	→	↑	Excess VLDL	FCH, FHTG
V	↑	→	↑	Excess chylomicrons and VLDL	FHTG, Apo CII deficiency

Total cholesterol (TC), LDL-cholesterol (LDL-C)

Beaumont, Carlson, Cooper, *et al*, 1970

1.6.1 Familial hypercholesterolaemia

Heterozygous FH (usually expressed as phenotype Type IIa) is seen in approximately 1 in 500 individuals in the UK, with homozygous FH being much more rare. Symptoms of CHD are seen by the mid twenties in heterozygotes, and in childhood in homozygotes, leading to premature death.

The molecular fault responsible for FH was first described in 1977 by Goldstein and Brown. A defect or deficit in the LDL receptor results in the inefficient catabolism of LDL and an increased plasma LDL concentration. A large number of mutations can cause the same effect, and can be broadly classified into those that affect receptor synthesis, those that affect the transport of the receptor from the RER to the golgi, those that interfere with the binding of LDL to the receptor, and those that affect the interaction of the LDL receptor with the clathrin coated pit important for its internalisation.

Heterozygotes have a plasma LDL fractional catabolic rate (FCR) that is approximately half of that in normal individuals, and only 20% of their LDL is cleared by receptors. Homozygotes have only trace amounts of LDL cleared by the receptor.

FDB is an example of why dyslipidaemia should be characterised by genotype rather than phenotype. It has the same phenotype as FH, but is due to a mutation in the apoB gene rather than the LDL receptor gene. Several different mutations have been described, all affecting the receptor binding region of apoB, rendering it incapable of binding to the LDL receptor (Myant 1993, Gaffney, Reid, Cameron, *et al*, 1995).

1.6.2 Familial hypertriglyceridaemia

Triglycerides are gaining credibility as an independent predictor of cardiovascular risk following results from large studies such as PROCAM (Assman and Schulte, 1992), and a meta-analysis of 17 prospective studies (Hokanson and Austin, 1996). Triglycerides are associated with atherogenesis by several mechanisms including effects on endothelial function, macrophage loading, thrombogenesis and LDL particle size. Hypertriglyceridaemic individuals are at an increased risk of type 2

diabetes. Raised triglycerides in association with small dense LDL and decreased levels of HDL cholesterol (the ALP) is the most common lipoprotein pattern seen in individuals who have had an MI.

Within the Fredrickson classification, there are two phenotypes associated with FHTG, type IV and type V, which differ in the extent to which triglycerides are elevated and can sometimes both occur in the one family. Type IV hyperlipoproteinaemia, FHTG, is characterised by increased levels of VLDL, and has a population frequency of 0.3%. It is not normally expressed in childhood. VLDL are larger than normal, and are triglyceride enriched, due to an increased triglyceride synthesis and a decreased FCR which is likely to be caused by saturation of the clearance mechanism. The catabolism of VLDL to LDL is lower than normal, therefore plasma cholesterol is not elevated. HDL cholesterol is also decreased. The underlying cause of FHTG is not known, but insulin resistance has been implicated in a number of studies (reviewed by Taskinen, 1995).

Type V hyperlipidaemia is a rare disease, sharing features of type I (chylomicronaemia) and type IV. These individuals are often obese, but evidence suggests that following weight loss the type V phenotype may change to type IV. The phenotype is not seen until adulthood. VLDL apoB synthesis is increased and the FCR is decreased to a greater extent than in type IV hyperlipidaemia. Severe hypertriglyceridaemia is a heterogeneous disorder, and is likely to have many causes. It may be due to LPL deficiency, the presence of an LPL inhibitor or a paucity of apoCII.

1.6.3 Familial combined hyperlipidaemia

FCH is a complex inherited disorder, defined by the presence of multiple phenotypes within a single family - elevated plasma cholesterol, elevated plasma triglyceride or both. It was first described in 1973 (Goldstein, Schrott, Hazzard, *et al*, 1973, Nikkilä and Aro, 1973, Rose, Kranz, Weinstock, *et al*, 1973) as being the most common genetic lipid disorder in a group of MI survivors and their relatives. The population frequency is 0.5%, with a frequency of about 10% in MI survivors aged under 60. Lipid levels are often normal in childhood, rising in the mid-twenties. FCH is

genetically distinct from FH and FHTG as the distribution pattern in affected families is different; children do not express hypercholesterolaemia in FCH although they do in FH, there is no abnormality in bile acid synthesis in FCH which is sometimes seen in FHTG, and FCH is a risk factor for premature CHD, whereas FHTG is apparently not.

Although FCH is expressed as a heterogeneous lipid phenotype there is thought to be an underlying common defect - increased plasma levels of apoB, probably due to an overproduction of this protein. VLDL and/ or LDL apoB can be overproduced (Kissebah, Alfarsi and Evans, 1984, Venkatesan, Cullen, Pacy, *et al*, 1993) in FCH and the particles are usually smaller than in normals (Chait, Albers and Brunzell, 1980, Hokanson, Krauss, Albers, *et al*, 1995). This makes the hypertriglyceridaemia different from that seen in FHTG, where there is a normal amount of VLDL particles, but they are large and triglyceride enriched. A subset of FCH may be due to the catabolism of VLDL and LDL at a slow rate (Chait *et al*, 1980, Aguilar-Salinas, Barrett, Pulai, *et al*, 1997). For example, studies in combined hyperlipidaemics of unknown genetics which may include subjects with FCH, have shown that these individuals most commonly exhibit a decreased catabolism of VLDL or LDL with or without an increased production (Millar, Watson, Stewart, *et al*, unpublished observations). Aguilar-Salinas *et al* (1997) have reported an FCH kindred with decreased VLDL and LDL apoB FCR, but no alteration in production rates, and Castro Cabezas, de Bruin, Jansen, *et al*, (1993a) have reported delayed postprandial chylomicron remnant clearance in combined hyperlipidaemia due to competition for lipolysis by LPL and for uptake by hepatic receptors. However when the familial form of combined hyperlipidaemia is studied, most investigators are convinced that the main defect is overproduction of apoB containing lipoproteins.

The underlying defect that causes overproduction of apoB is not known. It is generally accepted that apoB is synthesised at a constant rate, and alterations in output are not due to variation in expression of the apoB gene. It is considered likely that an increased availability of lipid in the hepatocyte leads to the secretion of more apoB with less being internally degraded. A number of investigators have examined FA metabolism in FCH and found evidence for specific abnormalities which would increase FA flux into the liver.

Insulin resistance can cause alterations in lipid metabolism (Figure 1.3.) such as those seen in FCH. Insulin resistance is often seen in FCH, particularly in obese individuals (Aitman, Godsland, Farren, *et al*, 1997). In his group of 8 FCH patients, Aitman reported an impaired insulin-mediated glucose uptake in peripheral tissues, and an impaired insulin-mediated suppression of serum FFAs compared to controls. It was postulated that a defect exists within the adipocyte, either a failure of insulin to inhibit HSL, or to stimulate the re-esterification of triglyceride. Prolonged postprandial plasma FFA concentration then results in increased FFA flux to the liver and stimulation of VLDL production.

Alternatively, the elevated plasma insulin levels seen in FCH individuals may be secondary to an impaired postprandial FFA clearance. This elevation in plasma FFA has been postulated to be the result of an impairment of uptake of FFA into the adipose tissue (Teng, Forse, Rodriguez, *et al*, 1988, Cianflone, Maslowska and Sniderman, 1990). Acylation stimulatory protein (ASP) mediates the intracellular re-esterification of FFA to triglyceride in the adipose tissue for storage and stimulates glucose transport in differentiated human adipocytes (Maslowska, Sniderman, Germinario, *et al*, 1997) (Figure 1.3.). ASP levels have been reported to be decreased in patients with hyperapobetalipoproteinaemia, a disorder similar to FCH (Cianflone *et al*, 1990, Cianflone, 1994). This may explain the increased plasma FFA seen in FCH. Increased FFA in turn may stimulate insulin secretion and therefore in FCH, increased fasting plasma insulin concentrations could be secondary to impaired postprandial FA metabolism. (Castro Cabezas, de Bruin, de Valk, *et al*, 1993b). Whether insulin resistance causes the elevated FFA or whether an impaired FFA metabolism causes increased insulin secretion is unknown, and may in fact vary in subsets of patients.

Obligate heterozygotes for LPL deficiency express a phenotype consistent with FCH. Approximately one third of patients with FCH have been found to have impaired LPL activity (Babirak, Brown and Brunzell, 1992). However severe defects in this gene have not been found to be important in FCH patients. Moderate variation in LPL activity due to gene polymorphisms may magnify the phenotype by impairing the clearance of chylomicrons and VLDL (de Bruin, Mailly, van Barlingen, *et al*, 1996,

Pajukanta, Porkka, Antikainen, *et al*, 1997). Evidence has also been found of a linkage between the AI-CIII-AIV gene cluster and FCH, prompting speculation of an association between apoCIII levels (an inhibitor of LPL) and LPL activity (Wojciechowski, Farrall, Cullen, *et al*, 1991, Patsch, Sharrett, Chen, *et al*, 1994,) with consequent effects on FFA concentration. Similarly, there may be an effect of a mutation in apoAIV. ApoAIV may facilitate the transfer of apoCII to chylomicrons, where it promotes the hydrolysis of triglyceride in the chylomicrons by LPL. Therefore a defect in apoAIV could cause hypertriglyceridaemia, and may explain the defect in chylomicron remnant clearance described above. A defect in HSL has also been implicated in the progression of FCH (Reynisdottir, Eriksson, Angelin, *et al*, 1995).

There is also a strong association between a preponderance of small dense LDL and FCH (Bredie, Demacker and Stalenhoef, 1997). Recent evidence suggests that 40% of variation in LDL subfraction distribution is due to genetic factors with the remainder due to environmental factors. The predominance of small dense LDL may play a large part in the development of premature cardiovascular disease. This may be a consequence of increased levels of CETP.

Therefore, FCH appears to be a heterogeneous disorder, with a variety of potential genetic and molecular defects. It may result from the inheritance of more than one minor defect, which combine to produce the FCH phenotype. Further understanding of the disease will enable a subclassification of FCH and a more straightforward diagnosis.

1.7. Rabbit models of genetic hyperlipidaemias

Lipoprotein metabolism in the rabbit is similar to that in humans, making them a suitable model for the study of lipid transport and its relation to atherosclerosis. Rabbits like humans have apoB containing lipoproteins as the major carriers of cholesterol in plasma (Chapman 1986). They have plasma volumes sufficiently large to look at lipoprotein subclasses, and to make turnover studies possible. They also have atherosclerotic plaques that are similar to human lesions. Unlike rodents, they, like humans express apoB48 in the intestine and apoB100 in the liver. The principal

difference from man is that rabbits have relatively greater concentrations of IDL, probably due to the fact that they express only low levels of HL.

Rabbits are susceptible to cholesterol feeding and develop atherosclerosis at an enhanced rate. Some strains hyper-respond to cholesterol in the diet and have substantially elevated plasma lipid levels (Meijer, van der Palen, Geelen, *et al*, 1992). However, rabbits appear to react in different ways to dietary intervention (Harris 1997), therefore it is important to ensure that the rabbit model mirrors the human situation as far as possible.

1.7.1 The Watanabe heritable hyperlipidaemic rabbit

The Watanabe heritable hyperlipdaemic rabbit (WHHL) was first described in 1980 by Watanabe as a model for FH since the animal had virtually no active LDL receptors. The condition is inherited as an autosomal recessive trait, with slight expression of the phenotype being seen in the heterozygous state. LDL are therefore cleared slowly from the circulation and accumulate in plasma. VLDL and IDL also appear to be cleared slowly, and are thus more likely to be transformed to LDL, also contributing to the rise in LDL levels (Kita, Brown, Bilheimer, *et al*, 1982). Homozygous WHHL rabbits have elevated plasma cholesterol (approximately 10 fold), LDL-cholesterol (20 - 50 fold) and triglyceride (2 - 4 fold) and decreased HDL levels (to one third of normal). Heterozygotes have LDL levels between homozygotes and normals (Goldstein, Kita and Brown, 1983). WHHL rabbits develop atherosclerosis and xanthomas. There are no differences in secretion of apoB containing lipoproteins (Hornick, Kita, Hamilton, *et al*, 1983), therefore like in humans, the FH exhibited by these animals derives entirely from a catabolic defect.

1.7.2 The St Thomas' hospital rabbit

This strain of NZW rabbit was first described in 1987 by Agnes La Ville and coworkers at St Thomas' Hospital London, as exhibiting an inherited hyperlipidaemia similar to that seen in FCH (La Ville, Turner, Pittilo, *et al*, 1987). The proband was discovered serendipidously when it was found that the addition of a small amount of cholesterol to the diet caused a large response in serum lipid levels. On breeding the

proband it was discovered that plasma cholesterol and triglyceride concentrations were elevated and variable, and were inherited. There was no discernible defect in the LDL receptor, though in LDL kinetic studies there was an impaired FCR, possibly due to saturation of clearance mechanisms. Kinetic studies also revealed increased production rates of both VLDL and LDL.

Genetic analyses have not been performed on the St Thomas' hospital rabbit due to the closed nature of the colony, although there is evidence suggesting Mendelian inheritance of a major gene defect affecting apoB production and a minor gene acting on triglyceride metabolism (Beaty, Prenger, Virgil, *et al*, 1992).

Investigation of arterial pathology concluded that extensive intimal lesions were present in rabbits from 6 months old (Seddon, Woolf, La Ville, *et al*, 1987). Lesion pathology generally resembled that seen in cholesterol-fed rabbits, with some features similar to the WHHL rabbit and humans.

1.7.3 Transgenic rabbits

Transgenic animals are providing new methods for the study of genes of interest in the development of atherosclerosis, and although rabbits are used rarely in comparison to transgenic mice, they are increasingly proving the animal of choice due to the similarities of lipoprotein metabolism with the human.

Rabbits overexpressing human apoAI have been shown to have higher plasma HDL cholesterol levels than non transgenic rabbits, and to develop a significantly smaller area of aortic lesion when compared to controls (Duverger, Kruth, Emmanuel, *et al,* 1996). Similarly LCAT transgenic rabbits had an increased HDL cholesterol concentration, and were protected from diet induced atherosclerosis (Hoeg, Santamarina-Fojo, Berard, *et al,* 1996). Brousseau, Santamarina-Fojo, Vaisman, *et al* (1997) showed that high expressors of LCAT had high levels of HDL cholesterol and low levels of non-HDL cholesterol when compared to low expressors or non expressors, and demonstrated that the mechanism by which LCAT regulates HDL and non-HDL cholesterol concentration is by modulating the FCR of these lipoprotein species.

Expression of human apoB in transgenic rabbits has been shown to increase plasma cholesterol (mainly LDL cholesterol) and triglyceride concentrations. LDL particles produced were smaller and triglyceride enriched compared to control rabbits, and contained increased amounts of apoE and apoCIII. HDL cholesterol and apoAI levels were decreased (Fan, McCormick, Krauss, *et al,* 1995).

Transgenic rabbits have been produced expressing $apoE_3$ and $apoE_2$. $ApoE_3$ rabbits showed an increased binding affinity of VLDL for the LDL receptor, therefore having lower VLDL and triglyceride concentrations. However plasma cholesterol concentration was increased as LDL was cleared more slowly in transgenic rabbits possibly because VLDL competes for LDL receptor mediated clearance (Fan, Ji, Huang, *et al,* 1998). $ApoE_2$ expressing rabbits had a phenotype similar to type III hyperlipoproteinaemia, with increases in plasma cholesterol and triglyceride concentration being much greater in males than females. This was shown to be mainly due to the effects of oestrogen on lipase and LDL receptor activity (Huang, Schwendner, Rall, *et al,* 1997).

Rabbits expressing human HL (Fan, Wang, Bensadoun, *et al,* 1994) had decreased plasma lipid levels compared to normal rabbits, with a 5-fold decrease in HDL cholesterol accounting for a large proportion of the decrease in plasma cholesterol concentration. IDL levels were decreased, and LDL was increased slightly, consistent with the role of HL in the conversion of IDL to LDL. Transgenic rabbits have also been reported expressing human apo(a) and apoB which have been shown to make Lp(a) (Rouy, Duverger, Lin, *et al,* 1998).

1.8. Methods of study of lipoprotein metabolism

To fully understand the complex system of lipoprotein metabolism, it is necessary to be able to study each part both in isolation, and as a part of the global mechanism. The use of animal models, especially of the size of the rabbit, allows a number of approaches to be investigated and then integrated.

1.8.1 Whole animal studies

Analysis of the metabolism of lipoproteins in the whole animal provides the most physiological model available for study. Early studies of the interconversion and catabolism of lipoprotein species involved the isolation of a particular lipoprotein species. These lipoproteins were then labelled with a radioactive tracer, reinjected, and the tracer was followed as it was cleared from the circulation. Labelling was of either protein or lipid moieties, depending on the tracer used. The problem with this *ex vivo* method of labelling was that production of lipoproteins could not be measured directly, but had to be inferred. The use of stable isotopes has negated the need for the *ex-vivo* labelling of lipoproteins, as the isotope can be injected and the lipoproteins are labelled *in vivo*. This method is preferable as *in vivo* labelling of lipoproteins means there should be minimal alteration in kinetic behaviour, but also means that all new proteins are labelled.

Labelling of apoB provides a handle by which the metabolism of particular lipoprotein particles can be followed in the whole animal, as apoB is known not to be transferred between particles. Whole animal studies of lipoprotein metabolism can be performed in the fed or fasted state. In the fed state, the production of chylomicrons complicates the study of VLDL metabolism, but it is the most physiological state. One of the main problems with turnover studies is the analysis of the data. Multicompartmental modelling, allowing the fitting of a multiexponential curve to the apoB decay is currently the method of choice. This requires complex mathematical procedures which are handled by computer programmes, such as the Simulation, Analysis And Modeling (SAAM) computer programme (SAAM Institute, 1997).

1.8.2 Organ perfusions

Organ perfusions are the most physiological method used to investigate the direct output of a particle of interest from an organ of choice and can also be used to measure catabolism by the organ. In terms of lipoprotein metabolism, the perfused liver gives useful information on the direct production of lipoproteins without the effects of lipolytic enzymes. Experiments can be performed *in vivo* or *in vitro*, with *in vitro* experiments providing scope for manipulations to the system. Organ perfusions

are also an ideal way to investigate effects of prior *in vivo* interventions (Marzolo, Amigo and Nervi, 1993).

As with all procedures where the body is not taken as a whole, there are opportunities for the system to produce erroneous results due to the inability of the experimental operator to maintain conditions for the the perfused organ exactly as *in vivo.* However, organ perfusions provide better results than cell cultures, as the different cell types are present in their correct spatial location and are more likely to respond in a physiological manner to external stimuli. For an organ like the liver, consisting of many different cell types, this topography is important for normal interaction with other hepatic cells and results may differ in isolated cell cultures.

The main disadvantage of organ perfusions is that the duration of experiment is limited as the organ can usually only be kept patent for a matter of hours. The experimental set up is technically demanding. Also a large number of animals are needed, compared to hepatocyte cultures where several experiments can be set up using cells from one organ.

1.8.3 Cell culture

Cells maintained in culture can be of two types, namely primary cell cultures where the cells are prepared from living tissue, and transformed (immortal) cell lines (Gibbons, 1994). Rabbit hepatocytes are a good model for human lipoprotein metabolism as they produce hepatic apoB100 only, and they secrete full sized VLDL particles.

Primary hepatocyte cultures are useful models of hepatic metabolism in that unlike perfused livers they remain viable for a few days and the yield of cells from one liver provides enough material for several experiments. *In vivo* pre-treatment of the animal donor can be used to provide different conditions in the cells. However culture of cells means the topography of the liver is lost, and usually only one type of hepatic cell is cultured, therefore there is no interaction with other hepatic cell types.

Transformed cell lines are easy to maintain in culture, and are immortal. There is no need for an animal donor, therefore the cells are much easier to prepare, and can be available whenever required and in whatever quantity. Long term experiments can also be performed. Further, laboratories around the world can work on the same cells and compare results. However, some differentiated functions of the cells are lost on transformation.

The human cell line HepG2 is the most commonly used model for hepatic metabolism, but has several shortfalls. These cells do not produce full sized VLDL particles possibly because they have lost the second step in assembly. They also have an unusual bile acid secretion, and poor capacity for FA oxidation.

When cell culture studies are performed, it is necessary to remember that results have been produced from a system that is not physiological, and vital cofactors or substrates may be missing, resulting in a response that is different to that seen from the whole organ.

1.8.4 Gene expression studies

Molecular biology provides an alternative to the traditional biochemical and physiological investigations into lipoprotein disorders. Discovery of a mutation in a gene of interest opens up the possibility of tracing the defect through families, and provides the potential of treatment with gene therapy. If the level of expression of a gene is found to be varied, this can provide information on the protein that is causing the disorder, and suggest new treatment strategies.

To measure levels of gene expression, cells can be transfected with the promoters of genes of interest, ligated to a reporter gene, and the relative expression of the reporter can be measured, thereby highlighting any differences in promoter efficacy. However, cell work can give false results due to absent spatial stimuli, or transcription factors.

The current method of choice involves measuring the levels of mRNA in the tissue of interest for the gene of interest. This provides information on genes that are transcribed at different rates, and narrows down the search for proteins that are

differentially expressed. However, levels of mRNA are not always directly associated with levels of protein.

1.9. Aims and objectives

The principal aim of the experiments contained within this thesis was to characterise the metabolism of apoB containing lipoproteins in a new colony of St Thomas' hospital rabbit, named the St Thomas' mixed hyperlipidaemic rabbit (SMHL). We wished to investigate the stability of the phenotype in the SMHL rabbits, and the similarities between the phenotype of these rabbits and the human disorder FCH.

We planned to investigate the effects of a low cholesterol diet on the lipids and lipoproteins of these rabbits, as such a diet enhances the expression of the FCH phenotype. We intended to perform liver perfusion experiments to examine the direct output of lipoproteins from the liver in order to define the exact cause of the increased lipoprotein concentrations in these rabbits and, complimentary to this, to study the kinetics of the lipoprotein classes using stable isotope labelling of apoB. And finally, using information gathered from the previous experiments, we planned to look at the expression levels of some candidate genes.

Chapter 2. General Methods

2.1. Materials

All chemicals used were of analytical grade. Names and addresses of suppliers of reagents, hardware and software are given in appendix 1.

2.2. Animals

Rabbits were obtained from Froxfield Farm and later from Harlan Interfauna Ltd. They were housed individually in cages at 18 - 20°C and 55% humidity, with 12 hours light per 24 hours. Unless stated, the rabbits were fed a diet supplemented with a low amount (0.075% wt/wt) cholesterol (0.08% total) (Special Diet Service). Food and water were freely available. All animal procedures in this thesis were approved by the Home Office, and carried out subject to their regulations.

2.3. Lipid and lipoprotein measurements

2.3.1 Preparation of plasma

Blood samples were collected from the marginal ear vein into K_3EDTA tubes (1 mg/ml) and centrifuged at 1780 *g* for 10 minutes at 4°C. The supernatant was aspirated off as plasma.

2.3.2 Cholesterol

Cholesterol was measured using an enzymatic colorimetric test kit (Boehringer Mannheim, Cat number 704121). The principle of the assay is described below:

$$\text{cholesterol esters} + H_2O_2 \xrightarrow{\text{cholesterol esterase}} \text{cholesterol} + \text{RCOOH}$$

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

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

Samples are either measured automatically on the Hitachi 717, or manually with the optical density measured at 500 nm on a Beckman DU70 spectrophotometer.

2.3.3 Triglyceride

Plasma triglycerides were measured using an enzymatic colorimetric test kit (Boehringer Mannheim, Cat number 704113). The assay is performed either automatically on the Hitachi 717, or manually following the manufacturers instructions, with the optical density measured at 340 nm. The principle of the test is described below:

$$\text{triglycerides} + 3\ H_2O \xrightarrow{\text{lipase}} \text{glycerol} + 3\text{RCOOH}$$

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

$$\text{ADP} + \text{PEP} \xrightarrow{\text{pyruvate kinase}} \text{pyruvate} + \text{ATP}$$

$$\text{pyruvate} + \text{NADH} + H^+ \xrightarrow{\text{lactate dehydrogenase}} \text{L-lactate} + NAD^+$$

PEP phospho-enol-pyruvate

Cholesterol and triglyceride were kindly measured by staff of the routine lipids section, Department of Biochemistry, Glasgow Royal Infirmary.

2.3.4 Glycerol

The triglyceride assay involves the conversion of all triglyceride to glycerol with subsequent measurement of glycerol whether derived from triglyceride or not. In order to obtain a true triglyeride value, it is necessary to subtract plasma glycerol from triglyceride.

Plasma glycerol is measured by a colorimetric kit (Randox Laboratories Ltd., Cat number GY105) following the manufacturers instructions, with the optical density measured at 520 nm. The principle of the assay is described below:

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

$$\text{glycerol-3-phosphate} + O_2 \xrightarrow{\text{glycerol phosphate oxidase}} H_2O_2 + \text{dihydroxyacetone-phosphate}$$

$$2\,H_2O_2 + \text{DCHBS} + \text{4-aminophenazone} \xrightarrow{\text{peroxidase}} \text{ACSB}$$

DCHBS 3,5-dichloro-2-hydroxybenzene sulphonic acid

ACSB n-(4-antipyryl)-3-chloro-5-sulphonate-p-benzoquinoneimine

2.3.5 Free cholesterol

Free cholesterol was measured using an enzymatic colorimetric kit (Boehringer Mannheim, Cat number 310328). The reaction is similar to that for cholesterol except that the cholesterol esterase is omitted, in order that only free cholesterol in the sample is oxidised and measured.

2.3.6 Phospholipid

Phospholipid was measured using an enzymatic colorimetric kit (Boehringer Mannheim, Cat number 691844). The principle of the assay is described below:

$$\text{phospholipid} \xrightarrow{\text{phospholipase D}} \text{choline} + \text{phosphatidic acid}$$

$$\text{choline} \xrightarrow{\text{choline oxidase}} \text{betaine} + H_2O_2$$

Free cholesterol and phospholipid measurements were performed by Mr Michael McConnell of the Department of Pathological Biochemistry, Glasgow Royal Infirmary.

2.3.7 Coating of Beckman Ultra Clear centrifuge tubes

To enable salt solutions to gravity feed smoothly down the side of the Beckman Ultra Clear centrifuge tubes used for density gradient ultracentrifugation, providing no disruption to the formation of density layers, it is necessary to coat the interior surface of the tubes with 2% polyvinyl alcohol in propan-2-ol. The tubes are filled with this solution for 15 minutes and dried overnight, and are subsequently washed with distilled H_2O overnight, rinsed once with H_2O and left to dry (Holmquist, 1982).

2.3.8 Separation of $VLDL_1$ (Sf 60 - 400), $VLDL_2$ (Sf 20 - 60), IDL (Sf 12 - 20) and LDL (Sf 0 - 12)

$VLDL_1$, $VLDL_2$, IDL and LDL were prepared from plasma or perfusate by a modification (Packard, Clegg, Dominiczak, *et al*, 1986) of the method of Lindgren, Jensen and Hatch (1972). Density (d) solutions were prepared from stock solutions at d = 1.006 g/ml (0.195 mol/l NaCl) and d = 1.182 g/ml (0.195 mol/l NaCl, 2.44 mol/l

NaBr). The densities were measured to 3 decimal places in a Paar Scientific density meter (model DMA 35).

The density of 2 ml of the plasma or perfusate was adjusted to 1.118 g/ml by the addition of NaCl according to the formula:

$$\text{Weight of salt} = \frac{V(d_2 - d_1)}{1 - \nu d_2}$$

where V is the volume of solution, d_1 is the density of the solution, d_2 is the required density and ν is the partial specific volume of the salt at the relevant temperature and concentration.

In a precoated Beckman Ultra Clear centrifuge tube the sample was layered over a cushion of 0.5 ml d = 1.182 g/ml solution using an AAII pump (Technicon), and a discontinuous gradient of salt solutions was formed over the top (1 ml d = 1.0988 g/ml, 1 ml d = 1.0860 g/ml, 2 ml d = 1.0790 g/ml, 2 ml d = 1.0722 g/ml, 2 ml d = 1.0641 g/ml, 2 ml d = 1.0588 g/ml). This was centrifuged in a Beckman SW40 rotor under vacuum at 23°C in a Beckman L8 ultracentrifuge according to the conditions described in Table 2.1. The rotors were decelerated without the brake. Lipoprotein fractions were removed from the top using a drawn out glass Pasteur pipette. After removing the $VLDL_1$ fraction, 1ml of d = 1.0588 g/ml was overlayered on the existing gradient.

Table 2.1. Centrifuge conditions for isolation of $VLDL_1$, $VLDL_2$, IDL and LDL

Fraction	Relative centrifugal field (*g*)	Time	Fraction volume (ml)
$VLDL_1$	272 000	1 hour 38 mins	1
$VLDL_2$	62 000	15 hours 41 mins	0.5
IDL	272 000	2 hours 35 mins	0.5
LDL	160 000	21 hours 10 mins	1

2.4. Measurement of protein

2.4.1 Isolation of apoB

ApoB was isolated from preparations of $VLDL_1$, $VLDL_2$, IDL and LDL. The samples were brought to room temperature and an equal volume of propan-2-ol was added and immediately mixed (Egusa, Brady, Grundy, *et al*, 1983). After an overnight incubation at 4°C the samples were spun at 1780 *g* for 30 minutes at 4°C and the supernatant was removed using a finely drawn glass Pasteur pipette. The supernatant contained isopropanol soluble proteins which in lipoprotein fractions are all proteins except for apoB.

The precipitate was redissolved in 3 ml ethanol/ether (3:1), mixed and incubated at 4°C overnight. The samples were centrifuged at 1780 *g* for 30 minutes at 4°C, and the supernatant was removed. Traces of ethanol were removed by redissolving the precipitate in 3 ml ether by mixing and incubating the samples for at least 1 hour. The samples were centrifuged as above and the supernatant was removed. The apoB pellets were air dried.

ApoB protein was measured by the method of Lowry, Rosebrough, Farr, *et al* (1951) (section 2.4.2) by subtracting the isopropanol soluble protein from total protein, or by measuring the protein content of the NaOH dissolved apoB pellet.

2.4.2 Modified Lowry protein assay

Protein is measured by a modification (Markwell, Hass, Bieber, *et al*, 1978) of the Lowry protein method (Lowry *et al*, 1951).

Reagents: Solution A - 2% (wt/v) Na_2CO_3 in 0.1 M NaOH

Solution B - 2% (wt/v) NaK tartrate in deionised H_2O

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

Folin Ciocalteu reagent (BDH, Prod 19058 3Q)

Working reagents:

Biuret reagent - 1 ml solution B and 1 ml solution C were added to 100 ml solution A. For apoB samples, 100 ml 2% (wt/v) Na_2CO_3 in deionised H_2O was used. If the samples to be analysed were turbid, sodium dodecyl sulphate (SDS) (1 mg/ml) was added to the Biuret reagent.

Folin Ciocalteu reagent was diluted 1:1 with deionised H_2O (freshly made).

Standards: A standard curve in the range 0 - 50 μg was made from a 1 mg/ml stock standard of human serum albumin (Sigma A-8763) in a final volume of 400 μl. For apoB samples, 0.5 M NaOH was also added.

Quality Control:

15 μg and 30 μg quality control samples (in 400 μl) were made from 150 μg/ml and 300 μg/ml stock solutions of bovine serum albumin (Sigma A-4503). For apoB samples, 0.5 M NaOH were also added.

Samples: Samples were used neat or diluted as necessary. Volumes were made up to 400 μl with deionised H_2O.

Procedure: 2 ml Biuret reagent was added to 400 μl standard, quality control or sample, mixed and incubated at room temperature for 10 minutes. Two hundred μl of working Folin Ciocalteu reagent was added and mixed immediately. The optical density was read at 750 nm after 30 minutes and within 2 hours. From the standard curve, unknown values were calculated.

2.5. Routine Biochemical tests

Urea was measured using a urease method on an Olympus 5231. Albumin was also measured on an Olympus 5231, using its affinity for bromo-cresol green at a neutral pH. Microalbumin was measured by an immunoturbidimetric assay on a Behring Laser Nephelometer. The antibody was manufactured by the Scottish Antibody

Production Unit. Aspartate aminotransferase (AST) was measured on an Olympus 5231 analyser using IFCC recommendations. The method links the conversion of alpha-oxoglutarate through oxaloacetate to malate with associated oxidation of NADH to NAD. Gamma glutamic acid (GGT) was measured on an Olympus 5231 using the conversion of gamma-glutamyl-3-carboxy-4-nitroanilide and glycylclycine to gamma glutamyl-glycylglycine and 5-amino-2-nitrobenzate. These measurements were performed by staff of the Department of Biochemistry, Glasgow Royal Infirmary.

2.6. Measurement of enzyme activities

2.6.1 CETP activity

CETP activity was measured in plasma samples using a fluorescence kit from Roar Biomedical, Inc., following the manufacturers guidelines. The assay measures the CETP transfer activity by measuring the fluorescence released when the CETP in the sample transfers fluorescent neutral lipid from the core of donor particles to acceptor particles. The fluorescence is quenched when within the donor core, and fluorescence increases as the labelled lipid is transferred from the donor to the acceptor. Briefly, a standard curve was prepared from donor particles in isopropanol at a concentration range of 0 - 430 pmol/ml. One µl of sample was incubated with 10 µl donor particles and 489 µl CETP buffer (10mmol/l, Tris base, 150 mmol/l NaCl, 2mmol/l Na_2EDTA, pH 7.4) for 3 hours at 37°C. (If the sample was not of plasma, 10 µl acceptor particles were also added in a total volume of 500 µl). The fluorescence was measured at excitation 465 nm/ emission 535 nm and the CETP transfer activity was calculated using the standard curve.

2.6.2 Measurement of lipase activity

Seventy iU heparin (Leo Laboratories Ltd.)/kg body weight was injected into the marginal ear vein. After 12 minutes, a blood sample was withdrawn and placed immediately on ice. Post heparin plasma samples were incubated with a ^{14}C-labelled triglyceride/gum arabic emulsion. FFA released by lipase activity were captured by

albumin and extracted into a solvent and the ratio of radioactivity in the extracted fraction to the total present in blank incubations enabled the calculation of the lipase activity using the following formula:

$$\text{Lipase Activity (}\mu\text{moles FFA released/ml/hour)} = \frac{\text{(cpm(sample) - cpm(blank))}}{\text{cpm(total) - background}}$$

Each sample was measured under 3 different conditions. The addition of SDS inactivated HL, and serum was added as it contains the LPL activator apoCII. HL was measured in the presence of a high salt concentration (1 M NaCl) to inactivate LPL. A third tube contained a low salt concentration, and no SDS, and total lipase activity was measured here.

CETP, HL and LPL were kindly measured by Mr Michael McConnell from the Department of Pathological Biochemistry, Glasgow Royal Infirmary.

2.7. Statistical analysis

Variables were tested for normal distribution. If necessary, samples were transformed to a normal distribution by taking the logarithm of their values. Two sample t-tests were performed to determine significance. Where the distribution of a variable or a series of variables could not consistently be converted to normal, a Mann-Whitney *U* test was performed. All statistics were carried out using Minitab version 10 (Minitab Inc.).

Chapter 3. The St Thomas' Mixed Hyperlipidaemic Rabbit

3.1. Introduction

The St Thomas' hospital rabbit, a strain of NZW rabbit was first described in 1987 (La Ville, *et al*) (Chapter 1) as exhibiting an inherited hyperlipidaemia similar to that seen in FCH. Both NZW and Dutch Belt rabbits were introduced into this closed colony.

In 1993, the remaining rabbits of the St Thomas' hospital colony were transferred to Froxfield Farm where they were bred to maintain a diversity of lipid levels. This new colony was named the St Thomas' Mixed Hyperlipidaemic rabbit (SMHL). As large a pool of rabbits as possible were mated, but some rabbits were poor breeders. The high degree of inbreeding, leading to an extremely complicated family tree has made the genetics of the disorder difficult to establish at present.

Rabbits were maintained on a vegetarian diet until required for experimental use, when they were fed specially prepared diets with varying cholesterol concentrations. La Ville *et al* (1987) had shown no effect on plasma cholesterol levels in St Thomas' hospital rabbits on feeding either a diet containing no cholesterol or a diet containing 0.053% (wt/wt) cholesterol for 6 weeks. We performed a similar study and looked at plasma lipid and lipoprotein parameters in more detail.

To characterise more carefully the hyperlipidaemia in SMHL rabbits, we undertook to measure plasma lipids, lipoproteins (and their compositions), insulin resistance status, and the levels of HL, LPL and CETP.

3.2. Methods

3.2.1 Animals

Three separate groups of animals were studied. Group 1 consisted of 60 young (< 4 months of age) SMHL, 12 young NZW, 16 mature (> 5 months of age) SMHL and 18 mature NZW male rabbits, all fed the 0.08% cholesterol diet since weaning. These were the group from which we later selected the rabbits for our perfusion experiments (chapters 5,6,7). We measured plasma cholesterol and triglyceride levels (section 2.3.2, 2.3.3) in these rabbits.

In group 2 were 9 SMHL (5 female and 4 male) and 9 age and sex matched NZW controls fed a chow diet (0.005% cholesterol wt/wt) (Stanrab diet, Special Diet Service) *ad libitum* for a minimum of 4 weeks, plus 10 SMHL and 10 age and sex matched NZW rabbits fed a diet supplemented with a low (0.075% wt/wt) level of cholesterol (0.08% total) for 12 weeks. These sets of animals were used to determine the effects of a diet with a slightly elevated cholesterol content on lipoprotein composition and mass. The experiments were carried out as a collaboration between Dr G. M. Benson of SmithKline Beecham Pharmaceuticals and Dr M. J. Caslake at the Department of Pathological Biochemistry, Glasgow Royal Infirmary.

A further 12 male and 12 female SMHL rabbits and 7 female NZW rabbits (group 3) fed on the 0.08% cholesterol diet since weaning were studied. Plasma cholesterol and triglyceride levels were measured at two week intervals from 9 to 27 weeks of age in order to determine the effects of age on plasma lipid levels. These experiments were performed by Dr G. M. Benson, Mr D. Grimsditch, Dr P. H. E. Groot and Dr K. E. Suckling at SmithKline Beecham Pharmaceuticals.

3.2.2 Glucose tolerance test

Six male SMHL rabbits aged approximately 11 months and 7 age and sex matched NZW control rabbits from group 1, fed on the 0.08% cholesterol diet since weaning were fasted overnight. A pretreatment blood sample was taken from the marginal ear

vein, and the animals were subsequently orally dosed with a solution of 1 g glucose/kg body weight. Blood samples were withdrawn at 15, 30, 45, 60 and 120 minutes after the glucose dose, and cholesterol, triglyceride, glucose, FFA and insulin were measured in these samples (sections 2.3.2, 2.3.3, 3.2.3, 3.2.4, 3.2.5). The area under the response curves for glucose, FFA and insulin were calculated by subtracting the baseline concentration from the concentration of the variable at each time point, and summing these values. Cholesterol, triglyceride and glucose concentrations were measured by staff of the Department of Biochemistry, Glasgow Royal Infirmary, and FFA and insulin concentrations were measured by Mr Michael McConnell of the Department of Pathological Biochemistry, Glasgow Royal Infirmary. The assistance of Mr D. Robertson, Mr D. McMurdo, Dr M. J. Caslake and Mrs M. Stewart is gratefully acknowledged.

3.2.3 Measurement of blood glucose concentrations

Glucose was measured on an Olympus 5231, using the hexokinase catalysed conversion of glucose to glucose-6-phosphate, which is then converted to gluconate-6-phosphate causing the reduction of NADP to NADPH, which is measured at 340 nm. These assays were performed by staff of the Department of Biochemistry, Glasgow Royal Infirmary.

3.2.4 Measurement of plasma FFA concentrations

Plasma free fatty acids were measured using an enzymatic colorimetric test kit (Wako Chemicals GmbH, Code No. 994-75409 E). The principle of the assay is described below, with the absorbance of the resultant adduct being measured at 550 nm.

acetyl-CoA synthetase

$$RCOOH + ATP + CoA \rightarrow \text{acetyl-CoA} + AMP + PPi$$

acetyl-CoA oxidase

$$\text{acetyl-CoA} + O_2 \rightarrow \text{2,3-trans-Enoyl-CoA} + H_2O_2$$

peroxidase

$$2\ H_2O_2 + MEHA + \text{4-aminoantipyrine} \rightarrow \text{adduct of MEHA and 4-aminoantipyrine} + 4H_2O$$

MEHA 3-methyl-N-ethyl-N-(β-hydroxy-ethyl)-aniline

3.2.5 Measurement of plasma insulin

Insulin was measured using a porcine radioimmunoassay kit (Linco Research, Inc.) which has been shown to cross react with rabbit insulin. Fasting plasma samples (100 µl) were incubated with 100 µl ^{125}I labelled insulin, 100 µl insulin antibody and 100 µl assay buffer (0.05 mol/l phosphosaline, pH 7.4, containing 0.025 mol/l EDTA, 0.1% sodium azide and 1% bovine serum albumin) for 18 hours at 4°C. One ml of precipitating reagent was added, mixed and incubated for 20 minutes at 4°C, before being centrifuged for 15 minutes at 2500 *g*. The supernatant was discarded and the pellet was counted in a gamma counter. The concentration of insulin was calculated with reference to a standard curve in the range of 2 - 200 µUnits/ml. These assays were performed with the help of Ms Carolyn Lister of SmithKline Beecham Pharmaceuticals.

3.2.6 SDS glycerol polyacrylamide gel electrophoresis

Half ml samples of lipoprotein fractions (prepared by density gradient ultracentrifugation as in 2.3.8), containing up to 1 mg protein (Table 3.1.) were shaken with 10 ml diethyl ether for 2 minutes (Mindham and Mayes, 1992); 0.2 ml of 3.6 mM sodium deoxycholate and 0.2 ml of 4.9 M trichloroacetic acid (TCA) were added and mixed briefly, and the top layer of diethyl ether was removed by aspiration

under a stream of nitrogen. The precipitated apolipoproteins in the lower layer were pelleted by centrifugation at 3070 *g* for 10 minutes at 4°C, and the supernatant was decanted. The apolipoproteins were dissolved in 0.1 ml sample buffer (0.087 M Tris base pH 6.8, 4% SDS solution, 0.002% bromophenol blue, 20% glycerol, 10% 2-mercaptoethanol), neutralised by the dropwise addition of 5 M NaOH, and incubated for 5 minutes at 80°C in a Techne heating block.

Table 3.1. Protein content (mg) loaded on to SDS glycerol polyacrylamide gels

Fraction	NZW	SMHL
$VLDL_1$	0.29	0.07
$VLDL_2$	0.43	0.05
IDL	0.28	0.06
LDL	0.03	0.02

The samples were loaded on to SDS polyacrylamide gels (3.5% acrylamide (acrylamide : bis-acrylamide ratio of 20 : 1), 18% glycerol, 0.064 M Tris/ phosphate pH 6.8, 0.05% tetramethylethylenediamine (TEMED), 0.1% SDS and 0.018% ammonium persulphate) prepared 18 hours in advance in order to allow full polymerisation and cross linking (Maguire, Lee and Connelly, 1989). The lower reservoir was filled with 0.1 M NaH_2PO_4 buffer, pH 7.0, and the upper reservoir was filled with the same buffer with 0.1% SDS added. Electrophoresis was carried out at a constant current of 20 mA for 30 minutes and then at 60 mA for 4.5 hours at room temperature, water cooled. Gels were fixed in 4% formaldehyde for 30 minutes, stained overnight in 0.025% coomassie brilliant blue G-250 in 45.4% methanol, 9.2% acetic acid, and destained in 7.5% acetic acid, 5% methanol.

High and low molecular weight markers (Table 3.2.) (Pharmacia Biotech) were loaded on each gel. Apolipoproteins were distinguished on the basis of their molecular weight.

Table 3.2. Molecular Weight markers

High Molecular Weight		Low Molecular Weight	
Protein	**Molecular Weight**	**Protein**	**Molecular Weight**
Thyroglobulin	669 000	Phosphorylase b	94 000
Ferritin	440 000	Albumin	67 000
Catalase	232 000	Ovalbumin	43 000
Lactate dehydrogenase	140 000	Carbonic Anhydrase	30 000
Albumin	67 000	Trypsin inhibitor	20 100
		α-lactalbumin	14 400

3.2.7 Statistical analyses

All statistics were performed in Minitab 10. Data was tested for normal distribution, and 2 sample t-tests were performed. If the data was not normally distributed, it was log transformed prior to 2 sample t-tests being performed (insulin, cholesterol and triglyceride concentrations from glucose tolerance tests, CETP). If the data was still not normally distributed, Mann Whitney *U* tests were performed (lipoprotein composition, lipase concentrations). Missing values in composition data were estimated using ratios of components calculated from complete data sets in the group. Results are presented as mean ± standard error of the mean (SEM) unless otherwise stated.

3.3. Results

3.3.1 Population lipids

A group of 60 young SMHL, 12 young NZW, 16 mature SMHL and 18 mature NZW male rabbits were screened for inclusion in the liver perfusion experiments (group 1). Plasma cholesterol levels of both young and mature SMHL rabbits were significantly

elevated (though variable) compared to NZW rabbits (5.97 ± 0.37 (mean ± SEM) vs 1.79 ± 0.14 mmol/l, $p < 0.001$ in young rabbits and 3.11 ± 0.95 vs 1.32 ± 0.23 mmol/l, $p = 0.04$ in mature rabbits, Figure 3.1.). Plasma triglyceride levels were significantly elevated in young SMHL rabbits compared to NZW controls (3.31 ± 0.25 vs 1.14 ± 0.14 mmol/l, $p < 0.001$, Figure 3.2.) but not in mature SMHL vs NZW animals. In the male SMHL rabbits, mature rabbits showed lower levels of plasma cholesterol ($p = 0.006$) and plasma triglyceride ($p < 0.001$) than young rabbits (Figure 3.1., 3.2.) but NZW rabbits did not.

3.3.2 Effects of age on plasma cholesterol and triglyceride concentrations

To explore further the influence of age on plasma lipids, 12 male and 12 female SMHL rabbits and 7 female NZW rabbits (group 3) were fed a diet containing 0.08% cholesterol and plasma cholesterol and triglyceride concentrations were followed from 9 - 27 weeks of age (Figure 3.3., 3.4.). Plasma cholesterol concentrations were similar for the 9 week old male and female SMHL rabbits (7.3 ± 0.89 and 8.8 ± 1.2 mmol/l respectively). However, by 26 (male SMHL) and 27 (female SMHL) weeks, plasma cholesterol had risen to 22 ± 2.2 mmol/l in the females, while in the males it had fallen to 2.5 ± 0.7 mmol/l. Cholesterol levels were significantly increased in female compared to male SMHL rabbits from 19 weeks of age, and were significantly increased in female SMHL compared to female NZW rabbits throughout the experiment. Plasma triglyceride concentrations fell from 4.5 ± 0.3 mmol/l in male and 4.3 ± 0.3 mmol/l in female SMHL rabbits to 2.7 ± 0.5 mmol/l in both sexes of SMHL rabbits, but in female SMHL rabbits, triglyceride concentrations remained significantly elevated when compared to female NZW rabbits throughout the period of the experiment.

Figure 3.1. Plasma cholesterol concentrations in a population of NZW and SMHL rabbits

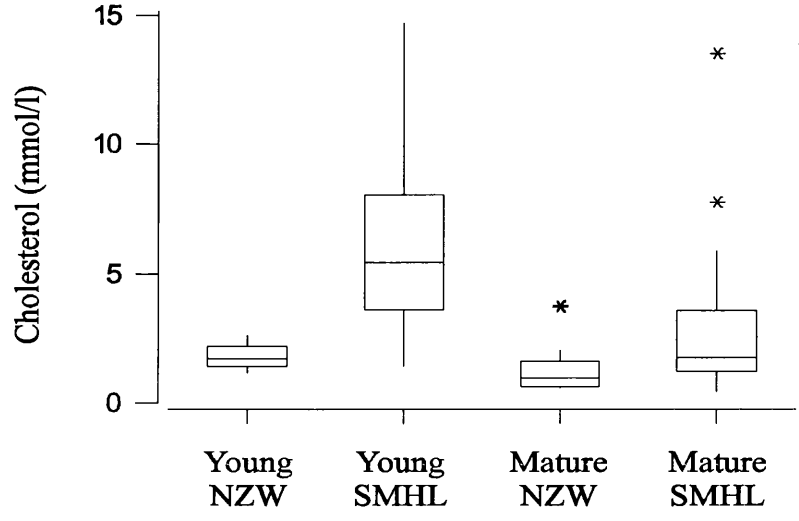
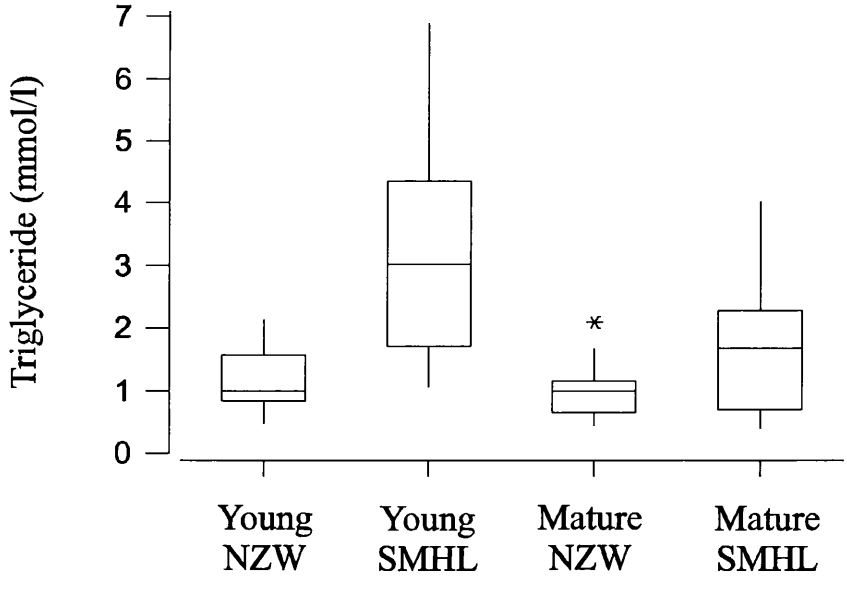


Figure 3.2. Plasma triglyceride concentrations in a population of NZW and SMHL rabbits



The horizontal line represents the median, the upper and lower limits of the box are the 75 and 25 percentile, the vertical lines show the range and asterisks represent outliers

Figure 3.3. Plasma cholesterol concentrations by age in NZW and SMHL rabbits

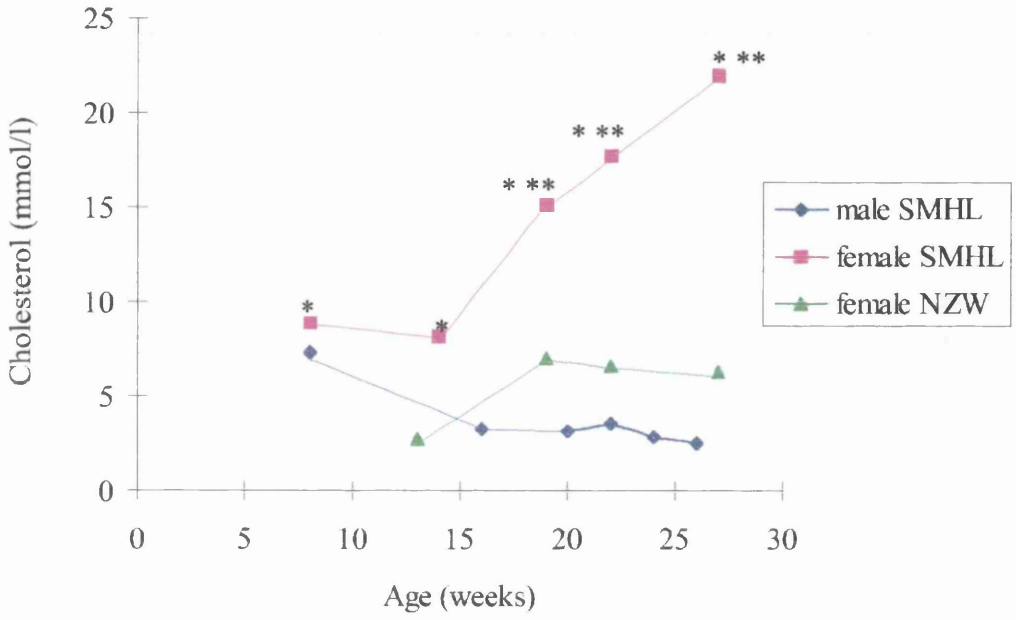
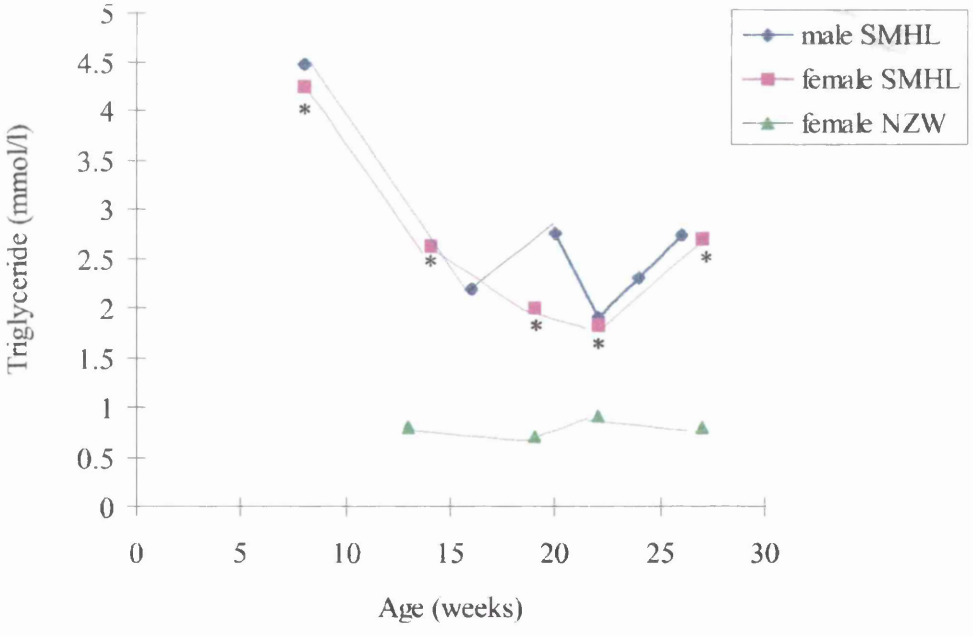


Figure 3.4. Plasma triglyceride concentrations by age in NZW and SMHL rabbits



* significant differences between female SMHL and female NZW rabbits. ** significant differences between female SMHL and male SMHL rabbits

3.3.3 Effect of 0.08% cholesterol diet on plasma lipids and lipoproteins

To uncover the hyperlipidaemic phenotype in SMHL rabbits it was necessary to add a small amount of cholesterol to the diet (Table 3.3., rabbits from group 2). Cholesterol supplementation at this level had no significant effect on plasma lipid levels in NZW rabbits, however plasma cholesterol was significantly elevated in SMHL rabbits on the 0.08% cholesterol diet compared to SMHL on normal chow and NZW fed either normal or supplemented diet. There were no significant differences in plasma triglyceride concentrations between any of the groups. However, it was noted that despite having normal total lipid levels, chow fed SMHL rabbits were dyslipidaemic. $VLDL_2$ and IDL mass levels tended to be elevated compared to NZW rabbits fed the same diet ($p = 0.08$, $p = 0.01$ respectively). When fed the cholesterol supplemented diet all four apoB containing lipoproteins were increased in SMHL compared to NZW rabbits fed either normal or supplemented diet.

Table 3.3. Effect of 0.08% cholesterol diet on plasma lipid and lipoprotein levels in SMHL and NZW rabbits

	Cholesterol diet		Chow diet	
	NZW	**SMHL**	**NZW**	**SMHL**
Number	10	10	9	9
Cholesterol (mmol/l)	1.6 ± 0.3	$3.8 \pm 0.7^{a,b}$	0.8 ± 0.1	1.1 ± 0.2
Triglyceride (mmol/l)	0.49 ± 0.04	1.3 ± 0.46	0.8 ± 0.2	1.2 ± 0.3
$VLDL_1$ mass (mg/dl)	16 ± 4	97 ± 35^{a}	38 ± 11	43 ± 16
$VLDL_2$ mass (mg/dl)	21 ± 5	$99 \pm 22^{a,b}$	10 ± 4	20 ± 4
IDL mass (mg/dl)	31 ± 10	$108 \pm 24^{a,b}$	7 ± 1	18 ± 3
LDL mass (mg/dl)	15 ± 4	$36 \pm 6^{a,b}$	4 ± 1	7 ± 2

Plasma lipid and lipoprotein measurements in NZW and SMHL rabbits fed either normal chow (0.005% cholesterol) or 0.08% cholesterol diet (mean ± SEM). Significant differences $p < 0.05$ using the Mann Whitney U test [a] SMHL vs NZW on 0.08% cholesterol diet, [b] SMHL on 0.08% cholesterol diet vs SMHL on normal diet.

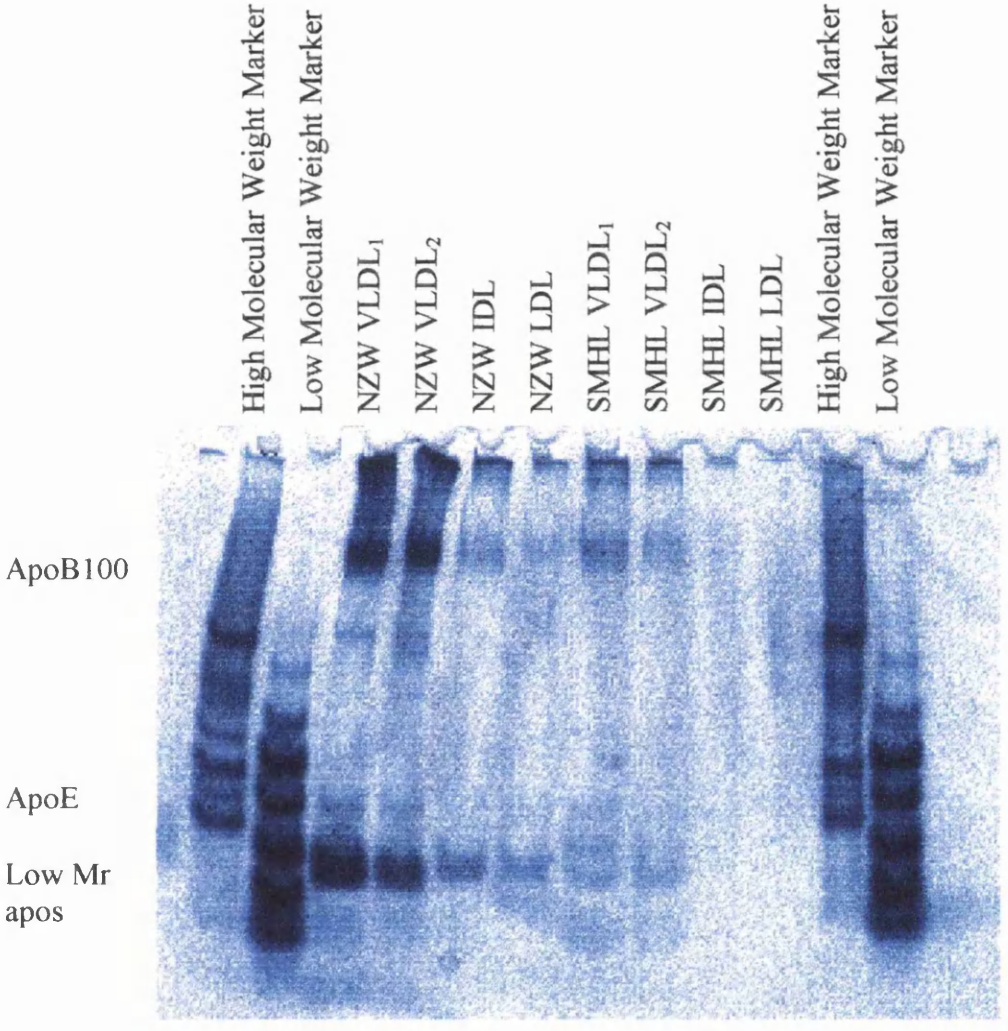
3.3.4 Lipoprotein composition

The content of protein, cholesterol, free cholesterol, triglyceride and phospholipid were measured (sections 2.4.2, 2.3.2, 2.3.5, 2.3.3, 2.3.6) in each lipoprotein fraction in the 10 NZW and 10 SMHL rabbits fed the 0.08% cholesterol diet above (section 3.3.3). Cholesterol ester was calculated by subtracting free cholesterol from total cholesterol. The contribution of each component as a percentage of the whole lipoprotein was calculated (Table 3.4.). The SMHL rabbits studied had a significantly increased plasma cholesterol concentration of 3.8 ± 0.7 mmol/l compared to 1.6 ± 0.3 mmol/l in the NZW rabbits ($p < 0.01$). The triglyceride concentrations were not significantly different (Table 3.3.). Percentage compositions of the lipoproteins were varied, but did not differ significantly between the two strains of rabbit, except in phospholipid content, which was significantly increased in SMHL rabbits in all four fractions. Additionally, the percentage of free cholesterol in $VLDL_1$ in SMHL rabbits was decreased compared to NZW controls (5.0 ± 0.7 vs 16.0 ± 6.8, $p < 0.05$).

3.3.5 Apolipoprotein content

Apolipoprotein content was examined by SDS glycerol polyacrylamide gel electrophoresis. Protein isolated from $VLDL_1$, $VLDL_2$, IDL and LDL fractions separated by density gradient ultracentrifugation of plasma was loaded on to the gel (see Table 3.1. for protein concentrations) and the apolipoproteins were separated. A representative gel picture is shown in Figure 3.5. There was no discernible difference in apolipoprotein content between NZW and SMHL rabbits.

Figure 3.5. Apolipoprotein content of NZW and SMHL rabbit $VLDL_1$, $VLDL_2$, IDL and LDL



ApoB100 and apoE bands are clearly seen in NZW and SMHL rabbit VLDL, with apoB100 bands visible in NZW and SMHL rabbit IDL and LDL, showing no difference between the two strains. The protein content loaded on to the gel is given in Table 3.1. Lower molecular weight (Mr) apolipoproteins were incompletely separated.

3.3.6 Insulin resistance status

Plasma insulin levels were measured as an index of the degree of insulin resistance present in 7 NZW and 7 SMHL rabbits from group 1 (aged 5 months), fed the 0.08% cholesterol diet (Table 3.5.). Insulin concentrations were variable, particularly in the SMHL group, but were significantly increased in the SMHL rabbits compared to the NZW ($p = 0.048$).

Table 3.4. % Composition of lipoproteins in 0.08% cholesterol diet fed SMHL and NZW rabbits

	NZW				SMHL			
	$VLDL_1$	**$VLDL_2$**	**IDL**	**LDL**	**$VLDL_1$**	**$VLDL_2$**	**IDL**	**LDL**
Protein	17.1 ± 4.8	16.8 ± 1.4	19.2 ± 1.0	19.9 ± 1.8	8.3 ± 0.9	14.0 ± 0.3	19.4 ± 0.6	25.6 ± 1.7
FC	16.0 ± 6.8	7.0 ± 1.8	7.9 ± 1.1	10.0 ± 2.2	5.0 ± 0.7[a]	7.2 ± 1.8	7.1 ± 1.7	6.0 ± 1.2
CE	28.1 ± 9.0	51.6 ± 4.0	49.1 ± 2.4	37.4 ± 5.8	37.5 ± 5.0	46.1 ± 3.8	42.4 ± 3.2	44.6 ± 3.2
TG	27.7 ± 5.9	8.5 ± 1.8	6.7 ± 1.7	8.3 ± 0.5	36.5 ± 5.2	13.8 ± 3.8	11.3 ± 3.1	11.3 ± 2.7
PL	11.0 ± 2.4	16.1 ± 0.8	17.1 ± 1.2	24.4 ± 3.0	12.8 ± 0.4[a]	18.8 ± 0.4[b]	19.8 ± 0.3[b]	12.4 ± 1.7[c]

Mean ± SEM

SMHL significantly different from NZW rabbits, [a] $p < 0.05$, [b] $p < 0.01$, [c] $p < 0.005$

Phospholipid (PL)

Table 3.5. Plasma insulin concentrations (pmol/l)

Rabbit	NZW	SMHL
1	9.3	344.3
2	7.3	21.5
3	11.8	83.6
4	18.8	23.3
5	27.4	17.0
6	15.5	49.4
7	14.7	15.5
Mean ± SEM	15.0 ± 2.5	79.2 ± 45.1[a]

Significant difference [a] $p < 0.05$

This prompted further investigation of the insulin resistance status of the SMHL rabbits, and oral glucose tolerance tests were performed in the same group of rabbits (now 6 SMHL and 7 NZW) when aged 11 months (section 3.2.2). Mean fasting plasma cholesterol and triglyceride concentrations were 3.5 ± 1.8 vs 1.4 ± 0.4 mmol/l (SMHL vs NZW), NS, and 1.3 ± 0.8 vs 0.3 ± 0.01 mmol/l (SMHL vs NZW), NS, and did not vary significantly over the 2 hours of the experiment. There was no difference in mean area under the curve for the glucose response (Table 3.6.), however the mean areas under the curve for both FFA (Figure 3.6.) and insulin (Figure 3.7.) were significantly elevated in the SMHL rabbits compared to the NZW rabbits (FFA area under the curve was 1.2 ± 0.2 vs 0.5 ± 0.1 mmol/l, SMHL vs NZW, $p < 0.05$; insulin area under the curve was 62.0 ± 10.0 vs 20.9 ± 4.0 μU/ml, SMHL vs NZW, $p < 0.01$, Table 3.6. Thus there was an abnormal response by the SMHL rabbits to the glucose dose, consistent with insulin resistance. Insulin concentrations were significantly increased in the SMHL rabbits at 15, 45, 60 and 120 minute time points ($p < 0.05$).

Figure 3.6.A Plasma FFA response of NZW rabbits to an oral dose of glucose

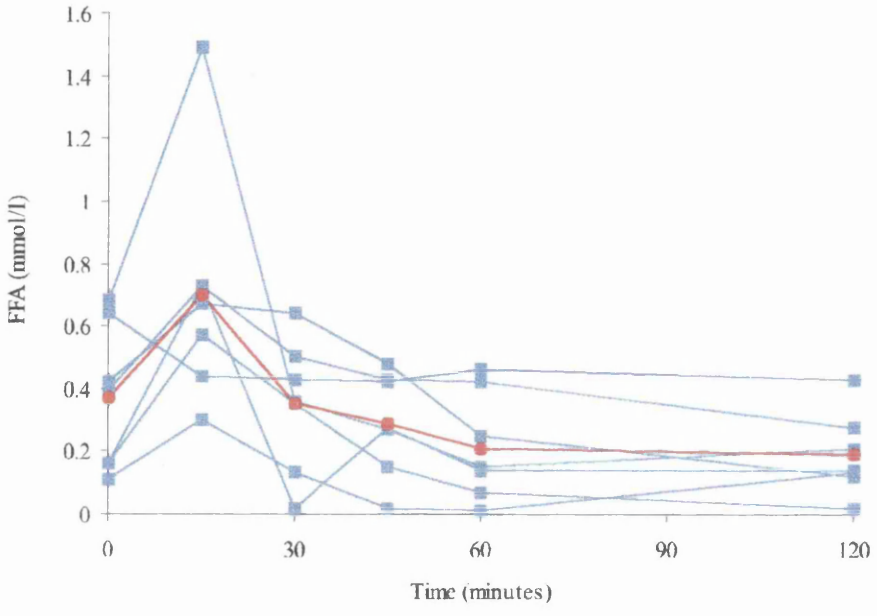
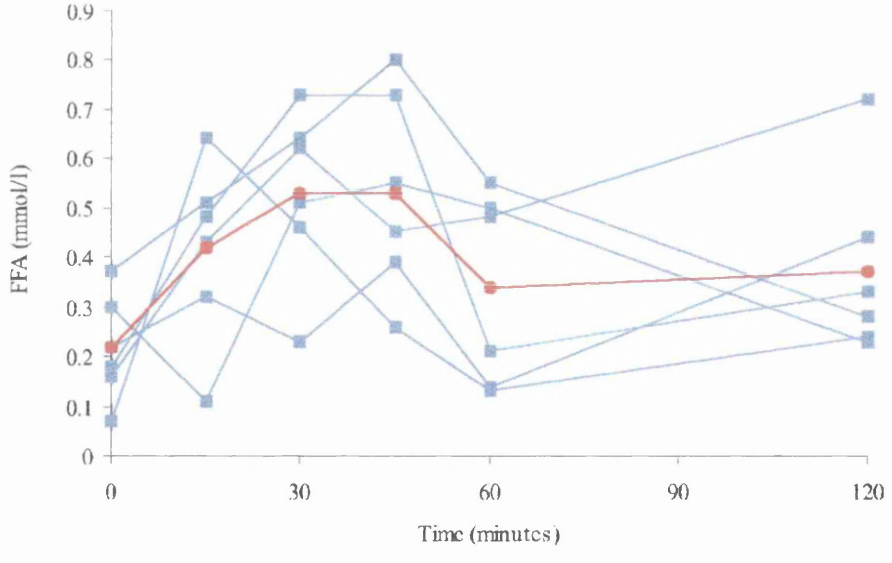


Figure 3.6.B Plasma FFA response of SMHL rabbits to an oral dose of glucose



Individual rabbit responses are shown in blue (■), and mean values are shown in red (●)

Figure 3.7.A Plasma insulin response of NZW rabbits to an oral dose of glucose

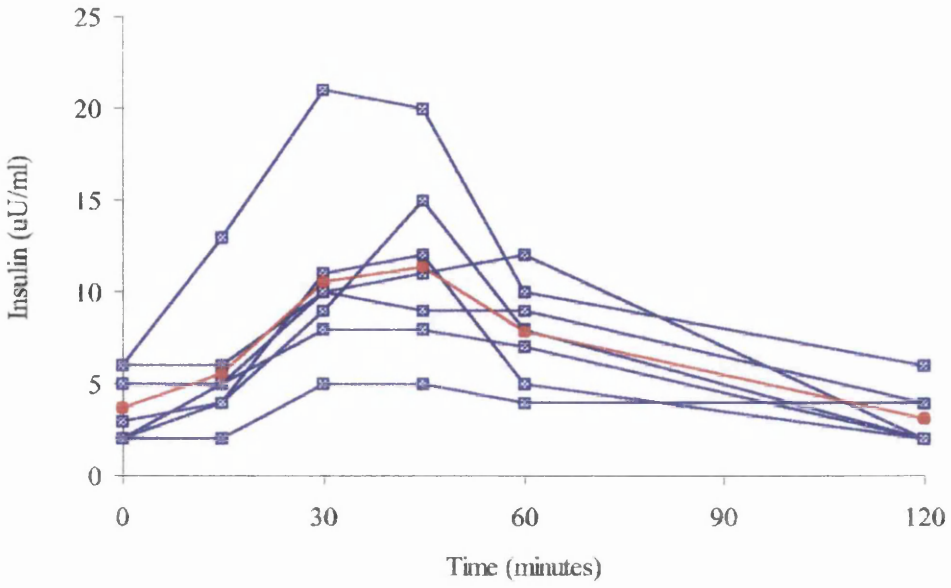
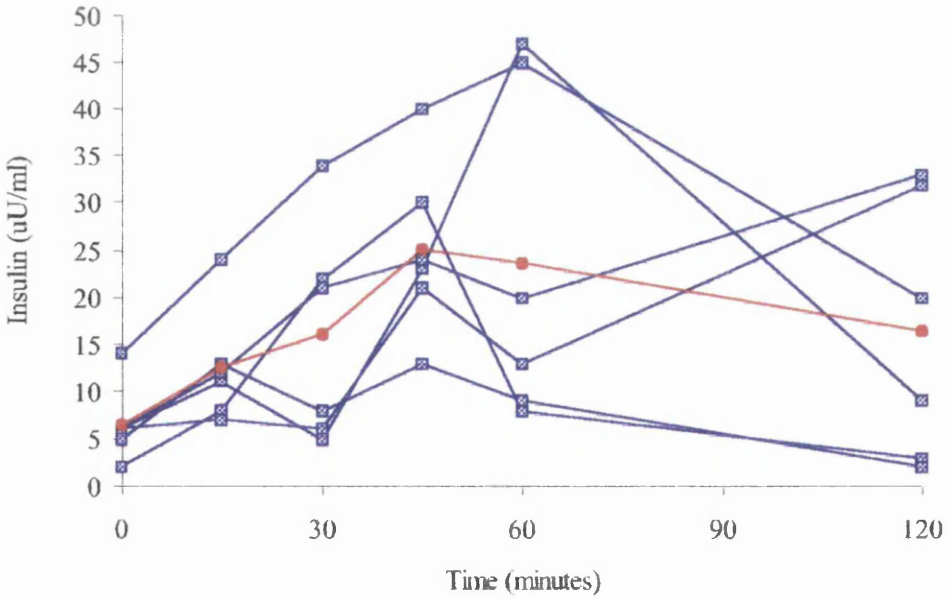


Figure 3.7.B Plasma insulin response of SMHL rabbits to an oral dose of glucose



Individual rabbit responses are shown in blue (■), mean values are shown in red (■)

We tested for correlations between variables within the whole group, and also within the NZW and SMHL populations individually. Significant positive correlations were seen between the insulin area under the curve and both the glucose area under the curve and (log) plasma triglyceride (Figure 3.8.). Additionally, a positive correlation was seen between FFA area under the curve and (log) plasma cholesterol but not plasma triglyceride (Figure 3.8.).

Table 3.6. Insulin, FFA and glucose area under the curve in response to an oral glucose dose

Mean area under the curve	NZW	SMHL	p-value
Insulin (μU/ml)	20.9 ± 4.0	62.0 ± 10.0	< 0.01
FFA (mmol/l)	0.5 ± 0.1	1.2 ± 0.2	< 0.05
Glucose (mmol/l)	14.6 ± 1.9	20.2 ± 3.1	NS

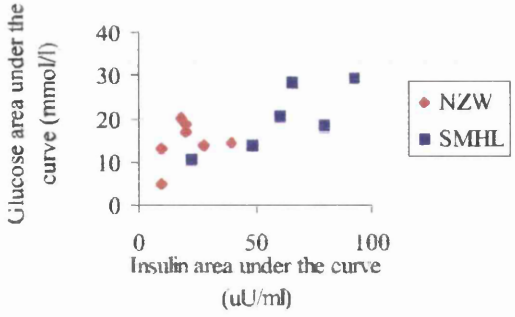
3.3.7 Lipoprotein lipase and hepatic lipase activities

LPL and HL activities in post heparin plasma were measured (section 2.6.2) in 7 NZW and 7 SMHL rabbits from group 1 (aged 4 months) fed the 0.08% cholesterol diet. There were no significant differences in lipase activities between the two strains of rabbit (Table 3.7.) by the Mann Whitney *U* test.

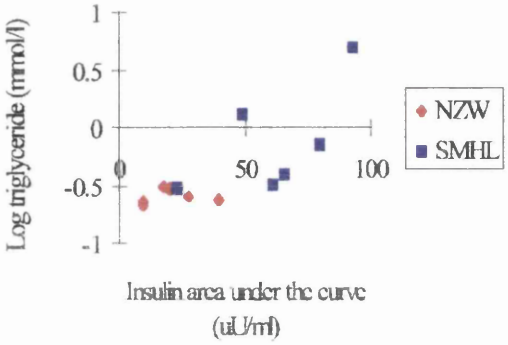
3.3.8 CETP activity

Plasma CETP activities were measured (section 2.6.1) in 7 NZW and 7 SMHL rabbits fed the 0.08% cholesterol diet from group 1. CETP activity was significantly increased in SMHL rabbits when compared to NZW rabbits (128.8 ± 16.9 vs 46.6 ± 9.5 pmoles substrate transferred per 1 μl plasma, $p < 0.01$, Table 3.7.).

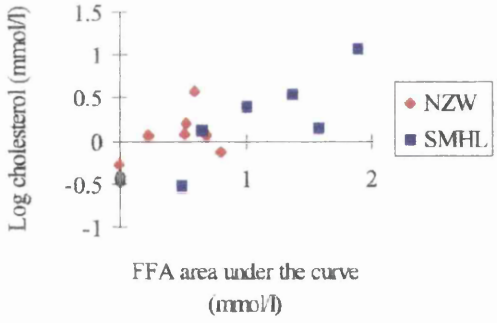
3.8. Correlations between glucose tolerance test variables



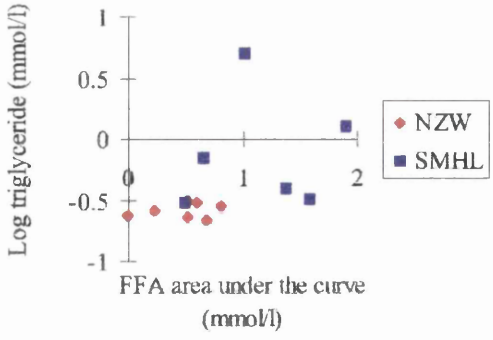
Whole group
r = 0.712
p = 0.006
SMHL rabbits
r = 0.808
p = 0.05



Whole group
r = 0.763
p = 0.002
SMHL rabbits
r = 0.64
p = 0.17



Whole group
r = 0.693
p = 0.009
SMHL rabbits
r = 0.824
p = 0.044



Whole group
r = 0.464
NS
SMHL rabbits
r = 0.094
NS

Table 3.7. Plasma lipase and CETP activities

	LPL activity (μmoles FFA released/ml/hour)		HL activity (μmoles FFA released/ml/hour)		CETP activity (pmoles substrate transfer/μl plasma)	
Rabbit	**NZW**	**SMHL**	**NZW**	**SMHL**	**NZW**	**SMHL**
1	8.9	3.3	1.0	1.0	51.2	81.0
2	8.2	8.1	0.9	4.1	22.8	88.6
3	19.6	6.1	1.4	1.0	64.0	176.0
4	13.7	16.3	4.6	2.7	80.4	165.6
5	15.2	11.3	1.3	1.0	60.6	133.8
6	20.7	18.6	1.2	3.8	40.1	175.7
7	25.5	23.5	1.4	1.3	7.2	81.0
Mean ± SEM	16.0 ± 2.4	12.4 ± 2.8	1.7 ± 0.5	2.1 ± 0.5	46.6 ± 9.5	128.8 ± 16.9[a]

Significant difference [a] $p < 0.01$

3.4. Discussion

The SMHL rabbit is a putative model for the human disorder FCH (La Ville *et al*, 1987). We performed some basic investigation to further characterise the phenotype shown by the SMHL rabbits.

The large population of male rabbits screened for possible use in the liver perfusion experiments showed that plasma cholesterol and triglyceride levels were variable, but higher in SMHL rabbits compared to NZW rabbits. Individually, SMHL rabbits showed elevated cholesterol or triglyceride levels, or, most commonly, increases in both cholesterol and triglyceride concentrations.

Male SMHL rabbits showed a decrease in plasma lipid levels with age, reaching a plateau at 14 - 16 weeks, while NZW rabbits did not change (Figure 3.1., 3.2., 3.3.,

3.4.). In contrast, plasma cholesterol concentrations in female SMHL rabbits increased with age (Figure 3.3.), while plasma triglyceride levels decreased (Figure 3.4.). The mechanisms responsible for this gender difference are unclear, however it is likely that for some reason the IDL and LDL accumulate to higher levels in the female compared to the male SMHL rabbits. Increased hepatic stores of cholesterol could lead to a shut down of LDL receptors and a rise in plasma LDL levels.

Appearance of the hyperlipidaemic phenotype of the SMHL rabbits is dependent on supplementing their diet with a small amount of cholesterol. NZW animals fed the same diet showed no change in plasma lipid levels. However, even on the chow diet, plasma $VLDL_2$ and IDL lipoprotein concentrations were elevated in SMHL rabbits indicating the presence of a lipoprotein disturbance. That La Ville *et al* did not find a difference in plasma lipid levels on feeding a cholesterol supplemented diet could be explained by the shorter duration of her study.

The composition of the lipoprotein particles present in plasma in SMHL and NZW rabbits was investigated. The content of protein, free cholesterol, cholesterol ester and triglyceride was variable, but not significantly different. This is in contrast with the original work performed by La Ville *et al* (1987) who found that free cholesterol and cholesterol ester content were significantly increased in VLDL, IDL and LDL fractions in the St Thomas' hospital rabbits compared to NZW controls, and that triglyceride content was significantly decreased in IDL and LDL in St Thomas' hospital rabbits compared to NZW controls. This may be explained by the fact that the rabbits included in the St Thomas' hospital study exhibited a greater hypercholesterolaemia than did our rabbits. From analysis of the apolipoprotein content of the lipoproteins by SDS glycerol polyacrylamide gel electrophoresis, we could detect no difference in the apolipoprotein distribution between the two strains of rabbit, in accordance with earlier work by La Ville.

The response of the SMHL rabbits to an oral glucose dose was characteristic of a degree of insulin resistance. Compared to the NZW rabbits (Figure 3.7.A), the response in SMHL rabbits was elevated and highly variable (Figure 3.7.B). In the NZW rabbits, insulin secreted in response to the glucose dose will have

downregulated HSL and reduced FFA release from triglyceride stores in the adipose tissue leading to the fall in plasma FFA levels (Figure 3.6.). However in the SMHL rabbits, there was resistance to the effects of insulin evidenced by the hyperinsulinaemia (Figure 3.7.B), and by the fact that the insulin release failed to lower plasma FFA, presumably due to more FFA release from adipose tissue (Figure 3.6.).

Insulin area under the curve was positively correlated with glucose area under the curve, demonstrating that an exaggerated insulin response is associated with impaired glucose disposal (Figure 3.8.); a correlation in the whole group that was driven by a positive correlation in the SMHL group. No correlation was seen between the insulin area under the curve and the FFA area under the curve, but there was a positive correlation between insulin area under the curve and (log) plasma triglyceride again primarily in the SMHL rabbits, explained possibly by an increased FFA delivery to the liver in insulin resistant animals which results in an increased triglyceride output. However, FFA area under the curve did not correlate with (log) plasma triglyceride as is the case in humans (Laws, 1996), but did show a positive correlation with (log) plasma cholesterol. This unusual finding may indicate that an increase in FFA due to insulin resistance is associated with the hyperlipidaemia but may not be the underlying driving force. If it was, we would have expected a tight correlation between FFA and plasma triglyceride and little association with plasma cholesterol.

Post heparin LPL and HL activities were measured in both strains of rabbit and did not show any significant differences. HL levels were low in both strains, in accordance with previous findings (Warren, Ebert, Mitchell, *et al*, 1991). This suggests that there is no underlying defect in the lipolytic cascade in the SMHL rabbits (Figure 1.1) and that any differential delipidation of lipoprotein species is due to altered affinity of the particles themselves for the lipase enzymes. This provides further evidence for the suitability of the SMHL rabbits as a model for FCH, as, although LPL activity has been reported to be impaired in approximately one third of patients with FCH (Babirak *et al*, 1992), severe defects in this gene have not been shown to be important in FCH patients, and moderate variations in LPL activity may

merely magnify the phenotype. Therefore LPL deficiency is not an obligate marker for FCH.

CETP activity has been documented to be increased in individuals with various forms of hyperlipidaemia, including combined hyperlipidaemia (Tatò, Vega, Tall, *et al*, 1995). Increased CETP activity may result in elevated cholesterol concentrations in lipoprotein remnants, which are then cleared less efficiently from plasma. In the presence of hypertriglyceridaemia, an increased CETP concentration can cause triglyceride transfer from VLDL to LDL ultimately resulting in the formation of small dense LDL particles, a risk factor for CHD. However, CETP deficiency can also be atherogenic as a lack of CETP results in large HDL particles that may not have anti-atherogenic functions, and is also associated with small LDL particles (Yamashita, Sakai, Hirano, *et al*, 1997). Therefore CETP levels require regulation within a narrow optimal level to exert anti-atherogenic effects.

Our findings of a significantly elevated CETP activity in the SMHL rabbits is intriguing and further confirms the rabbits as being a suitable model for FCH (Table 3.7.). Cholesterol feeding has been shown to increase CETP activity in rabbits (Quinet, Agellon, Kroon, *et al*, 1990) but whether the cholesterol level (0.08%) in the diet we fed to our rabbits was sufficient to induce an increase in CETP mRNA levels and mass is unclear, as Quinet *et al* supplemented their diet with 0.5% cholesterol. In any case, here we show an increased CETP activity in the cholesterol fed SMHL rabbits compared to the cholesterol fed NZW rabbits.

On balance, the SMHL rabbit exhibits many features that have been reported in the literature as being associated with FCH. The remainder of this thesis describes a series of experiments designed to take a more in depth look at the metabolic disorder in these animals.

Chapter 4. Stable Isotope Turnover Studies

4.1. Introduction

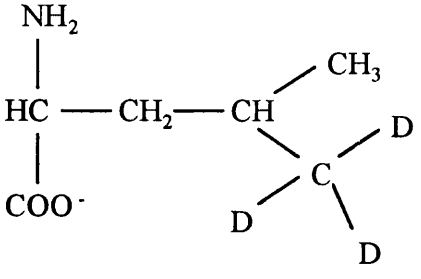
The use of stable isotopes to investigate lipid metabolism *in vivo* in humans was first described in 1935 by Schönheimer and Rittenberg. However, it is only recently that they have become widely used, due to the increased availability of stable isotopes and improvements in gas chromatography - mass spectrometry (GC-MS) detection systems.

The basis of the stable isotope turnover technique is the endogenous labelling of proteins with the injected tracer, usually a stable isotope enriched amino acid. The production and catabolism of the protein of interest can then be followed in several plasma samples by isolating the protein, in this case apoB, and analysing its isotopic enrichment by GC-MS. However the endogenous labelling of proteins with stable isotopes has a number of disadvantages, for example that all newly synthesised proteins are labelled with the stable isotope and therefore the one of interest must be purified, and that interconversion and recycling of label must be taken in to account. Stable isotopes are difficult to measure, however they are much safer than radio-isotopes. Endogenous labelling provides much clearer information on production rates (PR) of proteins than does exogenous labelling which infers PR from steady state turnover data.

The stable isotope turnover technique has been applied to a wide variety of lipid disorders in humans but has rarely been used in any animal species because radio-isotopes are cheaper and easier to detect. Nonetheless, we decided to apply the stable isotope turnover technique currently used in humans in our laboratory (Demant, Packard, Demmelmair, *et al*, 1996), to our rabbit population. We hoped that, if successful, this would enable a better comparison with humans and give a clearer picture of lipoprotein production, while proving a safer technique.

The choice of tracer depends on the protein of interest. The tracer amino acid must be abundant in the protein of interest. For apoB, L-[5,5,5 $^{2}H_3$]-leucine (d_3 leucine) is the tracer of choice (Figure 4.1.) as leucine is one of the most abundant amino acids in apoB, and it can be detected with precision in the mass spectrometer. Additionally, it is an essential amino acid, and is metabolised in the muscle, therefore the extent of recycling in the hepatic precursor pool is minimised. The stable isotope can be administered as a bolus or as a primed constant infusion. Generation of kinetic rate constants from the enrichment data is performed by multicompartmental modelling.

Figure 4.1. Structure of d_3 leucine



D = deuterium

We wished to use the stable isotope turnover technique in the SMHL rabbits to investigate the kinetics of the apoB containing lipoproteins in these rabbits in comparison to NZW controls. La Ville *et al* (1987) performed exogenous radiolabelled kinetic experiments on the St Thomas rabbits, and found that they had an increased VLDL and LDL apoB PR and a decreased LDL FCR. We planned a more detailed series of experiments to look at the metabolism of apoB in $VLDL_1$, $VLDL_2$, IDL and LDL to find potential mechanisms that could describe the hyperlipidaemia seen in these animals.

4.2. Methods

4.2.1 Selection of animals

Five male SMHL rabbits fed the 0.08% cholesterol diet since weaning and aged between 10 and 18 months were selected and five NZW rabbits of the same sex and similar age were used as control rabbits.

4.2.2 Calculation of standard apoB % in $VLDL_1$, $VLDL_2$, IDL and LDL

Human studies have demonstrated that the percentage of protein that is apoB in each lipoprotein fraction is relatively constant between individuals. Because of insufficient sample throughout the turnover we were unable to calculate apoB mass from the turnover samples. Therefore we determined the apoB % in $VLDL_1$, $VLDL_2$, IDL and LDL of 3 NZW and 3 SMHL rabbits to use as a standard. Ultracentrifugation of 2 ml fasted plasma samples from each rabbit to separate these lipoproteins (section 2.3.8) was followed by apoB precipiation (section 2.4.1). Protein content was measured in lipoprotein fractions and the isopropanol supernatant (non apoB protein), and this value was subtracted from total protein to give a value for apoB. ApoB protein was expressed as a percentage of total protein. The values obtained here did not vary greatly when compared to those in humans, except in $VLDL_2$ (Table 4.1.), and therefore, due to the small sample size we decided to use the widely accepted values determined from humans.

Table 4.1. Standard apoB%

	$VLDL_1$	$VLDL_2$	IDL	LDL
NZW mean	44.5 ± 3.1	74.3 ± 1.7	87.3 ± 0.7	86.3 ± 3.5
SMHL mean	50.4 ± 1.1	72.8 ± 1.7	82.8 ± 1.2	86.1
Whole group	46.8 ± 2.2	73.5 ± 1.2	84.6 ± 1.3	86.2 ± 2.0
Human	45	60	85	90

4.2.3 Testing of the separation of $VLDL_1$, $VLDL_2$, IDL and LDL from small volumes of plasma

Due to the small blood volume of the rabbits, it was only possible to take 2.5 ml blood samples at each time point. Therefore the separation of $VLDL_1$, $VLDL_2$, IDL and LDL from 2 ml of sample would have to be done on a 1:1 dilution of plasma with saline. To ensure that this was possible and did not affect the separation of the lipoproteins, we compared the separation of these lipoproteins (section 2.3.8) from 2 ml plasma and 1 ml plasma plus 1 ml saline from 1 NZW and 1 SMHL rabbit. We measured the content of total cholesterol, free cholesterol, triglyceride, phospholipid and protein in each fraction (sections 2.3.2, 2.3.5, 2.3.3, 2.3.6, 2.4.2 respectively).

4.2.4 Stable isotope turnover protocol

The rabbits were fasted overnight (> 15 hours). A 2.5 ml sample of blood was withdrawn from the marginal ear vein and subsequently a bolus dose of 10 mg d_3 leucine/kg body weight (Isotec Inc.) was injected. Two and a half ml blood samples were withdrawn periodically over 4 - 27 hours (Table 4.2.). Timing of samples was varied to obtain different levels of information on apoB particle secretion in the rabbit, and was a function of the size of the rabbit and the number of samples we were permitted to withdraw. One NZW and 1 SMHL rabbit followed protocol 1, 1 SMHL followed protocol 2, and the remainder, 4 NZW and 3 SMHL followed protocol 3. The majority of these experiments were performed by Mr D. Grimsditch and staff of L. A. S., Smithkline Beecham Pharmaceuticals.

Plasma was prepared from each sample and cholesterol and triglyceride concentrations were measured at time 0. $VLDL_1$, $VLDL_2$, IDL and LDL were separated at each time point (section 2.3.8) from 1 ml plasma plus 1 ml saline. ApoB was isolated as described previously (section 2.4.1).

Table 4.2. Sample protocol (minutes)

Protocol 1	Protocol 2	Protocol 3
0	0	0
15	30	30
30	40	40
40	50	50
50	60	60
60	75	75
75	90	90
90	105	105
105	120	120
120	180	180
180	240	240
240	360	360
		1440
		1620

4.2.5 Preparation of samples for GC-MS

ApoB pellets from $VLDL_1$, $VLDL_2$, IDL and LDL were hydrolysed by adding 2 ml of 6 M HCl and incubating at 110°C for 24 hours. The amino acid hydrolysate was concentrated in a vacuum concentrator centrifuge (Howe) and transferred to Chromacol vials (Chromacol Ltd.) before being dried completely.

4.2.6 Measurement of plasma leucine

One hundred µl of plasma from each time point was mixed with 100 µl 10% TCA and incubated at room temperature for 30 minutes. The samples were spun at 1780 *g* for 30 minutes at 4°C and the supernatant was loaded on to a cation exchange column (Dowex AG® 50W-X8 resin, 100 - 200 mesh, hydrogen form, Bio-Rad Laboratories)

pre-treated with 1 M HCl. The TCA was washed through with deionised H_2O, and the amino acids were eluted with 4 M NH_4OH. The samples were concentrated on the centrifugal evaporator , transferred to Chromacol vials and taken to dryness.

4.2.7 Sample analysis by GC-MS

The amino acids were converted to *tert*-butyl-dimethyl-silyl (TBDMS) derivatives, and the specific enrichment of tracer amino acid (the atom percent excess) in these derivatives was then measured on a Fisons Trio 1000 quadrupole system (Fisons Instruments). This work was performed by Mr J. P. Stewart and Mrs D. K. Bedford. The sensitivity of measurement was increased by comparing the d_3 leucine peak with a naturally occuring leucine (m + 2) peak, instead of the most common leucine (m = 0) peak The m + 2 peak occurs at a constant ratio with the m = 0 peak. To calculate the tracer to tracee ratio (the equivalent to specific activity in radioactive terms) the ratio of the sample and the naturally occurring leucine (m + 3) to leucine (m = 0) is used (Demant, Packard, Stewart, *et al*, 1994) (Figure 4.2.). The d_3 leucine peak (m + 3) is much closer in size to the m + 2 peak compared to the m = 0 peak, and this allows their ratio to be determined with greater precision.

Figure 4.2. Calculation of tracer/tracee ratios from mass ratios

$$\mathbf{E = (R - R_o)/[(1 + R) + (1 + R_o)]}$$

where E is the specific isotopic enrichment, R is the ratio of leucine (m = 3) : leucine (m + 0) (in sample), and R_o is the ratio of leucine (m = 3) : leucine (m + 0) (naturally occurring).

$$\mathbf{Z = E/(E_t - E)}$$

where Z is the tracer/tracee ratio and E_t is the specific enrichment of the d_3 leucine tracer.

4.2.8 Multicompartmental modelling

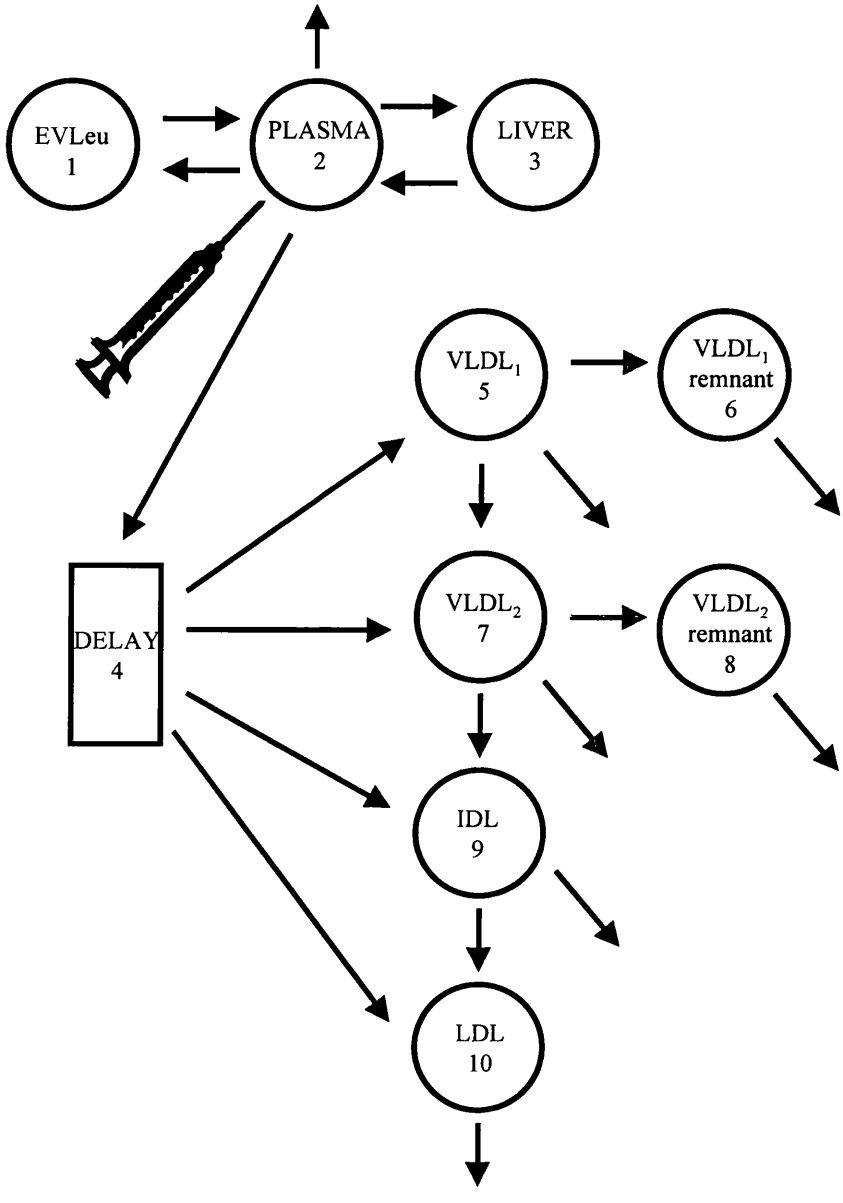
Using the tracer/tracee ratios from the plasma, $VLDL_1$, $VLDL_2$, IDL and LDL, and the apoB masses, the rates of production, transfer and catabolism of the lipoprotein species were calculated by creating a multicompartmental model.

Multicompartmental modelling was performed using the SAAM II programme. This software programme is a tool which creates models, and designs and simulates experiments, enabling the analysis of turnover data. The compartmental system is a visually interactive module, enabling the operator to graphically create an experimental model, from which the programme creates a series of differential equations. Experimental data is associated with specific compartments, and SAAM II fits the model to the data, generating rate constants.

Based on physiological knowledge and the ability to fit the data, we created a minimal model to express our results (Figure 4.3.). The plasma data is modelled in 3 compartments. The input of d_3 leucine is to the plasma pool (2), and this equilibrates with a hepatic (3) and an extravascular (1) leucine pool. A delay compartment accounts for the intrahepatic synthesis of apoB (4). From there, apoB can be channelled in to $VLDL_1$ (5), $VLDL_2$ (7), IDL (9) or LDL (10) compartments. Additionally there is a delipidation chain representing the stepwise delipidation of VLDL through to LDL. Finally, for some animals it was necessary to create compartments to describe more slowly metabolising lipoproteins, so called $VLDL_1$ and $VLDL_2$ remnants (6,8). We applied constraints to these compartments where the rate of catabolism of these remnants was equal ($k(0,6) = k(0,8)$).

Plasma data was fitted to the plasma model first. The parameters were then fixed, and the lipoprotein data was subsequently fitted, first in the absence of $VLDL_1$ and $VLDL_2$ remnant compartments, which were only added if they were required for best fit.

Figure 4.3. Compartmental Model



4.3. Results

4.3.1 Comparison of 2 ml plasma sample with 1 ml plasma sample plus 1 ml saline for separation of $VLDL_1$, $VLDL_2$, IDL and LDL

When we separated $VLDL_1$, $VLDL_2$, IDL and LDL from 2 ml plasma and compared them to the lipoproteins separated from 1 ml plasma plus 1 ml saline, we found that there was no difference in the composition of the lipoproteins, and that the contents were approximately half in the diluted sample compared to the neat sample (Table 4.3.). Therefore we concluded that the separation from diluted plasma was comparable to that from neat plasma and could be used for the turnover studies.

Table 4.3. Composition of $VLDL_1$, $VLDL_2$, IDL and LDL in samples prepared from neat or dilute plasma

	NZW									
	1 ml plasma + 1 ml saline					2 ml plasma				
	TC	FC	TG	PL	Prot	TC	FC	TG	PL	Prot
V1	0.09	0.09	0.07	9.4	4.6	0.21	0	0.13	4.1	6.2
V2	0.29	0.06	0.03	6.6	6.2	0.62	0.09	0.08	11.9	11.1
IDL	0.66	0.06	0.07	15.8	12.9	1.3	0.15	0.13	28.1	25.5
LDL	0.07	0	0.01	4.02	3.19	0.25	0	0.02	12.6	7.0
	SMHL									
	1 ml plasma + 1 ml saline					2 ml plasma				
V1	0.28	0.05	0.16	5.3	8.0	0.57	0	0.33	11.1	15.4
V2	0.53	0	0.06	11.3	10.1	1.2	0.24	0.13	19.2	22.6
IDL	0.51	0.03	0.04	13.0	12.0	1.1	0.02	0.09	23.36	17.0
LDL	0.08	0	0.01	3.3	3.6	0.24	0.05	0.03	6.0	6.5

TC - total cholesterol (mmol/l), FC - free cholesterol (mmol/l), TG - triglyceride (mmol/l), PL - phospholipid (mg/dl), Prot - protein (mg/dl)

4.3.2 Population lipids

Although there was a trend to higher lipid levels in SMHL rabbits, there was no significant difference in plasma cholesterol and triglyceride levels between the NZW and SMHL rabbits (Table 4.4.), ie SMHL rabbits in this small group of mature animals were not hyperlipidaemic.

Table 4.4. Mean plasma cholesterol and triglyceride concentrations

	NZW		SMHL	
Rabbit	**Cholesterol (mmol/l)**	**Triglyceride (mmol/l)**	**Cholesterol (mmol/l)**	**Triglyceride (mmol/l)**
1	0.90	0.45	1.17	0.47
2	2.03	0.99	2.83	1.87
3	0.98	0.54	2.28	2.29
4	1.52	0.67	1.26	0.40
5	0.64	0.78	0.72	0.86
Mean ± SEM	1.21 ± 0.25	0.69 ± 0.09	1.65 ± 0.39	1.18 ± 0.38

4.3.3 ApoB pool sizes

We calculated the apoB mass (mg) in each lipoprotein species by calculating the percentage of protein that was apoB using the standard values of 45, 60, 85 and 90% of the total protein in each fraction. IDL apoB mass and total apoB mass were significantly elevated in the SMHL rabbits compared to the NZW rabbits (Table 4.5.).

Table 4.5. ApoB mass (mg) in $VLDL_1$, $VLDL_2$, IDL, LDL and total lipoproteins

	$VLDL_1$	$VLDL_2$	IDL	LDL	Total
Rabbits	**NZW**				
1	0.9	1.3	2.5	1.8	6.5
2	1.7	3.1	2.9	5.2	12.9
3	4.0	3.0	2.6	8.7	18.3
4	10.2	2.0	3.9	2.7	18.8
5	1.0	1.1	3.8	1.2	7.1
Mean ± SEM	3.5 ± 1.6	2.1 ± 0.4	3.1 ± 0.3	3.9 ± 1.3	12.7 ± 2.4
	SMHL				
1	0.5	1.6	9.6	15.7	27.4
2	8.4	14.0	16.7	18.4	57.5
3	9.4	5.4	9.9	3.3	28.0
4	0.8	1.2	7.4	6.2	15.6
5	1.7	5.5	14.7	5.7	27.6
Mean ± SEM	4.2 ± 1.8	5.5 ± 2.1	11.7 ± 1.6[a]	9.9 ± 2.7	31.2 ± 6.3[a]

Significant differences (NZW vs SMHL) [a] $p < 0.05$

4.3.4 Kinetic parameters - NZW

Multicompartmental modelling was performed using tracer/tracee ratios and apoB masses of $VLDL_1$, $VLDL_2$, IDL and LDL. Rates of production, transfer and catabolism of each particle were defined (Table 4.6.).

Table 4.6. Kinetic results - NZW

Rabbits	$VLDL_1$ PR (mg/d)	$VLDL_1$ FDC (pools/d)	$VLDL_1$ FTR (pools/d)	$VLDL_2$ PR (mg/d)	$VLDL_2$ FDC (pools/d)	$VLDL_2$ FTR (pools/d)
NZW1	32.2	0	36.0	29.1	35.6	11.6
NZW2	61.3	36.7	0	12.1	3.6	1.55
NZW3	62.0	0	15.7	70.4	44.9	0
NZW4	127.0	1.0	40.1	0	56.5	0
NZW5	37.5	39.8	0	17.7	16.6	0
Mean ± SEM	64.0 ± 16.9	15.5 ± 9.3	18.4 ± 8.6	25.9 ± 12.1	31.4 ± 9.5	2.6 ± 2.3

Rabbits	IDL PR (mg/d)	IDL FDC (pools/d)	IDL FTR (pools/d)	LDL PR (mg/d)	LDL FCR (pools/d)
NZW1	10.0	6.4	3.8	4.6	8.1
NZW2	2.4	0	1.3	4.8	1.6
NZW3	16.8	0	6.6	16.8	4.0
NZW4	8.4	2.1	0	4.2	1.8
NZW5	12.0	3.1	0	3.5	2.8
Mean ± SEM	9.9 ± 2.4	2.3 ± 1.2	2.4 ± 1.3	6.8 ± 2.5	3.6 ± 1.2

FDC - fractional rate of direct catabolism

FTR - fractional transfer rate

d - day

A representative fit of the curves (solid lines) describing the kinetic parameters to the data points are shown in Figure 4.4.A. A schematic diagram of the kinetic parameters is shown in Figure 4.5.A.

Figure 4.4.A Representative tracer curves from a NZW rabbit

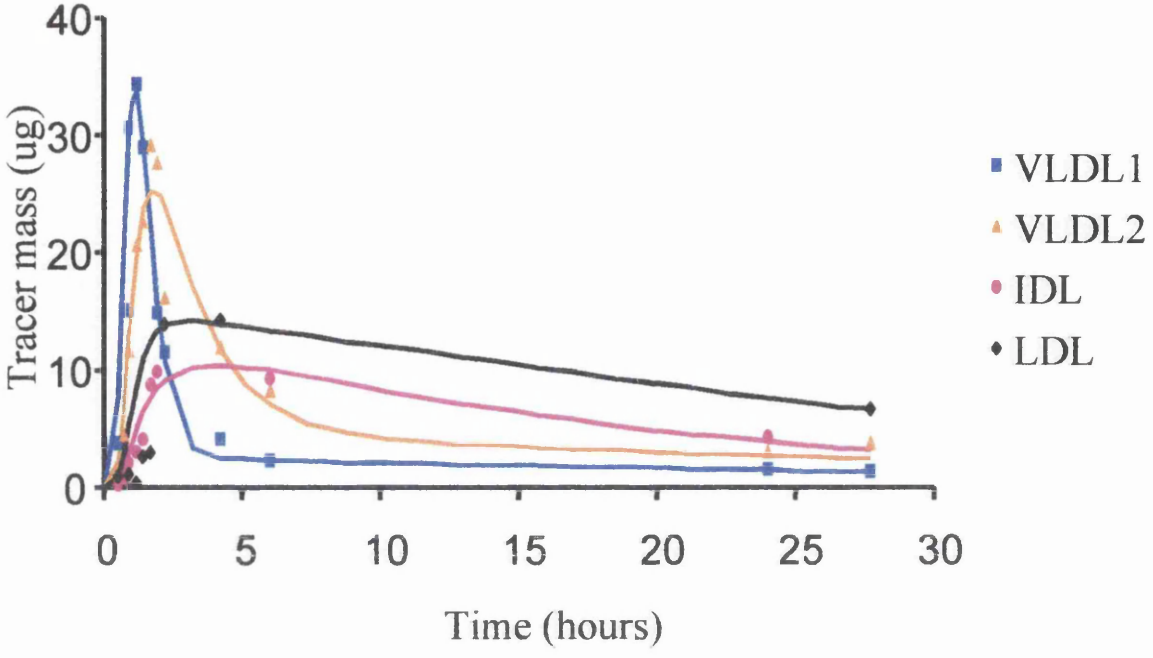


Figure 4.4.B Representative tracer curves from a SMHL rabbit

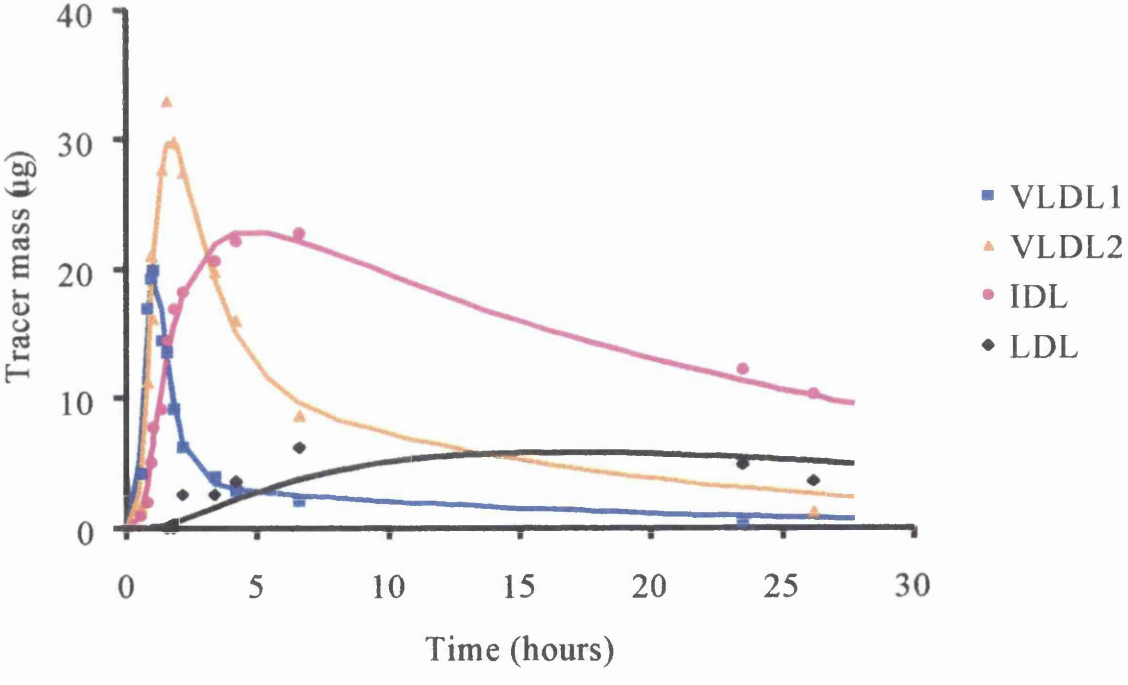


Figure 4.5.A Mean NZW kinetic parameters

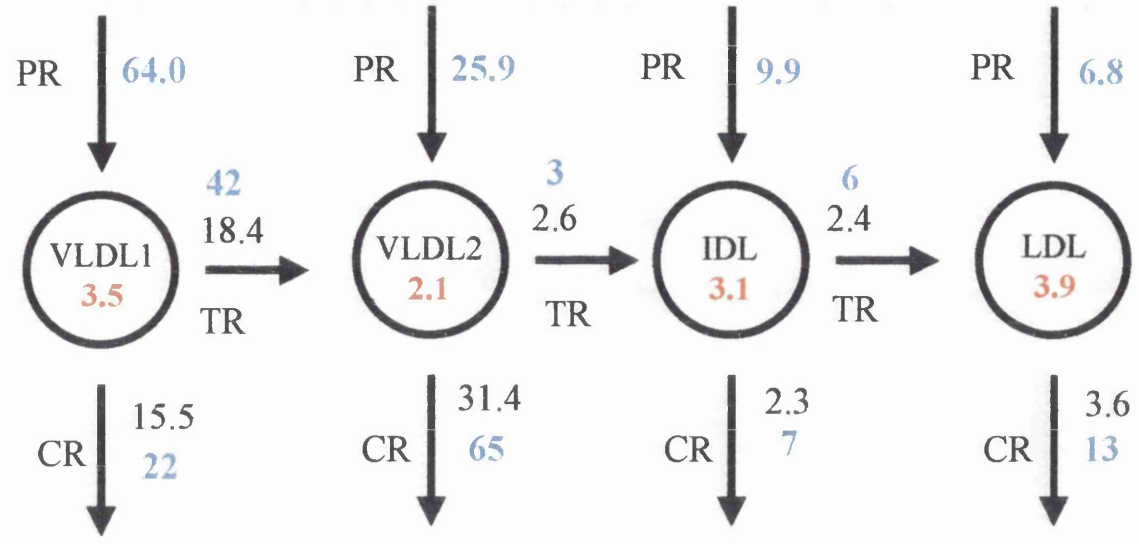
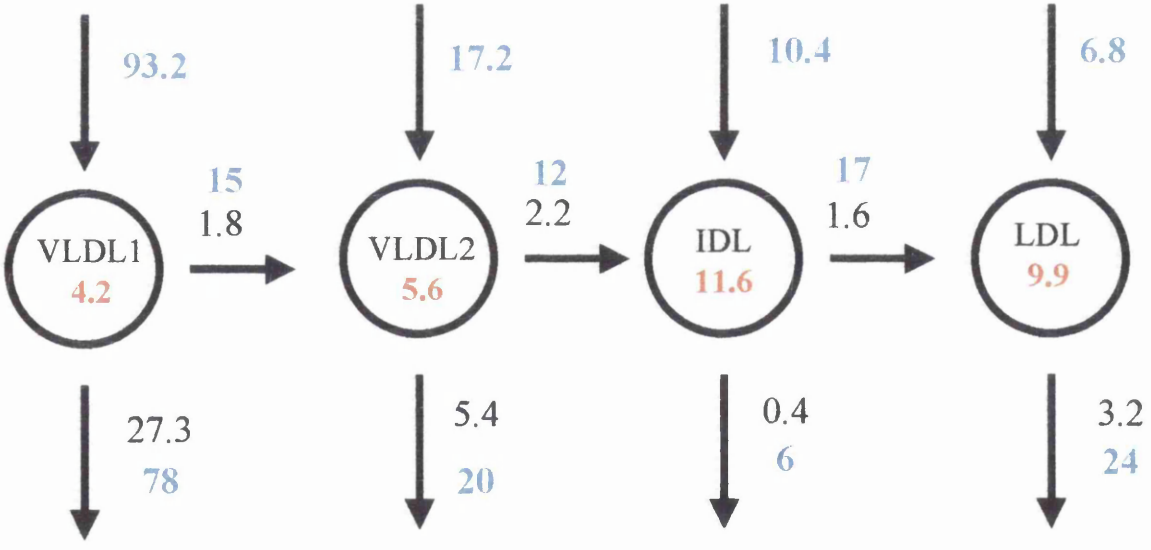


Figure 4.5.B Mean SMHL kinetic parameters



Production rates (PR), transfer rates (TR) and clearance rates (CR) of apoB.

mg apoB/day *mg apoB* *pools/day*

4.3.5 Kinetic parameters - SMHL

Rates of production, transfer and catabolism of lipoprotein particles in the SMHL rabbits were calculated from the multicompartmental model (Table 4.7.).

Table 4.7. Kinetic results - SMHL

Rabbits	$VLDL_1$ PR (mg/d)	$VLDL_1$ FDC (pools/d)	$VLDL_1$ FTR (pools/d)	$VLDL_2$ PR (mg/d)	$VLDL_2$ FDC (pools/d)	$VLDL_2$ FTR (pools/d)
SMHL1	11.8	21.5	0	13.9	8.7	0
SMHL2	133.2	7.0	9.0	15.5	3.7	2.8
SMHL3	209.4	22.6	0	16.3	0.4	2.6
SMHL4	34.5	40.8	0	12.7	10.6	0
SMHL5	77.7	45.0	0.02	27.8	3.4	5.6
Mean ± SEM	93.3 ± 32.5	27.4 ± 11.0	1.8 ± 1.6	17.2 ± 2.5	5.4 ± 2.1	2.2 ± 0.9

Rabbits	IDL PR (mg/d)	IDL FDC (pools/d)	IDL FTR (pools/d)	LDL PR (mg/d)	LDL FCR (pools/d)
SMHL1	24.3	0	2.5	19.4	2.8
SMHL2	4.6	1.5	1.2	0	1.1
SMHL3	2.3	0.2	1.5	4.7	5.9
SMHL4	14.1	0.2	1.7	9.9	3.6
SMHL5	6.7	0	1.0	0	2.6
Mean ± SEM	10.4 ± 3.6	0.4 ± 0.3	1.6 ± 0.2	6.8 ± 3.3	3.2 ± 0.7

Curves showing representative fits (solid lines) of the modelled kinetic curves to the data produced are shown in Figure 4.4.B. Kinetic parameters are shown in schematic form in Figure 4.5.B.

SMHL rabbits did not show a significant difference in any of the kinetic parameters when compared to NZW rabbits. However, there were clear differences in some $VLDL_1$ PR (for example SMHL 2 and 3), that may have reached significance given a larger sample size, or reduced variability.

4.3.6 Correlations

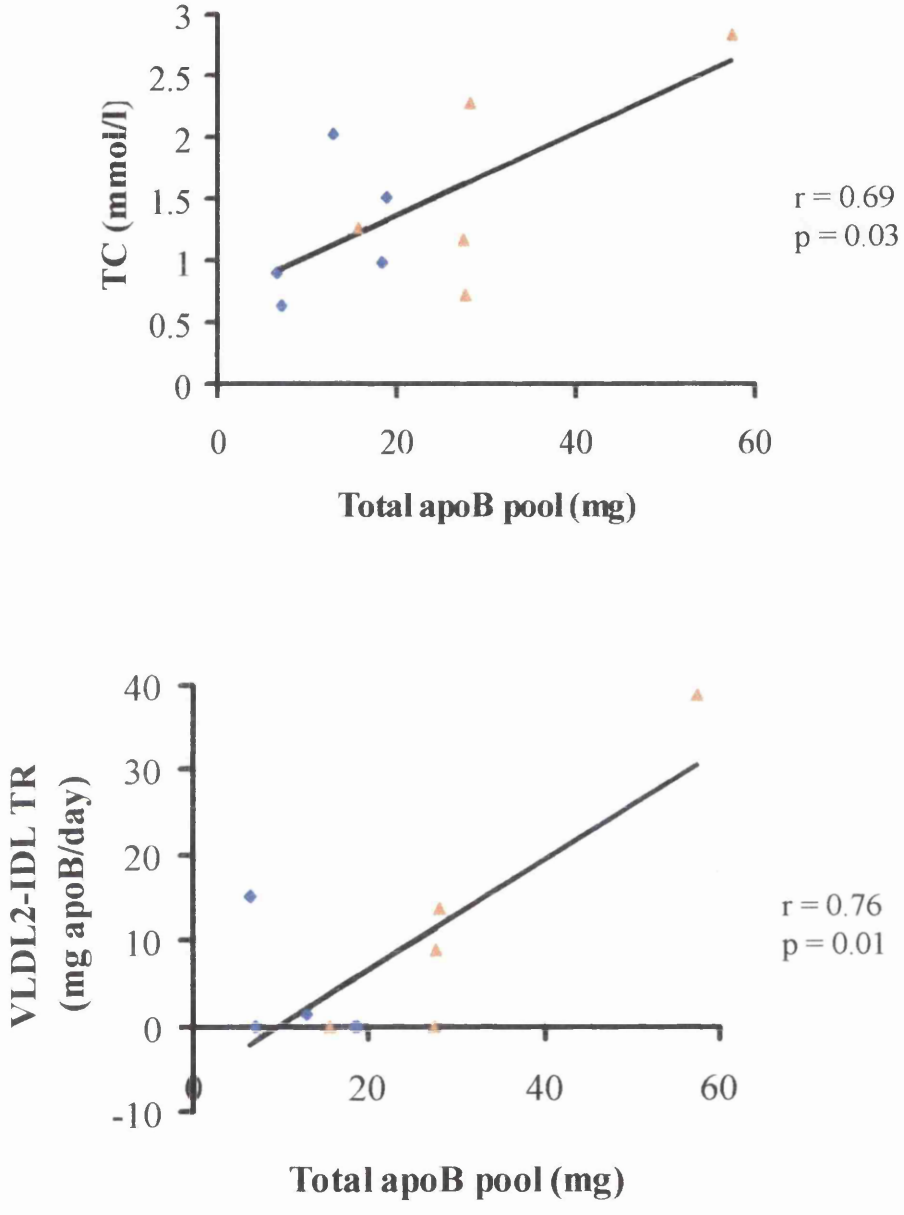
When parameters from both NZW and SMHL rabbits were combined, there were significant positive correlations between total apoB pool and plasma cholesterol concentration and $VLDL_2$ FTR (Figure 4.6).

4.4. Discussion

The method for human stable isotope turnover, adapted for the rabbit, was basically sound, but suffered as the levels of apoB isolated were small and levels of tracer enrichment were at the limit of detection by our GC-MS system. We were confident that our method of isolation of lipoproteins by density gradient ultracentrifugation of diluted plasma samples resulted in an accurate separation of lipoproteins, and that the recovery was similar to that from a non diluted plasma sample. However, the fact that the plasma was diluted 2 fold, added to the fact that the rabbits have a lower concentration of plasma lipid than do humans, meant that there was not much material in the samples (especially LDL) from which to isolate apoB. Thus obtaining precise enrichments was difficult despite great care being taken that no sample was lost during the isolation procedures and the loading of a larger amount of sample than usual on to the GC-MS.

With the small sample size in each group, and the large variance in kinetic parameters within group due partly to the problems with detection by the GC-MS, it was perhaps not surprising that there were no significant differences between NZW and SMHL rabbits. Also in this group of rabbits there was no significant difference in plasma lipids.

Figure 4.6. Correlations between total apoB pool size and plasma cholesterol and $VLDL_2$ - IDL transfer rate



The increased total apoB pool size confirmed work (section 3.3.3) which showed that the SMHL rabbits had a greater $VLDL_1$, $VLDL_2$. IDL and LDL mass compared to the NZW rabbits. IDL apoB pool size specifically was significantly larger in the SMHL rabbits than the NZW rabbits.

Although we did not see any significant differences between the rabbit groups in terms of production rates, transfer rates or catabolic rates, there were minor differences that would help to explain the difference in apoB pool size. The $VLDL_1$ and IDL PR were increased in the SMHL rabbits compared to the NZW rabbits. This would cause an increased flux of apoB into these compartments which may saturate the clearance mechanisms and result in an increased pool size. The $VLDL_1$ PR increase seems to be balanced by an increase in the direct catabolism of apoB from this fraction. This effect does not seem to carry on down the cascade. The NZW rabbits have a large proportion of their $VLDL_2$ cleared directly - 65 out of 68 mg/day (the remaining 3 mg are transferred to IDL). However, the SMHL rabbits have only 20 out of 32 mg/day cleared directly, with the remainder being fed to IDL, contributing to the increased IDL apoB pool size. Indeed, although SMHL rabbits transferred more apoB from IDL to LDL (17 vs 6 mg/day), this was a smaller proportion of the IDL pool size (1.6 vs 2.4 pools/day). Additionally, the IDL FDC was lower in the SMHL rabbits at 0.4 vs 2.3 pools/day, resulting in the clearance of the same absolute amount of apoB (6 vs 7 mg/day (SMHL vs NZW). Therefore, the SMHL rabbits were not able to cope with the increased amount of apoB entering the IDL compartment (both from direct synthesis, and from increased transfer from $VLDL_2$). The LDL apoB pool size is also increased in SMHL rabbits despite an identical PR to the NZW rabbits. Transfer from IDL is increased, and although the amount of apoB that is cleared per day is more in SMHL than in NZW, this is a smaller proportion of the pool size.

Both Venkatesan *et al* (1993) and Cortner, Coates, Bennett, *et al* (1991) found using stable isotopes that production rate of VLDL was increased in human patients with FCH. Additionally, Janus, Nicoll, Turner, *et al* (1980) found using exogenously labelled VLDL and LDL that there was an increased VLDL and LDL PR compared to normal controls in FCH patients. Aguilar-Salinas, *et al* (1997) described a kindred of FCH with an impaired catabolism of VLDL and LDL apoB, but also confirmed an

overproduction of VLDL apoB with normal FCRs in two FCH individuals from other kindreds. Therefore it appears that the human FCH population is heterogeneous, but that the main defect is an overproduction of VLDL and LDL apoB. These results from the SMHL rabbits neither confirm nor deny the strain as a potential model for FCH, exhibiting as they do, tendencies towards both overproduction and under catabolism.

In conclusion, while these experiments were probably a little too ambitious with respect to the measureable levels of apoB in the rabbits samples, these results gave ample cause for further investigation into the metabolic basis for the dyslipidaemia in the SMHL rabbits.

Chapter 5. Development of rabbit liver perfusion methodology

5.1. Introduction

La Ville *et al* (1987) described an overproduction of VLDL and LDL apoB in the St Thomas' hospital rabbits in association with a decreased LDL apoB FCR. In our kinetic studies (Chapter 4), we did not see any significant difference in apoB PR in SMHL rabbits. However, these studies were fraught with problems and, based on the findings of La Ville and the considerable evidence in the literature (Chait *et al*, 1980, Janus, *et al*, 1980, Venkatesan *et al*, 1993) that FCH is associated with an overproduction of apoB containing lipoproteins, we decided to try another approach to determine if the SMHL rabbits had a similar defect.

The perfused liver provides a more physiological approach than hepatic cell culture to the investigation of the action of the liver cells. It offers a flexible experimental system where the effects of various substrates can be studied in a situation where the anatomical structure is preserved, but where the confounding effects of other organs and plasma constituents are eliminated. Experiments can be performed immediately unlike cell culture where it can take days to isolate the cells.

This chapter describes the development of a method to perfuse the livers of the SMHL rabbits in order to characterise directly the lipoproteins secreted from the isolated liver in a system where there was little contribution of catabolism of the lipoproteins by lipolysis or by receptor clearance. The use of the perfused rabbit liver in the study of lipoprotein secretion has been previously documented (Mackinnon, Savage, Wishart, *et al*, 1986, Meijer, Geelen, van Herck, *et al*, 1990) and has provided valuable information on another rabbit model of hyperlipidaemia, the WHHL rabbit (Hornick *et al*, 1983).

5.2. Methods

The liver perfusion system requires the surgical attachment of the liver to a recirculating system of tubes and pumps, designed to oxygenate the perfusate and feed it through the liver. To perfuse the liver we planned to use Krebs Henseleit buffer, pH 7.4 (118 mM NaCl, 5 mM KCl, 1.2 mM $MgSO_4.7H_2O$, 12 mM $CaCl_2$, 1.2 mM KH_2PO_4, 25 mM $NaHCO_3$, 8 mM Glucose) (Krebs and Henseleit, 1932) containing a 20% haematocrit of washed human red blood cells (rbc).

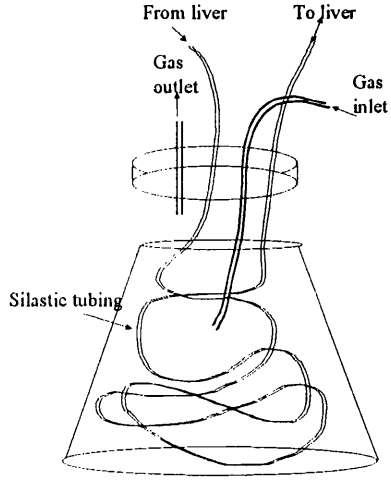
5.2.1 Oxygenation of perfusate

Firstly we wished to find a simple, effective method of oxygenating the perfusate. We were offered an unused human integral membrane oxygenator, a device used for the extra-corporeal oxygenation of blood during surgery. This consisted of a reservoir and a system of fine capillary tubes through which the perfusate passed. A mix of 95% O_2, 5% CO_2 would be passed round these tubes and the oxygen would diffuse into the perfusate. A 95% O_2, 5% CO_2 mix was used instead of 100% O_2 as continued aeration of a solution containing a bicarbonate buffer with 100% O_2 can cause it to lose CO_2 and become alkaline.

The integral membrane oxygenator was tested by pumping a mix of Krebs Henseleit buffer containing a 20% haematocrit of human rbc through it at 100 ml/minute. The partial pressure of O_2 (pO_2) in the perfusate was measured using an amperimetric electrode system where oxygen diffusing through a gas-permeable membrane is reduced at the cathode, and the current generated is proportional to the amount of O_2 reduced and thus to the pO_2 of the sample. These measurements were performed by staff of the Department of Biochemistry, Royal Infirmary, Glasgow. The integral membrane oxygenator oxygenated the buffer well, with pO_2 rising from 25.1 kPa before the gas supply was switched on, to 79.8 kPa after 10 minutes of gas supply with recirculation of buffer. Unfortunately as the oxygenator was meant to be for single use it proved difficult to clean with many capillary tubes becoming blocked, and it was also intended for a larger volume of blood than we wished to use. Therefore an alternative method of oxygenation had to be found.

Hamilton, Berry, Williams, *et al* (1974) described 'a simple and inexpensive membrane lung for small organ perfusion'. This apparatus, the Hamilton lung, was made from a jar into which a mix of 95% O_2, 5% CO_2 was fed through a tightly sealed opening in the lid, and vented through another outlet. Perfusate passed through the jar in 5 metres of gas permeable silastic tubing (OsteoTec Limited) (Figure 5.1.).

Figure 5.1. The Hamilton lung



The gas mixture enters the jar through the gas inlet and forms an O_2 rich environment within the sealed jar. Perfusate is pumped from the liver through silastic tubing within the lung, and O_2 exchange occurs across the tubing. Oxygenated buffer is pumped back in to the liver.

We checked the oxygenation of the perfusate (Krebs Henseleit buffer containing a 20% haematocrit of rbc) using the Hamilton lung. After 10 minutes of recirculation and gassing, pO_2 levels in the perfusate rose from 26.6 kPa to 93.1 kPa.

5.2.2 Perfusate composition

The majority of the literature describes the use of washed rbc as an oxygen carrier in the perfusate. Washed rbc were prepared from human cells. The cells were mixed gently with an equal volume of Krebs Henseleit buffer and centrifuged gently (1780 *g*,

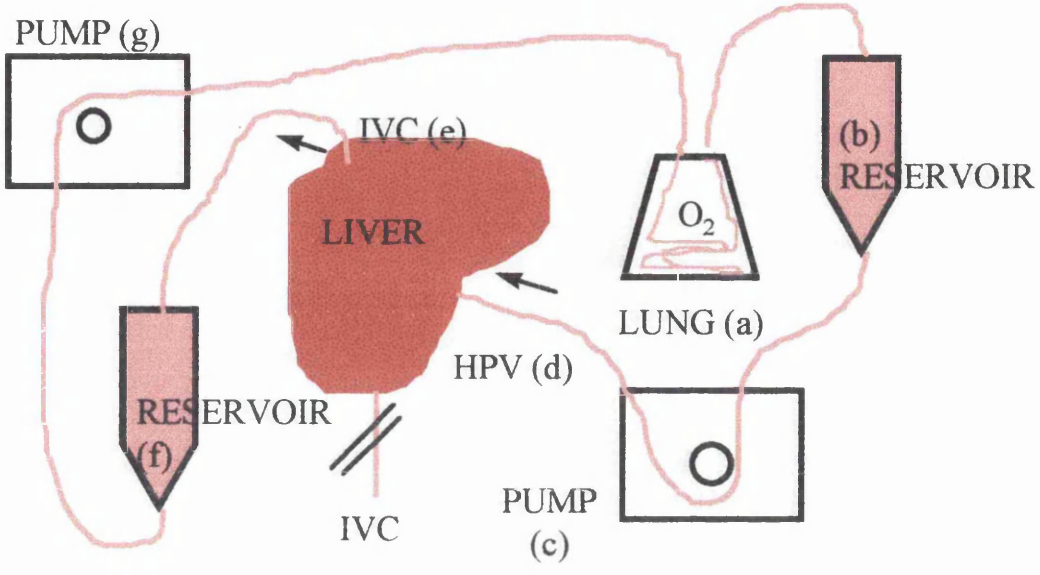
10 minutes). The supernatant was discarded, and the cells were again mixed with Krebs Henseleit buffer and spun. The cells were once again mixed with buffer (5 x volume) overnight, and then spun and washed again as above.

The washing of rbc in copious amounts of buffer was time consuming and the repeated centrifugation of the cells weakened them and caused some haemolysis. This haemolysis of rbc increased protein measurements. We compared pO_2 after oxygenating buffer and buffer plus rbc for 10 minutes and found there to be no difference, with levels rising from 26.6 to 93.1 kPa in the presence of rbc and from 26.0 kPa to 93.0 kPa in the absence of rbc, therefore potentially negating the need for rbc to be a constituent of the perfusate.

5.2.3 Perfusion system

To perfuse the livers we needed to create a system of tubes, pumps and reservoirs in series with the Hamilton lung. We experimented with a variety of systems and found that it was necessary to pump the perfusate both directly into the liver to ensure a constant speed and pressure, and through the Hamilton lung as the small bore of the silastic tubing, and its long length meant that a substantial pressure had to be given to enable the buffer to pass through. The system that was used for the experiments is depicted in Figure 5.2. Perfusate was pumped via a peristaltic pump (Watson Marlow 503U) at 100 ml/minute through the Hamilton lung where it was oxygenated. The perfusate gathered in the upper reservoir, where the pH was monitored (Jenway 3050 portable pH meter), and was then pumped by a second peristaltic pump (Watson Marlow MHRE) directly in to the liver. On efflux from the liver, the perfusate was collected in the lower reservoir from where samples were taken, and the perfusate was recirculated and reoxygenated in the lung. A 3.2 mm internal diameter, 6.4 mm external diameter tubing (AlteSil high strength tubing, Altec) was used throughout the system.

Figure 5.2.A Perfusion system



Krebs Henseleit buffer is oxygenated in the Hamilton lung (a), and pumped into the upper reservoir (b) where the pH is monitored. The buffer is pumped (c) directly through the HPV (d) into the liver, and exits through the IVC (e) into the lower reservoir (f). Buffer is then pumped by a second pump (g) back to the Hamilton lung.

Figure 5.2.B Overview of a perfusion experiment underway

Photograph of a perfusion experiment underway demonstrating the layout of the apparatus described in Figure 5.2.A

Figure 5.2.C Close up view of the perfused liver

Close up view of the perfused liver showing the inflow cannula into the HPV, the outflow cannula from the IVC and the collection of bile. For the purposes of the photograph, the buffer soaked gauze has been removed from the liver.

The entire experiment was performed in a thermostatically controlled room, set at 37°C and perfusate was prewarmed to this temperature, and pre oxygenated. The pH was monitored and maintained at 7.4 by the addition of 1M $NaHCO_3$.

5.2.4 Preparation of animals

On the day of the perfusion experiment the animal was terminally anaesthetised by the injection of 1 ml Euthatal (Rhône Mérieux Ltd.) per 1.4 kg body weight plus 1 ml heparin (1000 iU/ml) (Leo Laboratories Ltd.) into the marginal ear vein.

5.2.5 Surgical technique

A thoracotomy/laparotomy was performed along the line of the linea alba. The intestines were displaced to the left and the inferior vena cava (IVC) was cannulated cranial to the diaphragm using plastic tubing (internal diameter 2 mm, external diameter 3.2 mm). The hepatic portal vein (HPV) was cannulated (14 Gauge intra venous catheter; Vygon UK Ltd.), the IVC was ligated between the renal and hepatic veins (polyamide 66 suture, Ethicon Ltd.) and the common bile duct was cannulated (translucent vinyl tubing, internal diameter 0.63 mm, external diameter 1.4 mm, Portex Ltd.). We are grateful to Dr M. Hicks, Department of Medical Cardiology, Glasgow Royal Infirmary, for advice on the techniques used in this procedure.

5.2.6 Perfusion protocol

Livers were flushed with 750 ml of oxygenated Krebs Henseleit buffer (pH 7.4, 37°C) (some containing a 10% haematocrit of washed rbc) at 75 cm hydrostatic pressure prior to being connected to the perfusion apparatus. The livers were perfused with recirculating perfusate for 180 minutes. A 15 ml sample of perfusate was withdrawn at 0, 60, 120 and 180 minutes and 5 ml samples were removed at 30, 90 and 150 minutes. Samples were spun (1780 *g*, 10 minutes) to pellet any cellular material. Sample volume was replaced with an equal volume of oxygenated, warmed Krebs Henseleit buffer. The liver and intestines were kept moist by covering with gauze soaked in buffer. Cholesterol, triglyceride, glycerol, urea, albumin, AST and GGT

(section 2.3.2, 2.3.3, 2.3.4, 2.5) were measured in each sample. Albumin was below the level of detection, but towards the end of this group of experiments, a microalbumin method was used which enabled production of this protein to be determined. Samples from 0, 60, 120 and 180 minutes were concentrated 5 times (section 5.2.8) and $VLDL_1$, $VLDL_2$, IDL and LDL were isolated by density gradient ultracentrifugation of this lipoprotein concentrate. Cholesterol, triglyceride, total protein and apoB protein were measured in each fraction. The volume of bile produced was recorded. At the end of the experiment the livers were examined and found to be well perfused with no necrotic or hypoxic areas.

5.2.7 Testing of perfusion system

To test the perfusion system and the need for rbc to be part of the perfusate, livers from young (< 4 months) NZW rabbits fed normal rabbit chow, who had had their hearts removed for electrophysiology experiments were obtained. Prior to removing the heart, the IVC was clamped to maintain vascular pressure. We perfused 3 livers with buffer containing rbc (20% haematocrit) and 4 livers with buffer only for up to 3 hours each.

5.2.8 Concentration of lipoproteins at d = 1.065

From the perfusate samples we wished to isolate apoB containing lipoproteins $VLDL_1$, $VLDL_2$, IDL and LDL. However, the concentration of these lipoproteins in the perfusate was very small, so we performed an initial 5 fold concentration step. We measured the density of perfusate from each time point (0, 60, 120, 180 minutes) and obtained an average from the first 6 experiments, from which we calculated how much KBr to add to make the density up to 1.065 g/ml (Table 5.1.) using the following formula:

Weight of KBr (g) = $v(d_2 - d_1)/[1 - \upsilon d_2]$

where v - volume of solution

d_1 - actual density

d_2 - required density

υ - partial specific volume of KBr (0.2889)

Table 5.1. Density of perfusate at sample time points, and weight of KBr required to bring the density to 1.065 g/ml

Sample time (minutes)	Mean density (g/ml)	KBr (g)
0	1.005	0.866
60	1.006	0.851
120	1.007	0.837
180	1.008	0.823

We overlayered 5 ml of sample (d = 1.065) with 1 ml of NaCl solution at a density of 1.065 g/ml in duplicate, and spun in a centrifuge at 83150 *g* for 18 hours. The total lipoproteins obtained were harvested in 1 ml, and duplicates were combined giving 2 ml for isolation of $VLDL_1$, $VLDL_2$, IDL and LDL (section 2.3.8). The density of these 2 ml fractions of total lipoproteins were checked, and brought to 1.118 g/ml by the addition of NaCl using the above equation (substituting 0.3464 as the partial specific volume of NaCl). Again the average value from 5 experiments was calculated for each sample time point (Table 5.2.).

Table 5.2. Density of lipoprotein concentrate and weight of NaCl required to bring the density to 1.118 g/ml

Sample time (minutes)	Density (g/ml)	NaCl (g)
0	1.050	0.222
60	1.053	0.212
120	1.054	0.209
180	1.054	0.209

5.2.9 Measurement of $VLDL_1$ loss during perfusion

Total VLDL was isolated from 50 ml rabbit plasma at d = 1.006 g/ml (62918 *g*, 18 hours). From this, $VLDL_1$ was prepared by density gradient centrifugation as

described above. $VLDL_1$ was then labelled with Na[^{125}I] (Amersham International plc.) as described previously (Bilheimer, Eisenberg and Levy, 1972). Iodination was performed by Dr M. Caslake, Glasgow Royal Infirmary.

Livers from 2 NZW rabbits (fed standard rabbit chow) were prepared as above. After taking a pre-treatment sample at time 0, 5 μCi of ^{125}I-labelled $VLDL_1$ was added to the circulating buffer. $VLDL_1$, $VLDL_2$, IDL and LDL were isolated from samples as described above, apoB was isolated, resolubilised in 0.1 M NaOH and its specific activity was measured.

5.2.10 ^{3}H leucine experiments

To demonstrate that the accumulating lipoproteins in the recirculating perfusate represented newly synthesised products, the incorporation of radioactive leucine into apoB was measured during perfusion of livers from 2 mature NZW and 2 mature SMHL rabbits. One hundred μCi ^{3}H leucine (Amersham International plc.) (165 Ci/mmol) was added to the perfusate at time 0 and further doses of 100 μCi ^{3}H leucine were added at 30 minute intervals, just after each sample was removed for lipoprotein isolation. The specific activity of apoB isolated from $VLDL_1$, $VLDL_2$, IDL and LDL was then determined by radioactivity measurement in a β scintillation counter and assay of apoB protein as described above.

5.2.11 Measurement of lipase activity

To investigate whether lipase activity removed by the heparin flush (1000 iU) on anaesthetisation returns during the experiment, we measured lipase activity in a blood sample taken 15 minutes after anaesthetisation, and again 15 mins after a dose of heparin administered to the perfusate after the final 180 minute sample was taken from the perfusion experiment. This experiment was repeated 3 times. Post perfusion doses of heparin were 100 iU for NZW1 and NZW2 (based on 70 iU/kg for humans, calculated as an overdose for an average 100 g liver), and 5000 iU for NZW3.

5.2.12 Calculation of data

Raw data was corrected for all concentration factors introduced during the course of the experiment. Typically, free glycerol levels were subtracted from total perfusate triglyceride levels to give true triglyceride concentrations. $VLDL_1$, $VLDL_2$, IDL and LDL cholesterol, triglyceride and apoB levels were corrected to allow for the 5 fold concentration step prior to their isolation, and also for their concentration 2 fold ($VLDL_1$ and LDL) or 4 fold ($VLDL_2$ and IDL) during their isolation. Cholesterol, triglyceride and apoB levels in both total perfusate and lipoprotein isolates were corrected to allow for the dilution of the perfusate during the experiment. Values for $VLDL_1$, $VLDL_2$, IDL and LDL cholesterol, triglyceride and apoB levels at 30, 90 and 150 minutes which weren't measured were calculated by taking the mean of the previous and the following time points. Data was found not to be of normal distribution, and therefore Mann Whitney *U* tests were performed to determine significance in Minitab 10.

5.3. Results

5.3.1 Liver patency

Livers were kept warm (37°C) and moist during the perfusion by covering with gauze soaked in buffer. Only livers that were well perfused, reaching the tips of all lobes, were used for experiments. At the end of the perfusion, livers were examined and found to have remained healthy with no necrotic or hypoxic areas. Liver function tests were not grossly elevated (Table 5.3.), and urea production was similar to previously reported values (6 μmol/l/minute by MacKinnon *et al*, 1986). GGT and albumin were below the level of detection. Bile production was steady (Table 5.3.).

Subsequent to these initial experiments, we found a method to measure albumin on a micro scale (section 2.5.), and in the perfusion experiments described in Chapters 6 and 7, this method was used.

Table 5.3. Production rates of AST, urea and bile from buffer and rbc perfused NZW rabbit livers

	Buffer perfused	rbc perfused
AST (U/l/minute)	0.41 ± 0.15	0.8 ± 0.49
Urea (μmol/l/minute)	9 ± 2	17 ± 2
Bile (μl/minute)	20 ± 5	37 ± 2

5.3.2 Lipase activity pre and post perfusion

Lipase activity (HL and LPL) was measured in post heparin plasma and post heparin perfusate prior to and during 3 liver perfusion experiments. Results are given in Table 5.4. HL levels were low, a common feature of rabbits (Warren *et al*, 1991), but neither enzyme returned in any significant amount over the three hours of the perfusion.

Table 5.4. Pre and post perfusion HL and LPL activities (μmol/ml/hour) in 3 NZW rabbits

	Plasma		Perfusion medium	
	HL	**LPL**	**HL**	**LPL**
NZW 1	1.42	6.39	0	0
NZW 2	0.45	2.58	0	0
NZW 3	0.6	2.15	0.15	0

5.3.3 Cholesterol and triglyceride production from the livers of 4 NZW rabbits perfused with buffer, and 3 NZW rabbits perfused with buffer containing a 20% haematocrit

Cholesterol and triglyeride levels in the perfusate rose in an approximately linear manner in all animals studied (Figure 5.3.). When we compared the results from the

buffer perfused and the rbc perfused livers, there was a significant increase in cholesterol output at all time points and a significantly increased cholesterol output rate in the rbc perfused livers (Table 5.5.). We attributed this to cholesterol released from the membranes of haemolysed rbc, as it was noticable that the amount of haemolysis increased throughout the experiment. Similarly, when we pumped rbc round the system in the absence of a liver, there was an increase in the absorbance at 280 nm, a measure of protein, due to haemolysis. There was no significant difference in triglyceride output between the two perfusate contents.

Table 5.5. Cholesterol output (μg/g liver) and output rates (μg/g liver/minute) in 4 buffer perfused and 3 rbc perfused NZW rabbit livers

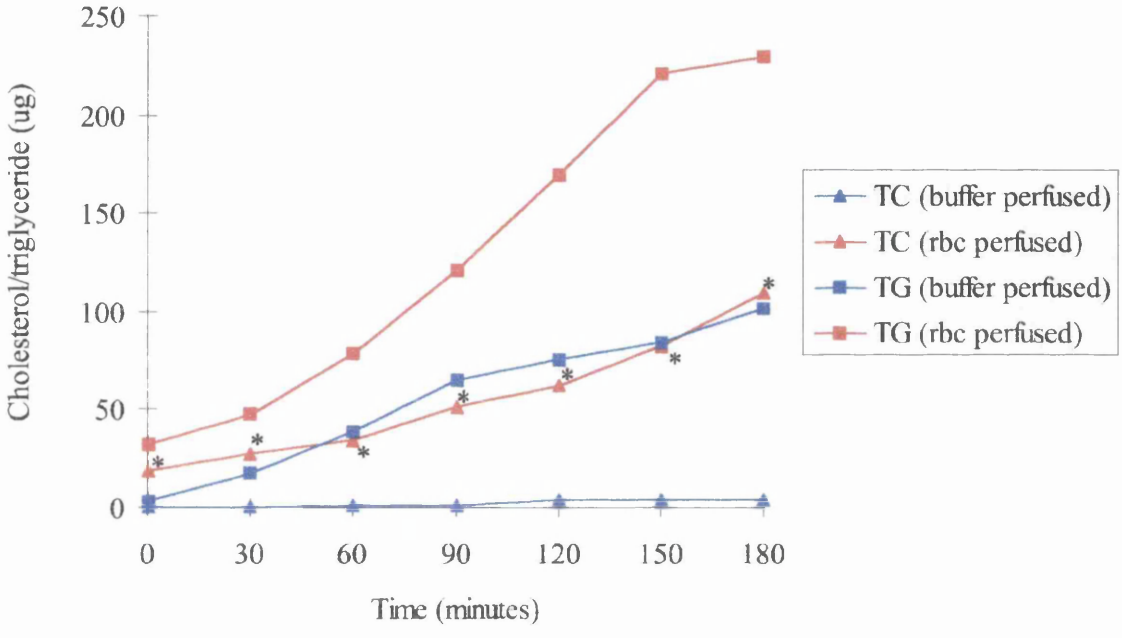
Sample (minutes)	Buffer perfused	rbc perfused
0	0	19 ± 5.0^{a}
30	0	27 ± 3.5^{b}
60	1.2 ± 1.2	34 ± 6.7^{a}
90	1.2 ± 1.2	51 ± 4.9^{b}
120	3.8 ± 3.8	62 ± 7.8^{b}
150	4.2 ± 4.2	82 ± 2.5^{b}
180	4.2 ± 4.2	110 ± 11.5^{b}
Output Rate	0.02 ± 0.02	0.43 ± 0.09^{a}

Significant differences at $^{a}\ p < 0.005$, $^{b}\ p < 0.001$

5.3.4 Lipoprotein output from 4 buffer perfused and 3 rbc perfused NZW rabbit livers

$VLDL_1$, $VLDL_2$, IDL and LDL were separated by density gradient ultracentrifugation of perfusate concentrated 5 fold at a density of 1.065 g/ml. Cholesterol and triglyceride levels rose approximately linearly over the 180 minutes of the experiment in the $VLDL_1$ fraction (Table 5.6., Figure 5.4.A, B). Cholesterol and triglyceride values were very low in the $VLDL_2$, IDL and LDL fractions, and were combined to

Figure 5.3. Cholesterol and triglyceride output in buffer and rbc perfused NZW livers



** indicates time points where TC output is significantly increased in rbc perfused rabbit livers compared to buffer perfused rabbit livers, $p < 0.05$*

make a non $VLDL_1$ fraction (Figure 5.4.A, B). There were no significant differences in the amount of cholesterol or triglyceride in any fraction when buffer perfused and rbc perfused experiments were compared.

Table 5.6. $VLDL_1$, $VLDL_2$, IDL, LDL and non $VLDL_1$ cholesterol and triglyceride output rates (ng/g liver/minute) from buffer perfused and rbc perfused NZW livers

	Buffer perfused		rbc perfused	
	Cholesterol	**Triglyceride**	**Cholesterol**	**Triglyceride**
$VLDL_1$ Output	26 ± 7	518 ± 125	65 ± 23	640 ± 225
$VLDL_2$ Output	9 ± 4	21 ± 4	9 ± 2	26 ± 5
IDL Output	4 ± 3	11 ± 1	4 ± 1	9 ± 3
LDL Output	8 ± 7	15 ± 7	4 ± 2	5 ± 2
non $VLDL_1$ *	21 ± 13	53 ± 12	16 ± 4	40 ± 10

** non $VLDL_1$ is the sum of $VLDL_2$, IDL and LDL cholesterol or triglyceride outputs*

5.3.5 $VLDL_1$, $VLDL_2$, IDL, LDL and non $VLDL_1$ apoB output in buffer and rbc perfused NZW rabbit livers

ApoB levels were measured in $VLDL_1$, $VLDL_2$, IDL and LDL perfusate samples. ApoB levels were low in $VLDL_2$, IDL and LDL fractions and these were combined to produce a cholesterol rich non $VLDL_1$ fraction. ApoB levels rose linearly in both $VLDL_1$ and non $VLDL_1$ fractions (Figure 5.5.). There was no significant difference in apoB output rate between buffer perfused and rbc perfused livers in any of the fractions (Table 5.7.), indicating that both types of perfusate were equally good at perfusing the liver, but that rbc caused an unrepresentative increase in cholesterol concentration (Figure 5.6.).

Figure 5.4.A $VLDL_1$ and non $VLDL_1$ cholesterol output in buffer and rbc perfused NZW livers

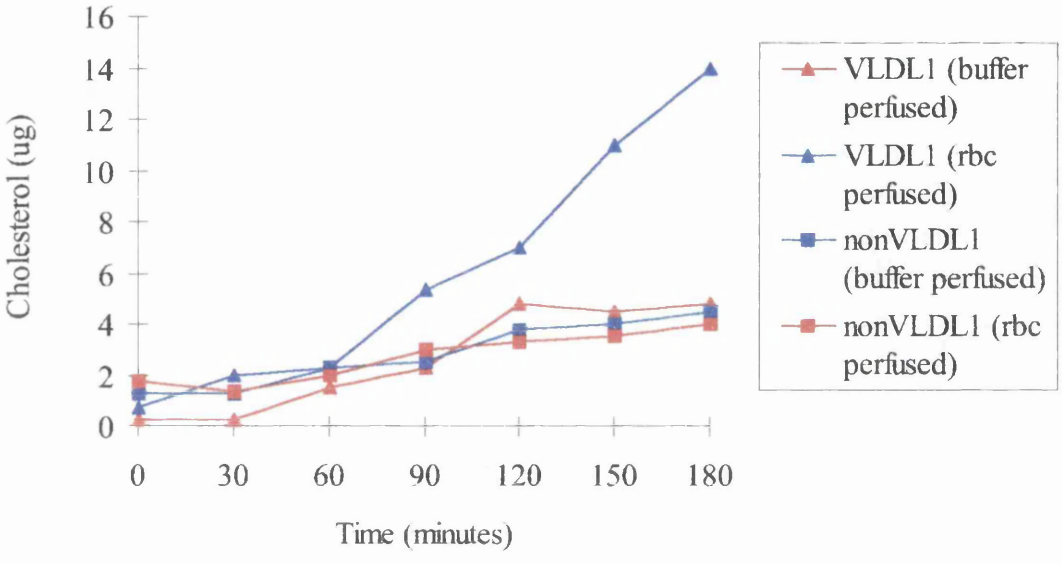


Figure 5.4.B $VLDL_1$ and non $VLDL_1$ triglyceride output in buffer and rbc perfused NZW livers

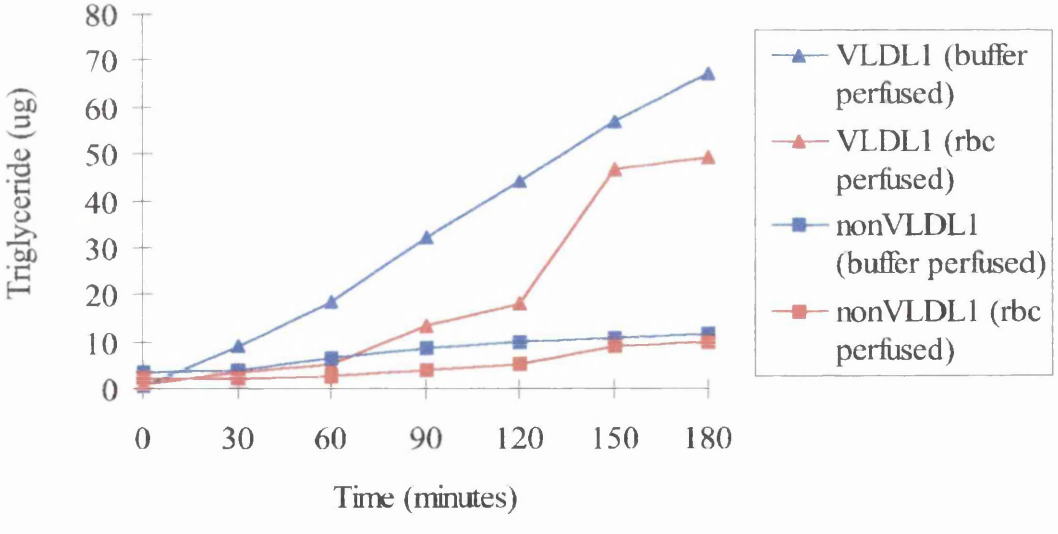


Figure 5.5. $VLDL_1$ and non $VLDL_1$ apoB output from buffer and rbc perfused NZW livers

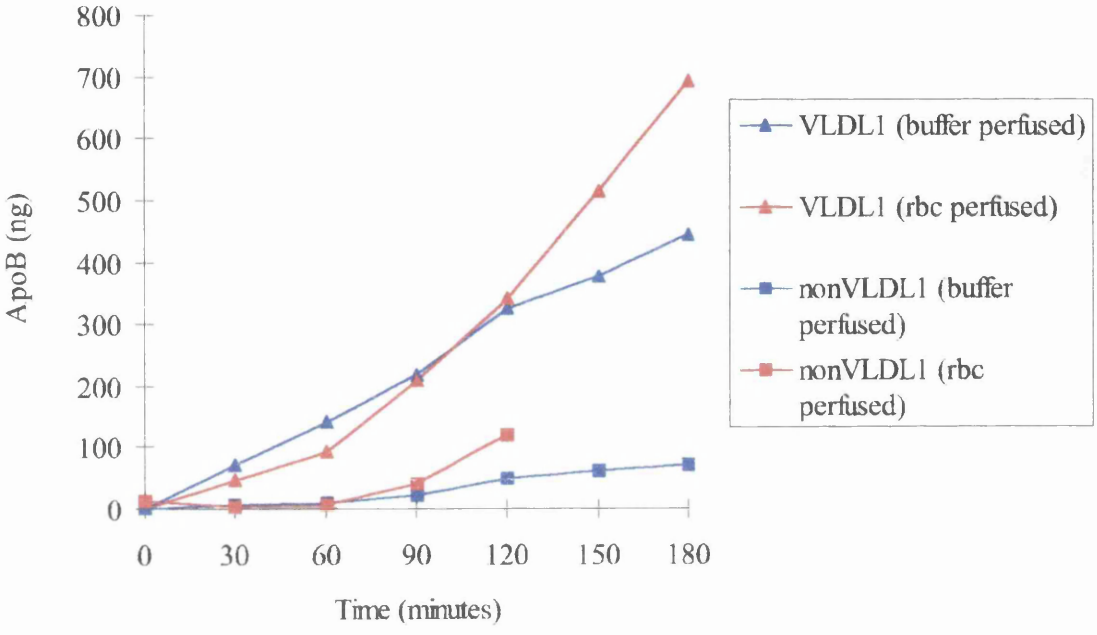


Figure 5.6.A Total cholesterol output from buffer and rbc perfused NZW livers

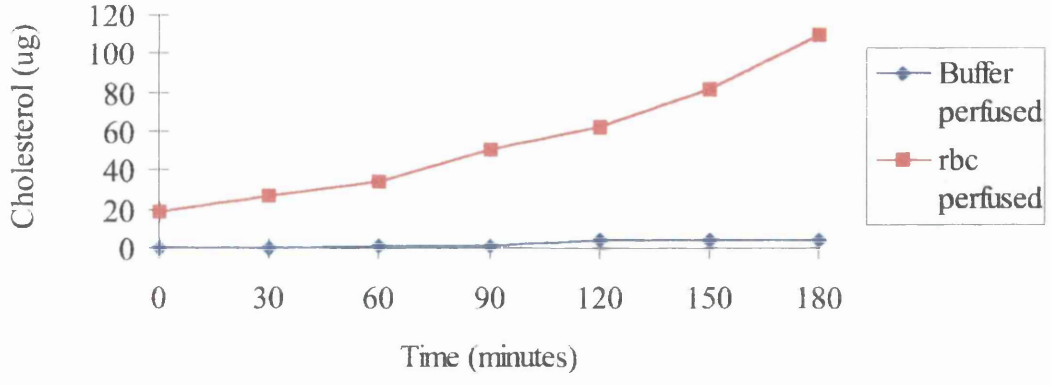


Figure 5.6.B $VLDL_1$ cholesterol output from buffer and rbc perfused NZW livers

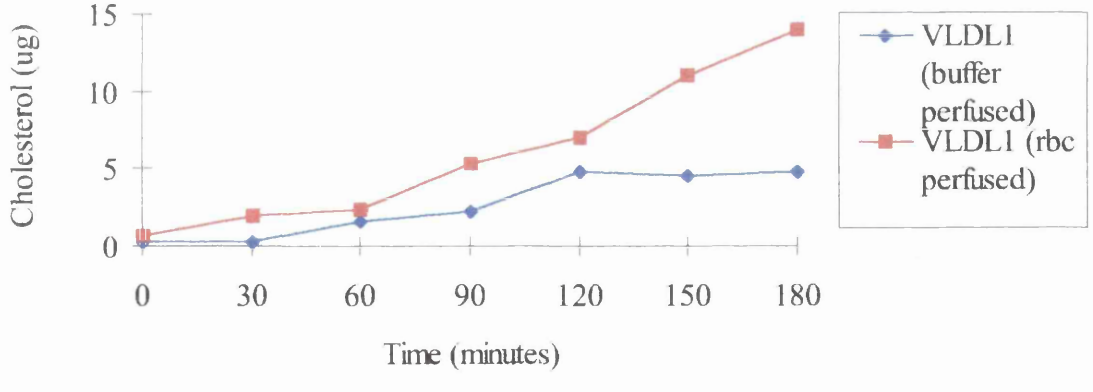
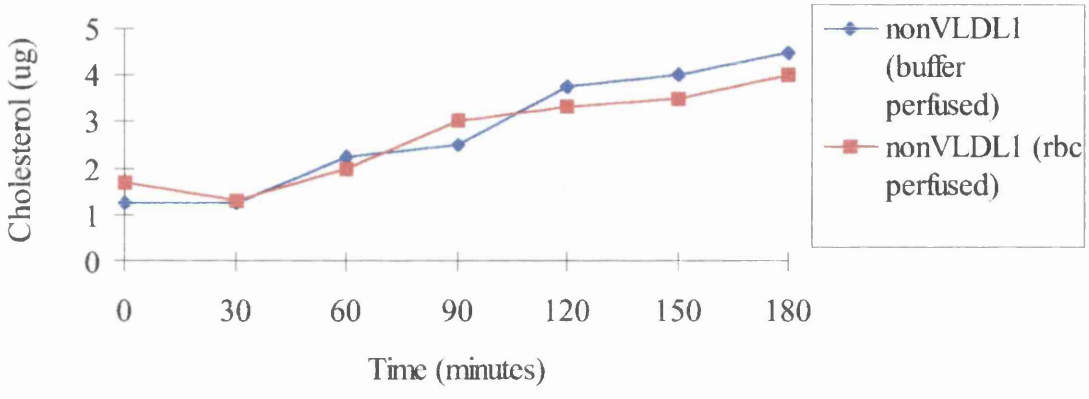


Figure 5.6.C Non $VLDL_1$ cholesterol output from buffer and rbc perfused NZW livers



Total, $VLDL_1$ and non $VLDL_1$ cholesterol output from buffer and rbc perfused NZW livers demonstrating that the increased production of total cholesterol in the rbc perfused livers is due to non-lipoprotein cholesterol

Table 5.7. ApoB output rates (ng/g liver/minute) from 3 buffer perfused and 3 red blood cell perfused NZW rabbit livers

Output rates	Buffer perfused	rbc perfused
$VLDL_1$	2.5 ± 0.6	3.4 ± 1.0
$VLDL_2$	0.23 ± 0.01	0.26 ± 0.22
IDL	0.18 ± 0.09	0.33 ± 0.19
LDL	0	0.21 ± 0.21
non $VLDL_1$	0.41 ± 0.10	0.81 ± 0.59

5.3.6 Measurement of $VLDL_1$ loss during perfusion

To examine the extent to which $VLDL_1$ was converted to denser lipoproteins during the 3 hour perfusion, ^{125}I labelled $VLDL_1$ was added to the perfusate (buffer only) of 2 NZW rabbits 10 minutes prior to the beginning of the experiment. $VLDL_1$, $VLDL_2$, IDL and LDL apoB was isolated to follow the fate of the particles. There was no difference in the cholesterol and triglyceride output nor in the subfraction cholesterol, triglyceride or apoB output in these two experiments from those buffer perfused experiments described above.

At time 0, a mean of 93% of apoB radioactivity was found in the $VLDL_1$ fraction, with 6% in $VLDL_2$ and 1% in IDL. After 3 hours 77% of the counts remained in $VLDL_1$, 7.5% were found in $VLDL_2$, 2.5% in IDL and 1.5% in LDL. The lack of increase in $VLDL_2$, IDL and LDL radioactivity indicated that delipidation was limited in this system.

5.3.7 ^3H leucine experiments

In order to show that the lipoproteins that accumulated were derived by *de novo* production of apoB, ^3H leucine was added to the perfusate of 2 mature NZW and 2 mature SMHL rabbits. Again, cholesterol and triglyceride output and subfraction

cholesterol, triglyceride and apoB output were not significantly different from buffer perfused experiments described above.

By 3 hours, leucine radioactivity in $VLDL_1$ apoB rose by an average of 5 fold above the zero time value in NZW and 15 fold in SMHL rabbits, in $VLDL_2$ it rose 2 and 6 fold; in IDL, 2 and 2 fold and in LDL, 2 and 3 fold respectively showing that the liver was synthesising these lipoproteins (Table 5.8.).

Table 5.8. % increase in specific activity resulting from *de novo* production of $VLDL_1$, $VLDL_2$, IDL and LDL apoB during 2 mature NZW and 2 mature SMHL rabbit liver perfusions

	NZW mean	SMHL mean
$VLDL_1$	554	1544
$VLDL_2$	224	640
IDL	178	206
LDL	188	276

5.4. Discussion

The development of the rabbit liver perfusion method described in this chapter was a complex and time-consuming process, pooling the knowledge and resources of a large number of people. The main difficulty in perfusion studies has been the development of a method of oxygenation of the perfusate without the introduction of air bubbles. Hamilton's lung, developed for use in rats, is certainly the best method of oxygenating perfusate for small animal organs, having been adapted previously for use in rabbits by several investigators (Hornick *et al*, 1983, De Parscau and Fielding, 1984, Meijer *et al*, 1990). The advantage over other types of oxygenation is that there is no air-liquid interface at which gas exchange could occur. This means that there is less opportunity for evaporation of the medium, less mechanical trauma, and there are no air bubbles introduced into the circulation. It is a cheap and simple device, and can handle small volumes of perfusate.

Only livers that were fully perfused in all lobes were used for experiments. All livers were examined on completion of the experiment, and were found to have been well perfused with no necrotic or hypoxic areas. Measurement of AST and GGT revealed that, while these enzymes accumulated throughout the perfusion, there was no abnormally high secretion indicative of the liver being under stress. Urea levels rose at a rate compatible with good liver function. Albumin levels were below the level of detection, but towards the end of the trial period, a method for measuring micro albumin was used, and this showed an approximately linear increase in albumin of the order of 410 μg/l/minute. The synthesis of albumin is one of the main functions of the liver, and therefore the secretion of albumin in to the perfusate throughout the experiment further demonstrated that the liver was viable. Finally, we measured bile flow (Table 5.3.) which was of the same magnitude as previously published data such as 45 μl/minute (De Parscau and Fielding, 1984) and 33 μl/minute (Hornick *et al*, 1983).

Cholesterol and triglyceride accumulated in the perfusate in an approximately linear manner (Figure 5.3.). Cholesterol output rate (Table 5.5.) was similar to values in the literature of 0.05 - 0.52 μg/g liver/minute (various strains of rabbit) (Meijer *et al*, 1990), and 0.5 μg/g liver/minute in NZW rabbits (Hornick *et al*, 1983). The rate of output of triglyceride (0.6 ± 0.2 μg/g liver/minute for buffer perfused and 1.0 ± 0.2 μg/g liver/minute for rbc perfused livers) is of the same order as that reported by Hornick *et al* (1983) of 4 μg/g liver/minute (NZW rabbits), and 0.6 - 1.8 μg/g liver/minute (various strains of rabbit) reported by Meijer *et al* (1990).

These experiments showed that we were able to isolate $VLDL_1$, $VLDL_2$, IDL and LDL by density gradient ultracentrifugation of perfusate, providing the opportunity to look directly at the hepatic output of these lipoproteins. Lipid levels in these lipoproteins accumulated linearly throughout the 3 hours of the perfusion. We compared total perfusate cholesterol content with $VLDL_1$ plus non $VLDL_1$ cholesterol content in buffer and rbc perfused livers, and found that in buffer perfused livers total cholesterol was accounted for by $VLDL_1$ and non $VLDL_1$ cholesterol. However in rbc perfused livers, total perfusate cholesterol was 6 fold higher than $VLDL_1$ plus non $VLDL_1$ cholesterol (Figure 5.6.). Clearly, the cholesterol in the total

fraction of rbc perfused livers contained non-lipoprotein cholesterol, such as that from the haemolysed membranes of rbc. Therefore we decided that as the buffer perfused livers performed as well as the rbc perfused livers, we would perfuse with buffer only to eliminate contamination with non-lipoprotein cholesterol.

The liver perfusion and lipoprotein separation techniques were sensitive enough for us to be able to isolate apoB in each lipoprotein fraction. Levels of this protein rose linearly throughout the experiments, and did not differ between buffer perfused and rbc perfused livers.

We found little evidence for extensive delipidation in our perfusion system. HL and LPL were removed by the heparin flush during anaesthetisation, and did not return to any appreciable extent during the perfusion (Table 5.4.). Furthermore ^{125}I labelled $VLDL_1$, when added to the perfusate, was not substantially delipidated (section 5.3.6). This means that our results are not complicated by the delipidation of lipoproteins as is the case *in vivo*, however it must be borne in mind that we have made no measure of lipoprotein catabolism during the experiment. We also demonstrated linear ^{3}H leucine incorporation into the apoB of denser lipoproteins throughout the 3 hour period (section 5.3.7) indicating that these species did not derive from wash out of the space of Disse as has been previously suggested (Hornick *et al*, 1983). Therefore, although $VLDL_1$ was by far the major species produced by these rabbit livers during the perfusion, in terms of cholesterol, triglyceride and apoB, the small amount of non $VLDL_1$ lipoproteins produced were due to direct production and not delipidation of $VLDL_1$.

Therefore we developed a method for rabbit liver perfusion that enabled us to study the output of lipid from the liver over a period of 3 hours without any noticeable degeneration of liver function. This method enabled us to look not only at cholesterol and triglyceride output, but more specifically at lipoprotein cholesterol, triglyceride and apoB output. The perfused liver provided a method of study of lipoprotein metabolism in a physiological system with the option of experimental manipulation, and we planned to use the system to investigate the mechanism of the dyslipidaemia in SMHL rabbits.

Chapter 6. Liver perfusion studies in mature NZW and SMHL rabbits

6.1. Introduction

The SMHL rabbit is a putative model for FCH, a disorder commonly thought to be due to an overproduction of apoB containing lipoprotein particles. To investigate the lipoprotein disorder exhibited by the SMHL rabbits in more detail, we performed a series of liver perfusion experiments. This method allowed the measurement of the direct output of lipoprotein particles from the liver in a system free from FFA flux from other tissues, and from lipolytic activity.

We have previously described the effect of age on plasma lipids in the SMHL rabbits (Chapter 3). Because of the decrease in plasma lipid concentration that occurs during the first 4 - 5 months of life, we decided to first study rabbits in which lipid levels had become stable, ie greater than 5 months old.

Studies of the output of apoB-100 containing lipoprotein particles from the liver of SMHL rabbits were compared with that of NZW rabbits of the same sex and similar age to investigate the hypothesis that, as suggested in FCH, the hyperlipidaemic phenotype of the SMHL rabbits was due to an overproduction of apoB containing lipoproteins.

6.2. Methods

6.2.1 Animal selection

Eleven male SMHL rabbits (aged > 5 months) fed the 0.08% cholesterol diet were selected on the basis of their moderate (cholesterol range 0.8 - 13.6 mmol/l, triglyceride range 0.4 - 4.0 mmol/l) hyperlipidaemia. The rabbit with a cholesterol of 13.6 mmol/l was an outlier, with all rabbits being below 4.1 mmol/l except for one at

7.8 mmol/l and one at 5.9 mmol/l. Twelve NZW controls of the same sex and similar age (cholesterol range 0.6 - 3.8 mmol/l, triglyceride range 0.5 - 2.1 mmol/l) were also selected. Mean age at sacrifice was 10.2 vs 9.8 months and mean body weight was 3.6 vs 4.0 kg (SMHL vs NZW, both NS).

6.2.2 Perfusion method

The method used for the perfusion experiments is described in Chapter 5, and is summarised here. The animal was terminally anaesthetised. To perfuse the liver, the IVC was cannulated cranial to the diaphragm, the HPV and the common bile duct were cannulated and the IVC was ligated between the renal and hepatic veins. The liver was flushed with 750 ml oxygenated Krebs Henseleit buffer (pH 7.4, 37°C) prior to being connected to the perfusion apparatus in a room thermostatically controlled at 37°C. The buffer was pumped by two peristaltic pumps in a recirculating system at 100 ml/minute through the liver, with inflow via the HPV and outflow through the IVC. The buffer was oxygenated by a Hamilton lung, and the pH was maintained at 7.4. The total volume of the perfusate was 120 ml, and was maintained thus by replacing sample volume with an equal volume of oxygenated warmed Krebs Henseleit buffer. Samples of perfusate were taken at 0, 30, 60, 90, 120, 150 and 180 minutes, and cholesterol, triglyceride, glycerol, protein, urea, microalbumin, AST and GGT were measured (Chapter 2, sections 2.3.2, 2.3.3, 2.3.4, 2.4., 2.5.). Lipoprotein subfractions were separated, and cholesterol, triglyceride and apoB were measured in each fraction (Chapter 2, sections 2.3.8, 2.3.2. 2.3.3, 2.4.). The volume of bile produced by the liver was also measured.

6.2.3 Extraction of lipid from liver samples

Samples of liver were removed and frozen immediately after the perfusion experiments were completed in 5 mature SMHL and 5 mature NZW rabbits. Once all samples were collected, they were thawed and 0.25 - 0.5 g wet weight pieces dissected for measurement of lipid content following a modification (Naito and David, 1984) of the method of Folch, Lees and Sloane Stanley (1957). Samples were homogenised (Janke & Kunkel KG homogeniser) in small volumes of methanol

(BDH) and the volume adjusted to 50 ml with methanol. After standing for 30 minutes with occasional mixing, 100 ml chloroform (BDH) was added. Following an overnight extraction at 15°C the samples were filtered (Whatmans No. 1 filter paper) and protein precipitated by the addition of 50 ml 0.05% calcium chloride solution. Samples were again maintained overnight at 15°C and the chloroform (bottom) layer was removed and dried on a centrifugal evaporator (Howe). The pellet was redissolved in isopropanol (2 ml) and total cholesterol, triglyceride, free cholesterol and phospholipid contents were measured (Section 2.3.2, 2.3.3, 2.3.5, 2.3.6).

6.2.4 Statistics

Variables were tested for normal distribution. Plasma cholesterol and triglyceride concentrations were transformed to a normal distribution by taking the logarithm of their values. Two sample t-tests were performed to determine significance. Where the distribution of a variable or series of variables could not consistently be converted to normal, as was the case with all perfusate concentration measurements, a Mann-Whitney *U* test was performed. Data are expressed as mean ± SEM unless otherwise stated.

6.3. Results

6.3.1 Population lipids and biochemical parameters

In these mature rabbits selected for perfusion experiments, plasma cholesterol was 4.1 ± 1.3 mmol/l in SMHL vs 1.4 ± 0.3 mmol/l in NZW rabbits ($p < 0.05$) and plasma triglyceride was 2.0 ± 0.4 mmol/l in SMHL vs 1.2 ± 0.1 mmol/l in NZW rabbits (NS).

Liver function tests were normal throughout the perfusions. The livers remained patent and produced bile at 15 ± 5 and 34 ± 6 µl/liver/minute in SMHL and NZW rabbits respectively. Examination of the livers at the end of the experiment revealed no necrotic or hypoxic areas. Mean liver weight was not different (92 ± 12 vs 103 ± 11 g, SMHL vs NZW).

6.3.2 Cholesterol and triglyceride production

Mean perfusate cholesterol and triglyceride levels rose linearly in both groups of rabbits over the 180 minutes of the experiment (Figure 6.1.). Cholesterol levels rose from 6 ± 4 to 96 ± 21 μg/g liver in SMHL rabbits and from 7 ± 5 to 41 ± 11 μg/g liver in NZW rabbits. The cholesterol content of the perfusate was significantly increased in SMHL rabbits in samples taken at 60, 90 and 150 minutes and the calculated cholesterol output was 510 ± 101 vs 220 ± 67 ng/g liver/minute in SMHL vs NZW rabbits (NS).

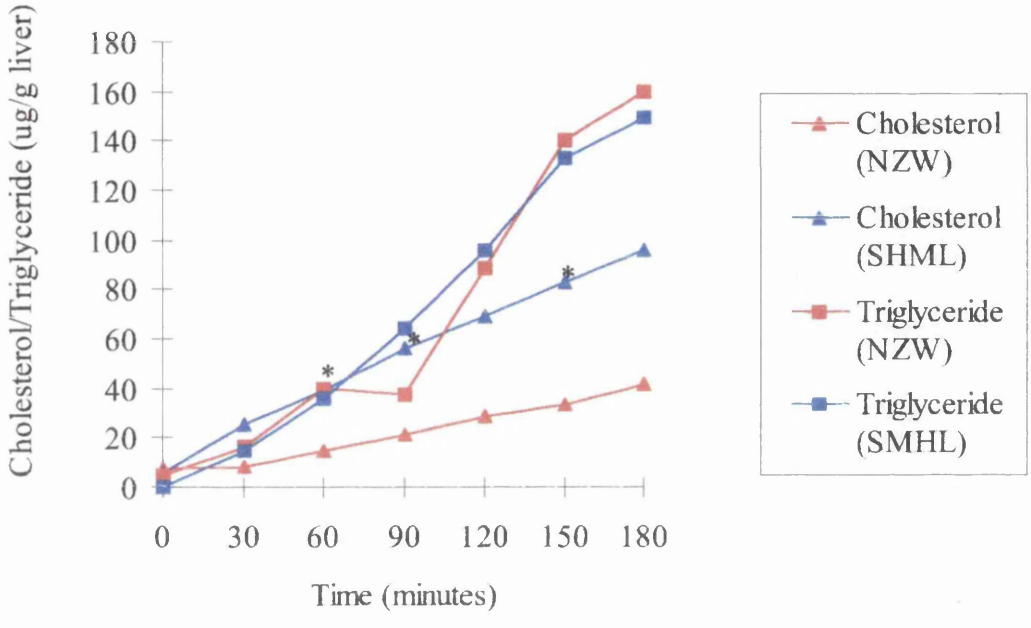
Triglyceride levels in the perfusate rose from 0 to 149 ± 29 μg/g liver in SMHL rabbits, and from 5 ± 4 to 160 ± 50 μg/g liver in NZW rabbits. Both groups had a similar triglyceride output: 860 ± 130 vs 890 ± 246 ng/g liver/minute in the SMHL and NZW rabbit groups respectively (NS).

6.3.3 Lipoprotein production during perfusion

The lipoproteins in the perfusate were separated and their cholesterol and triglyceride contents were analysed. Data are shown in Table 6.1. Mean perfusate IDL triglyceride output was increased in SMHL rabbits compared with NZW rabbits ($p < 0.05$), but no other significant differences were observed in individual fractions.

We then compared the output of triglyceride rich and cholesterol rich lipoproteins from the liver. The cholesterol and triglyceride contents of $VLDL_2$, IDL and LDL (i.e. non $VLDL_1$) were combined as these represent cholesterol rich lipoproteins and the values obtained were compared between groups as were those for the triglyceride rich $VLDL_1$ (Figure 6.2.A,B, Table 6.1.). There was no difference in mean $VLDL_1$ cholesterol output between the two strains of rabbit (229 vs 160 ng/g liver/minute, Table 6.1.). In NZW rabbits, $VLDL_1$ perfusate cholesterol levels rose from 0 to 25 ± 8 μg/g liver (Figure 6.2.A) and in SMHL rabbits from 0 to 46 ± 11 μg/g liver during the experiment. Although there was a 3.5 fold increase in SMHL rabbit non $VLDL_1$ cholesterol output when compared to NZW rabbits, this was not significant (Table 6.1.). Non $VLDL_1$ cholesterol levels increased from 0 to 21 ± 7 and 1 ± 1 to 5 ± 2

Figure 6.1. Cholesterol and triglyceride output from the perfused liver of mature NZW and SMHL rabbits



** indicates time points where cholesterol output is significantly increased in SMHL rabbits compared to NZW rabbits, $p < 0.05$*

μg/g liver in SMHL and NZW rabbits respectively during the 180 minute experiment. SMHL values were significantly different from NZW at 30, 90 and 150 minutes.

Table 6.1. $VLDL_1$, $VLDL_2$, IDL and LDL cholesterol and triglyceride output from the livers of 12 mature NZW and 11 mature SMHL rabbits

	Cholesterol (ng/g liver/minute)		Triglyceride (ng/g liver/minute)	
	NZW n = 12	**SMHL** n = 11	**NZW** n = 12	**SMHL** n = 11
$VLDL_1$	160 ± 45	229 ± 52	443 ± 92	439 ± 94
$VLDL_2$	18 ± 6	65 ± 20	13 ± 3	56 ± 30
IDL	8 ± 2	26 ± 10	5 ± 2	19 ± 6[a]
LDL	2 ± 1	7 ± 3	4 ± 2	8 ± 3
non $VLDL_1$	28 ± 8	98 ± 28	22 ± 6	82 ± 34[a]

[a] *Significant difference between NZW and SMHL rabbits, $p < 0.05$*

There were no significant differences in $VLDL_1$ triglyceride output in mature SMHL and NZW rabbits (Table 6.1., Figure 6.2.B). $VLDL_1$ triglyceride levels rose from 16 ± 11 to 84 ± 18 vs 5 ± 5 to 76 ± 18 μg/g liver in SMHL and NZW respectively. Non $VLDL_1$ triglyceride output was significantly increased 4 fold in SMHL compared to NZW rabbits (Table 6.1., $p < 0.05$). Non $VLDL_1$ triglyceride levels rose from 6 ± 6 to 26 ± 11 vs 0 to 4 ± 2 μg/g liver in SMHL vs NZW rabbits, being significantly higher ($p < 0.05$) in SMHL rabbits compared to NZW at 180 minutes.

Figure 6.2.A $VLDL_1$ and non $VLDL_1$ cholesterol output from the liver of mature NZW and SMHL rabbits

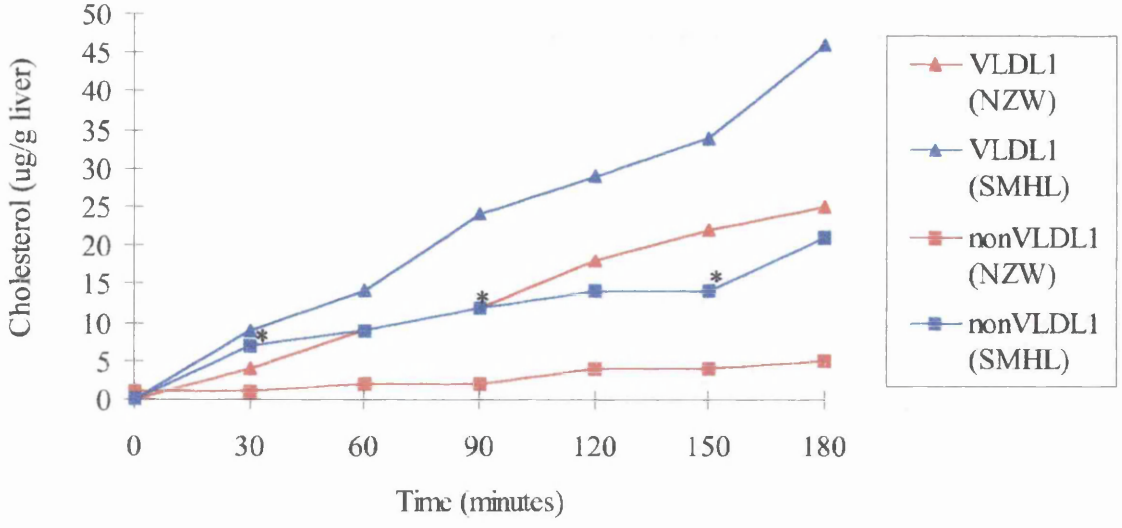
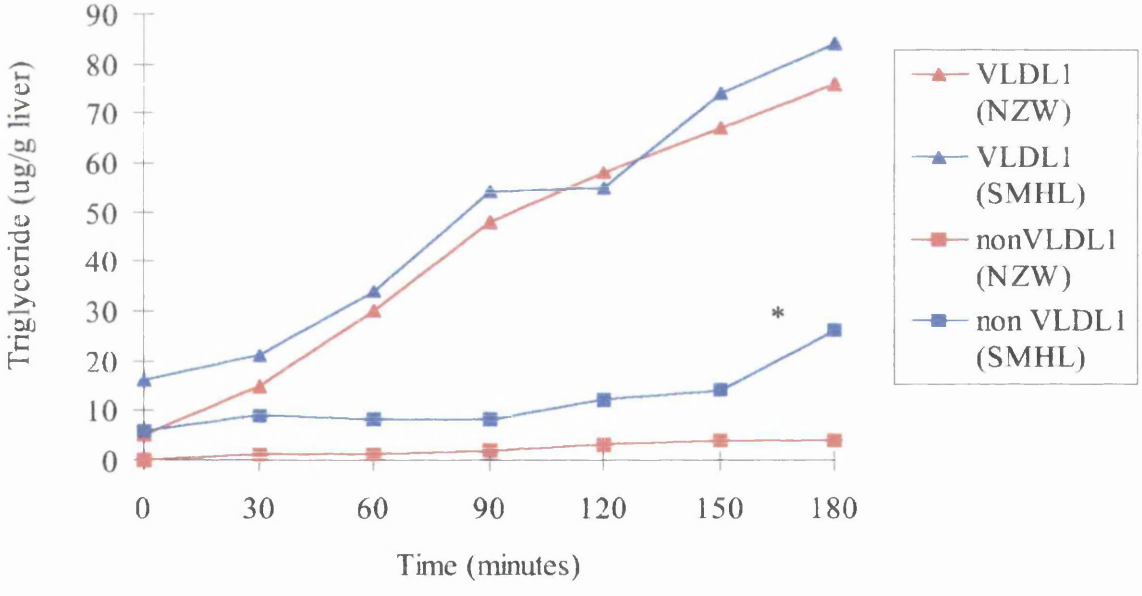


Figure 6.2.B $VLDL_1$ and non $VLDL_1$ triglyceride output from the liver of mature NZW and SMHL rabbits



** indicates time points where SMHL non $VLDL_1$ cholesterol and non $VLDL_1$ triglyceride outputs are significantly increased compared to NZW control rabbit data, $p < 0.05$*

6.3.4 ApoB output from perfused livers

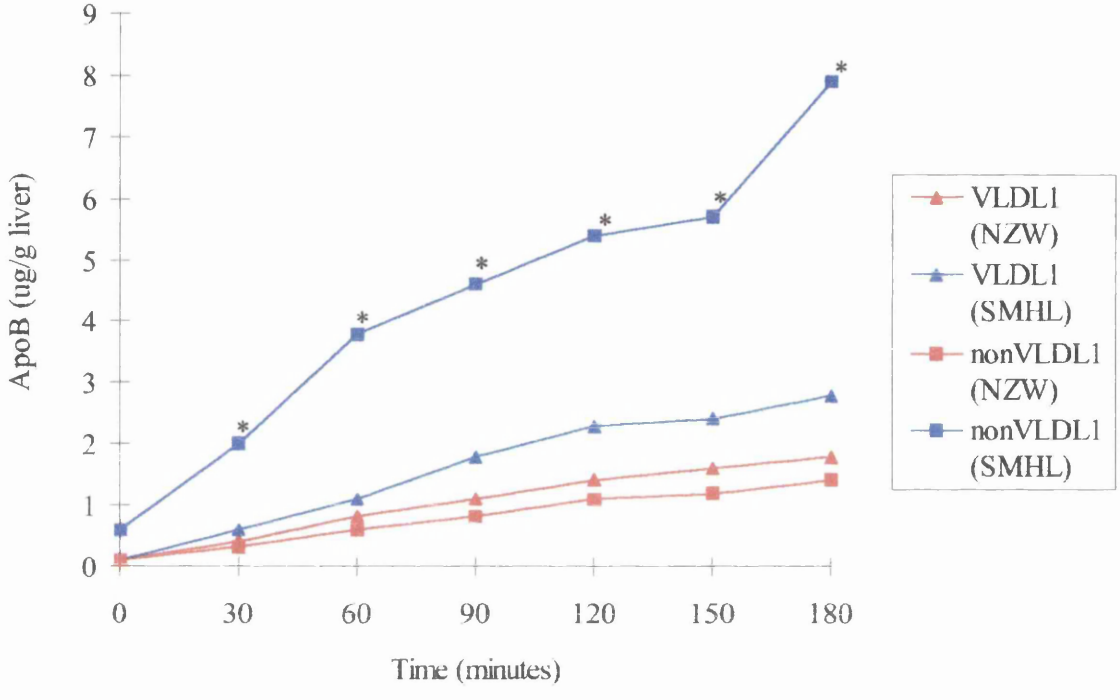
ApoB was measured in the individual lipoproteins secreted into the hepatic perfusate. Results are shown in Table 6.2. and Figure 6.3. Although apoB values particularly in the LDL fractions were very small, we were confident that they were accurate as apoB as a percentage of total lipoprotein protein was similar in SMHL and NZW rabbits. In $VLDL_1$ it comprised 41% of the total protein in SMHL vs 38% in NZW rabbits, in $VLDL_2$ 60% vs 64%, in IDL 79% vs 82% and in LDL 75% vs 76%. These values were similar to values we determined for use in the kinetic studies (Chapter 4, Table 4.1.). $VLDL_1$ apoB output was not significantly different between SMHL and NZW rabbits, but $VLDL_2$ and IDL apoB outputs were both significantly increased in SMHL compared to NZW rabbits (Table 6.2.). Mature NZW rabbits secreted $VLDL_1$ and non $VLDL_1$ apoB at similar rates (Table 6.2., Figure 6.3.). $VLDL_1$ apoB output from SMHL rabbits was not different from that in NZW rabbits (Table 6.2., Figure 6.3.). However, non $VLDL_1$ apoB concentrations were significantly increased in SMHL rabbits compared to NZW rabbits at all time points from 30 minutes until the end of the experiment (Figure 6.3.). Non $VLDL_1$ apoB output was increased 6 fold in SMHL rabbits relative to NZW, $p = 0.01$ (Table 6.2.). In SMHL rabbits the non $VLDL_1$ apoB output accounted for 75% of the total apoB output (48 ± 14 ng/g liver/minute).

Table 6.2. $VLDL_1$, $VLDL_2$, IDL and LDL apoB output rates (ng/g liver/minute) in mature NZW and SMHL rabbits

	NZW	SMHL
$VLDL_1$	10 ± 3	15 ± 4
$VLDL_2$	2 ± 1	14 ± 5[a]
IDL	3 ± 1	16 ± 6[a]
LDL	0	6 ± 2
non $VLDL_1$	6 ± 3	36 ± 12[b]

Significant differences between NZW and SMHL rabbits, [a] $p < 0.05$, [b] $p = 0.01$

Figure 6.3. $VLDL_1$ and non $VLDL_1$ apoB output from the liver of mature NZW and SMHL rabbits



** indicates time points where non $VLDL_1$ apoB output is significantly increased in SMHL rabbits compared to NZW rabbits, $p < 0.05$*

We calculated the ratio of the gram weight of cholesterol + triglyceride to the gram weight of apoB for each of $VLDL_1$, $VLDL_2$, IDL and LDL. This gave an indication of the amount of lipid (cholesterol and triglyceride) that was associated with an amount of apoB, and therefore a measure of particle size. There were no significant differences between the two groups, although SMHL particle size was consistently smaller than NZW (Table 6.3.).

Table 6.3. Cholesterol + triglyceride to apoB ratios (g/g)

	NZW	SMHL
$VLDL_1$	56.1	45.4
$VLDL_2$	12.4	8.8
IDL	3.8	2.9
LDL	/ *	2.5

* *there was no consistent value for LDL apoB in the NZW rabbits, therefore a ratio has not been calculated.*

6.3.5 Liver lipid content

Hepatic lipid concentrations were measured after the perfusion experiments (Table 6.4.). Phospholipid concentrations were significantly higher in mature SMHL rabbits compared to NZW rabbits (Table 6.4.), $p < 0.05$, but there were no differences in any other parameters.

When we compared the amount of lipid in the liver to the amount released into the perfusate during the experiments, the amount secreted was approximately 1% or less of that stored in the liver. In the SMHL rabbits, on average, 96 ± 21 μg of cholesterol were secreted per gram of liver, from a store of 12.2 ± 2.3 mg cholesterol/g liver. In NZW rabbits, 41 ± 11 μg of cholesterol/g liver were secreted, from a store of 15.4 ± 1.9 mg/g liver. Similarly for triglyceride, in SMHL rabbits, 149 ± 29 μg/g liver were secreted from a store of 11.4 ± 2.2 mg/g liver, and in NZW

rabbits 160 ± 50 μg/g liver were secreted into the perfusate from a store of 13.8 ± 1.3 mg/g liver.

Table 6.4. Liver lipid content (mg/g wet weight liver)

	NZW	SMHL
Total Cholesterol	15.4 ± 1.9	12.2 ± 2.3
Free Cholesterol	7.4 ± 1.3	8.2 ± 1.5
Triglyceride	13.8 ± 1.3	11.4 ± 2.2
Phospholipid	15.4 ± 0.6	17.3 ± 0.5[a]

Significant difference NZW vs SMHL [a] $p < 0.05$

6.4. Discussion

The rabbits used for these experiments were more than 5 months of age and had stable plasma lipid concentrations. SMHL rabbits were hyperlipidaemic compared to the NZW, and as such were representative of the colony as a whole.

Cholesterol, triglyceride and apoB accumulated in the perfusion medium in an approximately linear manner over the 180 minutes of the perfusion experiment, suggesting that the liver remained patent and fully functioning throughout. Normal liver function tests and linear bile production also suggested that the livers were functioning in a physiological manner during the perfusion. Output rates for cholesterol of 510 ± 101 and 220 ± 67 ng/g liver/minute and for triglyceride of 860 ± 130 and 890 ± 246 ng/g liver/minute were of the same order as those previously published for perfused rabbit livers of 380 ng/g liver/minute for cholesterol and 4000 ng/g liver/minute for triglyceride (Hornick *et al*, 1983). Comparison of hepatic triglyceride output in the four apoB containing lipoproteins in SMHL and NZW animals revealed no difference in $VLDL_1$ despite the fact that this was the most abundant triglyceride carrying particle released from the liver. $VLDL_2$ and IDL triglyceride outputs tended to be increased in SMHL rabbits, reaching significance in

the IDL fraction, and overall, non $VLDL_1$ triglyceride output was significantly elevated (Table 6.1.). Cholesterol output tended to be increased in SMHL rabbits in all fractions with a 3.5 fold increase in non $VLDL_1$ output, but these differences did not reach statistical significance.

In these mature animals, apoB was overproduced mainly in both the $VLDL_2$ and IDL density classes. This is an interesting observation given the marked difference in plasma cholesterol but not plasma triglyceride in these animals. Given that 75% of apoB released from the livers of the SMHL rabbits appeared in the non $VLDL_1$ fraction, and that there is minimal lipase activity, this having been removed by the initial heparin flush (Chapter 5), this strongly suggests that the SMHL rabbits overproduce denser lipoproteins. Output rates of cholesterol, triglyceride and apoB were not different in the $VLDL_1$ fraction between mature NZW and SMHL rabbits. However in the non $VLDL_1$ fraction, cholesterol output was increased 3.5-fold, triglyceride 3.5 fold and apoB 6 fold, suggesting that an increased number of lipoprotein particles were produced. These results are consistent with the metabolic studies of La Ville *et al* (1987) which showed an increased PR of VLDL and LDL apoB; but do not preclude an additional effect from a decreased clearance rate. The lipid to apoB ratios in the SMHL rabbits were lower than in the NZW rabbits, indicating that the particles secreted from the livers of the SMHL rabbits had a tendency to be smaller (Table 6.3.). Therefore like in FCH (Brunzell, Albers, Chait, *et al*, 1983, Hokanson *et al*, 1995), a larger number of relatively lipid poor lipoprotein particles were produced in the SMHL rabbit, further substantiating its use as a model for the disorder.

There were no differences in hepatic stores of free cholesterol, cholesteryl ester or triglyceride in the two strains of rabbit (Table 6.4.) suggesting that lipid availability is not driving lipoprotein production, although critical lipid pools more closely associated with lipoprotein assembly were not measured. However the livers of the SMHL rabbits were shown by the perfusion experiments to secrete greater amounts of cholesterol and, to a certain extent, triglyceride. In order to maintain hepatic cholesterol homeostasis, there must be an alteration in another aspect of its control, for example an upregulation in the uptake by the LDL receptor, either due to the

receptor itself or an increase in circulating lipoproteins; an upregulation in cholesterol synthesis, or a decrease in the conversion of cholesterol into bile acids for excretion.

In conclusion, the results of these experiments suggest that apoB is overproduced in the mature SMHL rabbit, particularly in the $VLDL_2$ and IDL density classes. This, rather than increased hepatic lipid stores, may be the driving force for the secretion of an elevated amount of lipid, resulting in the hyperlipidaemia characteristic of these rabbits. The fact that the lipoprotein overproduction in the SMHL rabbits can be seen across the range of apoB containing lipoproteins is a possible explanation for the inter- and intra-individual variability of the lipid phenotype in these rabbits and in FCH.

Chapter 7. Liver perfusion studies in young NZW and SMHL rabbits

7.1. Introduction

Concurrent with the experiments described in Chapter 6, we performed an investigation of the output of apoB100 containing lipoprotein particles from the liver of a group of young NZW and SMHL rabbits. Preliminary results from experiments performed during the development of the liver perfusion protocol suggested that NZW rabbits secrete mainly $VLDL_1$ particles when young, and that more mature rabbits secrete a greater amount of non $VLDL_1$ particles. This fact, combined with the effect of age on plasma lipids in SMHL rabbits reported in Chapter 3, led us to believe that there may be some differences between the output of apoB containing lipoprotein particles in young and mature SMHL rabbits.

7.2. Methods

7.2.1 Animal selection

We selected 6 SMHL and 7 NZW male rabbits aged between 11 and 16 weeks of age, that had been fed the 0.08% cholesterol diet since weaning (for at least 2 weeks before sacrifice). SMHL rabbits expressed a moderate hyperlipidaemia compared to controls (cholesterol range 2.7 - 5.6 mmol/l, triglyceride range 1.4 - 7.55 mmol/l in SMHL and cholesterol range 0.4 - 2.0 mmol/l, triglyceride range 0.4 - 1.0 mmol/l in NZW). Mean age at sacrifice was 2.8 vs 3.0 months and mean body weight was 2.0 vs 2.5 kg (SMHL vs NZW, both NS).

7.2.2 Perfusion method

The method used for the perfusion experiments is described in Chapter 5. Briefly, the animal was terminally anaesthetised and the IVC was cannulated cranial to the

diaphragm. The HPV and the common bile duct were cannulated and the IVC was ligated between the renal and hepatic veins. The liver was flushed with 750 ml oxygenated Krebs Henseleit buffer (pH 7.4, 37°C) prior to being connected to the perfusion apparatus in a room thermostatically controlled at 37°C. The buffer was oxygenated by a Hamilton lung, and was pumped by two peristaltic pumps in a recirculating system at 100 ml/minute through the liver, with inflow via the HPV and outflow through the IVC. The pH was maintained at 7.4. The total volume of the perfusate was 120 ml, and was maintained thus, with sample volume replaced with an equal volume of oxygenated warmed Krebs Henseleit buffer. Samples of perfusate were taken at 0, 30, 60, 90, 120, 150 and 180 minutes, and cholesterol, triglyceride, glycerol, protein, urea, microalbumin, AST and GGT were measured (Sections 2.3.2, 2.3.3, 2.3.4, 2.4.,2.5.). Lipoprotein subfractions were separated by density gradient ultracentrifugation, and cholesterol, triglyceride and apoB were measured in each fraction (Sections 2.3.8, 2.3.2, 2.3.3, 2.4). Bile production by the liver was also measured.

7.2.3 Extraction of lipid from liver samples

Samples of liver were removed and frozen from 5 young SMHL and 5 young NZW rabbits immediately after the perfusion experiments were completed. The lipid content of these samples was measured by the method described in section 6.2.3.

7.2.4 Statistics

Variables were tested for normal distribution. Plasma cholesterol and triglyceride concentrations were transformed to a normal distribution by taking the logarithm of their values. Two sample t-tests were performed to determine significance. Where the distribution of a variable or series of variables could not consistently be converted to normal, as was the case with all perfusate sample concentrations, a Mann-Whitney U test was performed. Data are expressed as mean ± SEM unless otherwise stated. Data was unavailable for SMHL rabbits at 180 minutes due to insufficient samples.

7.3. Results

7.3.1 Population lipids and biochemical parameters

The plasma cholesterol and triglyceride concentrations in the young SMHL rabbits selected for perfusion experiments were significantly elevated compared to NZW rabbits (plasma cholesterol 3.7 ± 0.4 mmol/l in SMHL vs 1.0 ± 0.2 mmol/l in NZW, $p = 0.0001$; plasma triglyceride 3.6 ± 0.9 mmol/l in SMHL vs 0.7 ± 0.1 mmol/l in NZW, $p < 0.005$).

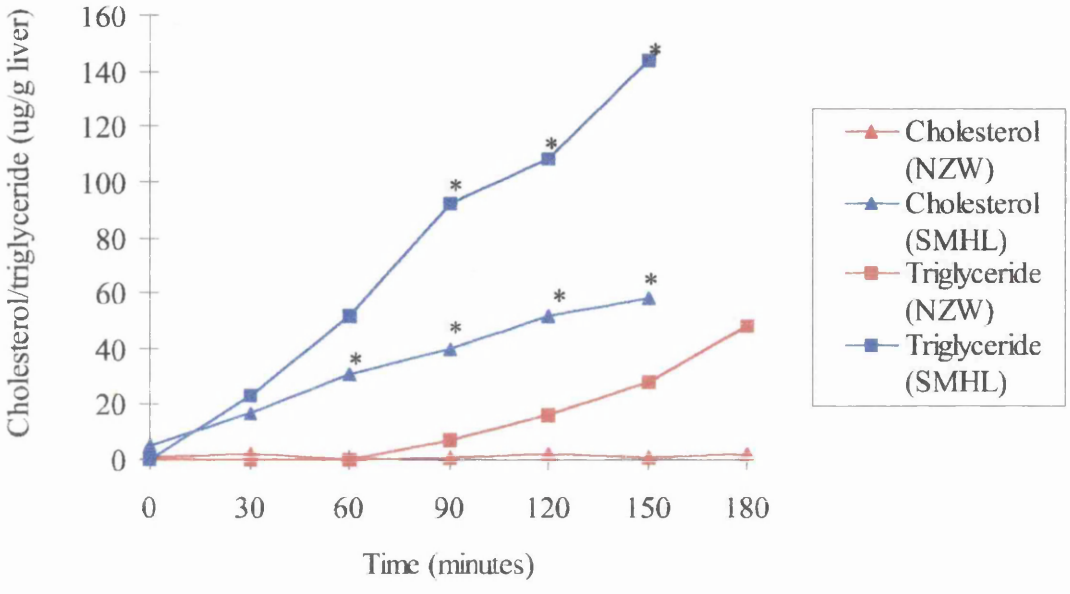
Liver function tests were normal throughout the perfusions. The livers remained patent and produced bile at 28 ± 4 and 40 ± 5 μl/liver/minute in SMHL and NZW rabbits respectively (NS). Examination of the livers at the end of the experiment revealed no necrotic or hypoxic areas. Mean liver weight was not different (76 ± 6 vs 71 ± 7 g, SMHL vs NZW, NS).

7.3.2 Cholesterol and triglyceride production

Mean perfusate cholesterol and triglyceride levels rose linearly in both groups of rabbits over the 180 (150) minutes of the experiment (Figure 7.1.). Cholesterol levels rose from 5 ± 5 to 58 ± 31 μg/g liver at 150 minutes in SMHL rabbits and from 0 to 2 ± 0.5 μg/g liver after 180 minutes in NZW rabbits. The cholesterol content of the perfusate was significantly elevated in SMHL rabbits in all samples from 60 to 150 minutes and the calculated cholesterol output was 380 ± 170 vs 10 ± 6 ng/g liver/minute in SMHL vs NZW rabbits ($p < 0.01$).

Triglyceride levels in the perfusate rose from 0 to 144 ± 63 μg/g liver at 150 minutes in SMHL rabbits and from 0 to 48 ± 24 μg/g liver at 180 minutes in NZW rabbits, with SMHL triglyceride levels being significantly higher at 90, 120 and 150 minutes when compared to NZW rabbits. Mean triglyceride output was 810 ± 290 vs 270 ± 100 ng/g liver/minute in SMHL vs NZW rabbits, $p = 0.12$.

Figure 7.1. Cholesterol and triglyceride output from the liver of young NZW and SMHL rabbits



** indicates time points where SMHL cholesterol and triglyceride outputs are significantly increased when compared to NZW outputs, $p < 0.05$*

7.3.3 Lipoprotein production during perfusion

The lipoproteins in the perfusate were separated and their cholesterol and triglyceride contents were analysed (Table 7.1.). Mean perfusate $VLDL_1$, $VLDL_2$ and IDL cholesterol and $VLDL_2$ triglyceride outputs were significantly increased in SMHL rabbits compared with NZW.

Table 7.1. $VLDL_1$, $VLDL_2$, IDL and LDL cholesterol and triglyceride output from the livers of 7 young NZW and 6 young SMHL rabbits

	Cholesterol (ng/g liver/minute)		Triglyceride (ng/g liver/minute)	
	NZW n = 7	SMHL n = 6	NZW n = 7	SMHL n = 6
$VLDL_1$	60 ± 19	237 ± 68[a]	416 ± 105	658 ± 177
$VLDL_2$	10 ± 4	72 ± 25[b]	20 ± 5	87 ± 44[a]
IDL	4 ± 2	40 ± 26[a]	3 ± 2	55 ± 40
LDL	5 ± 3	10 ± 7	3 ± 2	9 ± 7
non $VLDL_1$	21 ± 8	119 ± 56[a]	23 ± 7	150 ± 89[a]

Significant differences between NZW and SMHL rabbits [a] $p < 0.05$, [b] $p < 0.005$

As in Chapter 6, we compared the output of triglyceride rich $VLDL_1$ and cholesterol rich non $VLDL_1$ between the two strains (Table 7.1., Figure 7.2.A,B). $VLDL_1$ cholesterol output was elevated 4 fold in SMHL, $p < 0.05$ (Table 7.1.). In SMHL rabbits, $VLDL_1$ cholesterol levels rose from 1 ± 0.3 to 38 ± 12 μg/g liver after 150 minutes, and in NZW rabbits, $VLDL_1$ cholesterol levels rose from 0 to 13 ± 4 μg/g liver after 180 minutes with $VLDL_1$ cholesterol levels being significantly higher in SMHL rabbits at all time points from 30 to 150 minutes. Non $VLDL_1$ cholesterol output was elevated 6 fold in SMHL rabbits (Table 7.1., Figure 7.2.A), $p < 0.05$, rising from 1 ± 0.7 to 19 ± 10 μg/g liver in SMHL rabbits and from 0.6 ± 0.4 to 5 ± 2 μg/g liver in NZW. Levels of non $VLDL_1$ cholesterol were significantly higher in SMHL rabbits between 90 and 150 minutes.

Figure 7.2.A $VLDL_1$ and non $VLDL_1$ cholesterol output from the liver of young NZW and SMHL rabbits

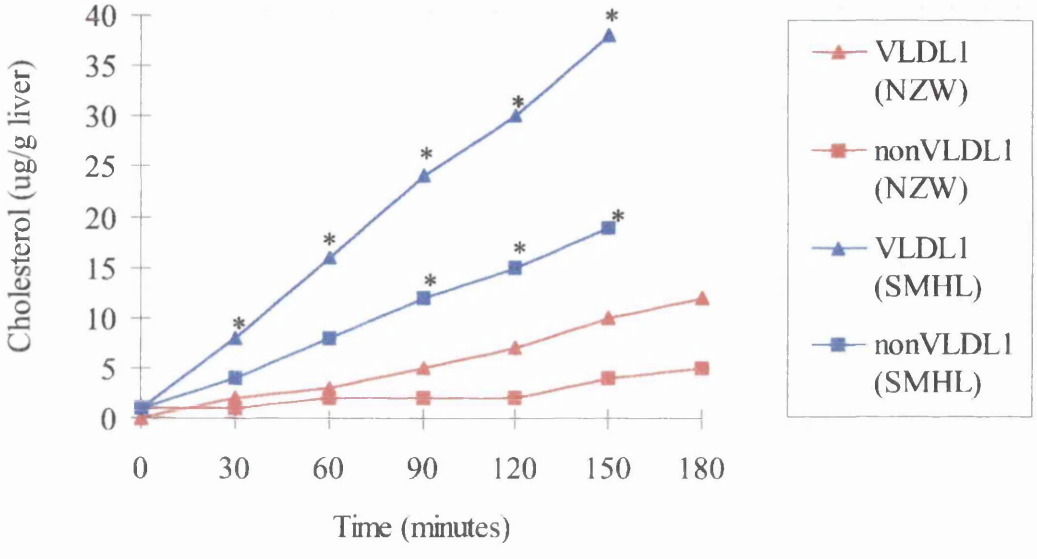
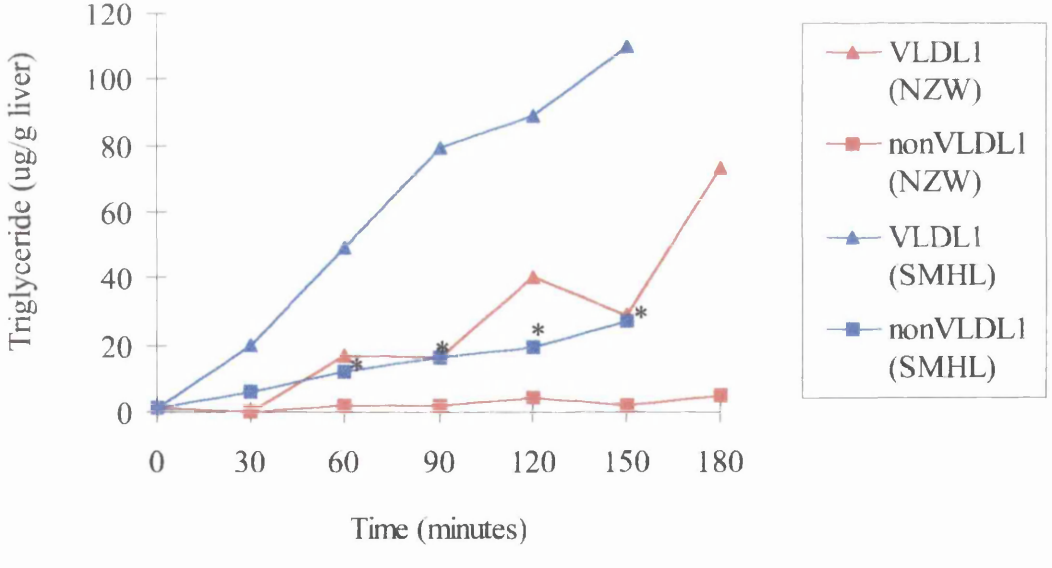


Figure 7.2.B $VLDL_1$ and non $VLDL_1$ triglyceride output from the liver of young NZW and SMHL rabbits



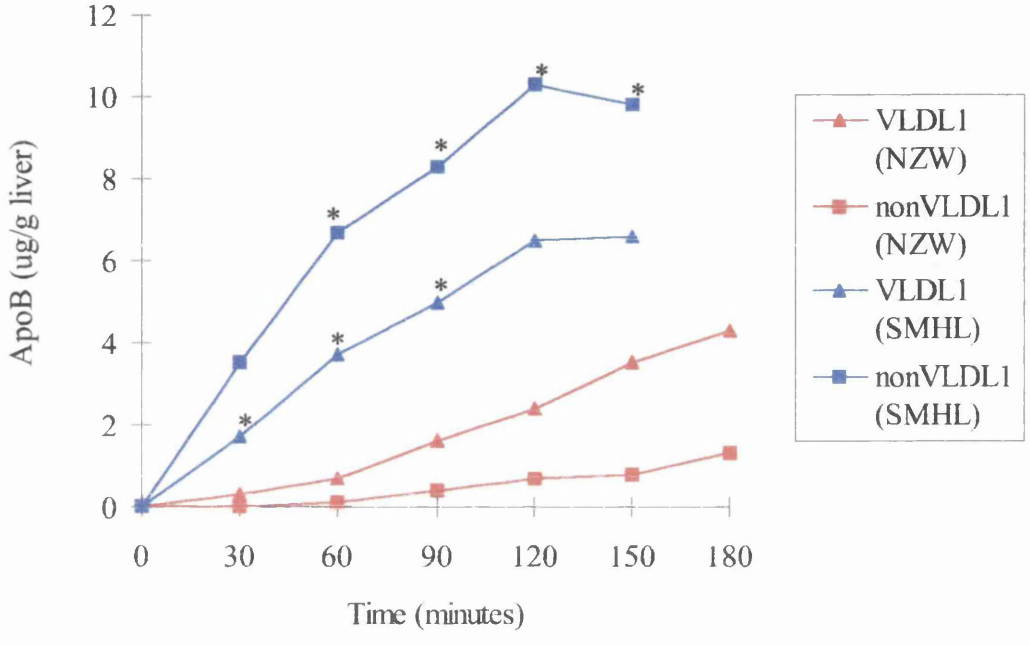
** indicates time points where SMHL rabbit parameters are significantly increased compared to corresponding NZW rabbit parameters $p < 0.05$*

There was no significant difference in $VLDL_1$ triglyceride output between the two strains of rabbit, due in part to the large inter-animal variablilty (Table 7.1., Figure 7.2.B). $VLDL_1$ triglyceride levels rose from 1 ± 0.5 to 110 ± 29 μg/g liver at 150 minutes in SMHL rabbits, and from 1 ± 0.6 to 73 ± 18 μg/g liver at 180 minutes in NZW rabbits. Non $VLDL_1$ triglyceride output was significantly increased more than 6 fold in SMHL rabbits (Table 7.1.), $p < 0.05$, rising from 1 ± 0.6 to 27 ± 16 μg/g liver at 150 minutes in SMHL rabbits and from 1 ± 0.4 to 5 ± 2 μg/g liver in NZW rabbits (Figure 7.2.B). SMHL rabbits had significantly higher non $VLDL_1$ triglyceride levels from 60 to 150 minutes.

7.3.4 ApoB output from perfused livers

ApoB was measured in each lipoprotein fraction secreted into the perfusate (Table 7.2., Figure 7.3.). $VLDL_1$, $VLDL_2$ and IDL apoB outputs were significantly elevated in this group of young SMHL rabbits when compared to young NZW rabbits (Table 7.2.). $VLDL_1$ apoB levels rose from 0 to 7 ± 1 μg/g liver at 150 minutes in SMHL rabbits and from 0 to 4 ± 0.8 μg/g liver at 180 minutes in NZW rabbits and were significantly increased in SMHL rabbits compared to NZW rabbits at 30, 60 and 90 minutes. Non $VLDL_1$ apoB output was increased 13 fold in the SMHL rabbits (Table 7.2.), $p < 0.005$, rising from 0 to 10 ± 3 μg/g liver at 150 minutes in SMHL rabbits and from 0 to 1.3 ± 0.6 μg/g liver at 180 minutes in NZW rabbits. Non $VLDL_1$ apoB levels were significantly elevated compared to NZW at all time points from 60 to 150 minutes. Total apoB output was significantly elevated 6 fold in the SMHL rabbits (159 ± 56 vs 28 ± 7 ng/g liver/minute, $p < 0.01$). In the young SMHL rabbits, the contribution of $VLDL_1$ and non $VLDL_1$ apoB to the total apoB output was virtually identical, whereas in the young NZW rabbits, 80% of the apoB was in the $VLDL_1$ fraction.

Figure 7.3. $VLDL_1$ and non $VLDL_1$ apoB output from the liver of young NZW and SMHL rabbits



** indicates time points where SMHL apoB output is significantly increased compared to NZW rabbit output $p < 0.05$*

Table 7.2. $VLDL_1$, $VLDL_2$, IDL and LDL apoB output (ng/g liver/minute) in young NZW and SMHL rabbits

	NZW	SMHL
$VLDL_1$	22 ± 4	79 ± 29[a]
$VLDL_2$	3 ± 1	36 ± 13[c]
IDL	1 ± 1	37 ± 23[b]
LDL	2 ± 1	7 ± 5
non $VLDL_1$	6 ± 3	80 ± 41[c]

Significant difference between NZW and SMHL rabbits [a] $p < 0.05$, [b] $p < 0.01$, [c] $p < 0.005$

There was no major difference between the cholesterol + triglyceride to apoB ratios for each lipoprotein fraction between the NZW and SMHL rabbits (Table 7.3.), although as with the mature rabbits, the SMHL rabbits showed a tendency towards smaller particles.

Table 7.3. Cholesterol + triglyceride to apoB ratios (g/g) in young NZW and SMHL rabbits

	NZW	SMHL
$VLDL_1$	19.7	11.3
$VLDL_2$	9.0	4.4
IDL	7.0	2.6
LDL	5.0	2.8
non $VLDL_1$	6.7	3.4

These values give an indication of the relative amount of cholesterol + triglyceride present in relation to apoB.

7.3.5 Liver lipid content

Hepatic lipid concentrations were measured after the perfusion experiments (Table 7.4.). Young SMHL rabbits had a significantly lower hepatic triglyceride concentration than did NZW rabbits ($p = 0.05$), but no other differences were seen.

Table 7.4. Liver lipid content (mg/g wet weight liver)

	NZW	SMHL
Total cholesterol	6.0 ± 1.1	7.9 ± 2.1
Free cholesterol	3.3 ± 0.8	5.0 ± 1.3
Triglyceride	13.0 ± 1.0	9.7 ± 1.0[a]
Phospholipid	16.2 ± 0.8	15.7 ± 1.5

Significant difference [a] $p < 0.05$

As before we compared the amount of lipid secreted from the liver into the perfusate with the amount of lipid in the hepatic store and it was clear that the amount of lipid output was a very small fraction (1% or less) of that stored. In SMHL rabbits 58 ± 31 μg cholesterol/g liver was secreted from a total hepatic store of 7.9 ± 2.1 mg/g liver, and in NZW rabbits 2 ± 0.5 μg cholesterol/g liver was secreted from a hepatic store of 6.0 ± 1.1 mg/g liver. In SMHL rabbits, 144 μg triglyceride/g liver was secreted from a hepatic pool of 9.7 ± 1.0 mg/g liver, and in NZW rabbits, 48 ± 24 μg triglyceride/g liver was secreted from a hepatic store of 13.0 ± 1.0 mg/g liver.

7.3.6 Young versus mature rabbits: cholesterol and triglyceride output

To investigate the effects of age on the output of cholesterol and triglyceride from the perfused liver, we compared young and mature rabbits (Chapter 6) of each strain. Mature NZW rabbits had a significantly greater cholesterol output compared to young NZW of 220 ± 67 vs 10 ± 6 ng/g liver/minute ($p < 0.005$), with cholesterol levels in the perfusate being significantly higher in mature rabbits from 60 minutes onwards.

Mature NZW also had a significantly greater triglyceride output of 888 ± 246 vs 274 ± 100 ng/g liver/minute (mature vs young), $p < 0.05$, with perfusate triglyceride levels being significantly higher from 120 minutes until the end of the experiment. SMHL rabbits did not show any effects of age.

7.3.7 Young versus mature rabbits: lipoprotein production

There were no differences in $VLDL_1$ or non $VLDL_1$ outputs with age in either the NZW or the SMHL rabbits.

7.3.8 Young versus mature rabbits: apoB production

In contrast to the lipoprotein output, $VLDL_1$ apoB output was significantly increased in the young NZW compared to the mature NZW (22 ± 4 vs 10 ± 3 ng/g liver/minute respectively, $p < 0.05$) with $VLDL_1$ apoB levels being significantly elevated in young rabbits at 150 and 180 minutes (Table 6.2., 7.2.). Young SMHL also had a significantly increased $VLDL_1$ apoB output compared to the mature SMHL (79 ± 29 vs 15 ± 4 ng/g liver/minute respectively, $p < 0.01$), with $VLDL_1$ apoB levels being significantly increased at 60 and 150 minutes in the young SMHL rabbits. No differences in non $VLDL_1$ apoB output were found between the young and mature rabbits of either strain.

7.3.9 Young versus mature rabbits: liver lipid content

Liver cholesterol stores showed a significant increase with age in NZW rabbits (Table 6.3., 7.3.). Total and free cholesterol were significantly increased in mature NZW compared to young NZW (15.4 ± 1.9 vs 6.0 ± 1.1 mg/g wet weight liver, $p = 0.015$ and 7.4 ± 1.3 vs 3.3 ± 0.8 mg/g wet weight liver, $p = 0.017$, respectively). There were no differences with age in triglyceride and phospholipid concentrations in NZW rabbits, and no significant differences in any hepatic lipid concentration in SMHL rabbits.

7.4. Discussion

The young SMHL rabbits used in these experiments were representative of the young rabbits in the colony. They were moderately hyperlipidaemic compared to the NZW control rabbits and were in good health at the time of sacrifice.

The experiments produced results consistent with those described in Chapter 5 and 6. Cholesterol, triglyceride and apoB accumulated in the perfusion medium in an approximately linear manner throughout the experiment (Figure 7.1., 7.3.). Indeed, cholesterol and triglyceride output rates from the 4 groups of rabbits were very similar in 3 of those groups (510 ± 101, 220 ± 67, 380 ± 170 ng cholesterol/g liver/minute and 860 ± 130, 890 ± 246, 810 ± 290 ng triglyceride/g liver/minute in mature SMHL, mature NZW and young SMHL respectively) and comparable to data produced by Hornick *et al* (1983) of 380 ng cholesterol/g liver/minute and 4000 ng triglyceride/g liver/minute. Liver function tests and bile production were concordant with those described in Chapter 5 and 6.

Consistent with the results obtained in the mature rabbits, there was a significantly increased cholesterol output in the young SMHL rabbits compared to the young NZW rabbits and no significant difference in triglyceride output. However, the triglyceride output was actually elevated 3 fold in the young SMHL rabbits, but due to the small group numbers, just failed to reach significance. $VLDL_1$, $VLDL_2$ and IDL cholesterol and $VLDL_2$ triglyceride outputs were increased in the young SMHL rabbits. Again, it was unexpected that the difference in total triglyceride output was explained by an increase in $VLDL_2$ and non $VLDL_1$ triglyceride production, however this may be partially explained by the large inter-animal variability and the fact that most of the triglyceride was found in the $VLDL_1$ fraction. $VLDL_1$ cholesterol was elevated 4 fold and non $VLDL_1$ cholesterol was elevated 6 fold in young SMHL rabbits, while $VLDL_1$ triglyceride was elevated 1.6 fold (NS) and non $VLDL_1$ triglyceride was elevated 6.5 fold. Together with the data on apoB which showed a significant 4 fold increase in $VLDL_1$ apoB and a 13 fold increase in non $VLDL_1$ apoB in SMHL rabbits, this suggests that the young SMHL rabbits overproduce lipoproteins in both the $VLDL_1$ and non $VLDL_1$ density fractions. The cause of this is unclear, but may be

driven by the apoB output. Again, similar to the case in the mature rabbits, compared to $VLDL_1$, more of the total apoB in the young SMHL rabbits is released from the liver in the non $VLDL_1$ fraction than in NZW rabbits, and this together with the fact that lipase activity is minimal, suggests that the young SMHL rabbits are also able to directly produce an increased amount of these denser lipoproteins compared to the young NZW rabbits.

The lipid to apoB ratios for $VLDL_1$ $VLDL_2$, IDL and LDL particles were lower in the young SMHL rabbits compared to the young NZW rabbits. Additionally, the ratios for $VLDL_1$ of all the young rabbits were substantially lower in this fraction than in the mature rabbits, suggesting, in conjunction with the increased apoB outputs and similar lipid outputs in the young rabbits, that the particles resulting in the increased amount of apoB being secreted are perhaps released earlier with less lipid associated. Therefore, both young NZW and SMHL rabbits secreted lipid poor $VLDL_1$ particles as compared to mature rabbits.

When the rabbits in the young and mature groups were pooled together, significant positive correlations were seen between plasma cholesterol and triglyceride levels and apoB output rates from the perfused livers (Figure 7.4.). Taken with the results of the analyses of the hepatic lipid stores which showed few differences between strains, this suggests that an increased apoB output rate is driving the hyperlipidaemia in the SMHL rabbits.

When young and mature rabbits of each strain were compared to each other, there was a significant increase in both cholesterol and triglyceride output overall in mature NZW rabbits compared to young NZW rabbits. This was likely to be due in part to the increased hepatic total and free cholesterol content which was seen in mature compared to young NZW rabbits. There were no significant differences in cholesterol or triglyceride output when young and mature SMHL rabbits were compared, nor did they show any differences in hepatic lipid concentrations. There were no significant differences in $VLDL_1$ and non $VLDL_1$ cholesterol and triglyceride outputs with age. With a larger sample number, we may have been able to distinguish if the overall differences in cholesterol and triglyceride outputs in the NZW rabbits were due to the

Figure 7.4.A Correlation of plasma cholesterol and apoB output in all perfused rabbit livers

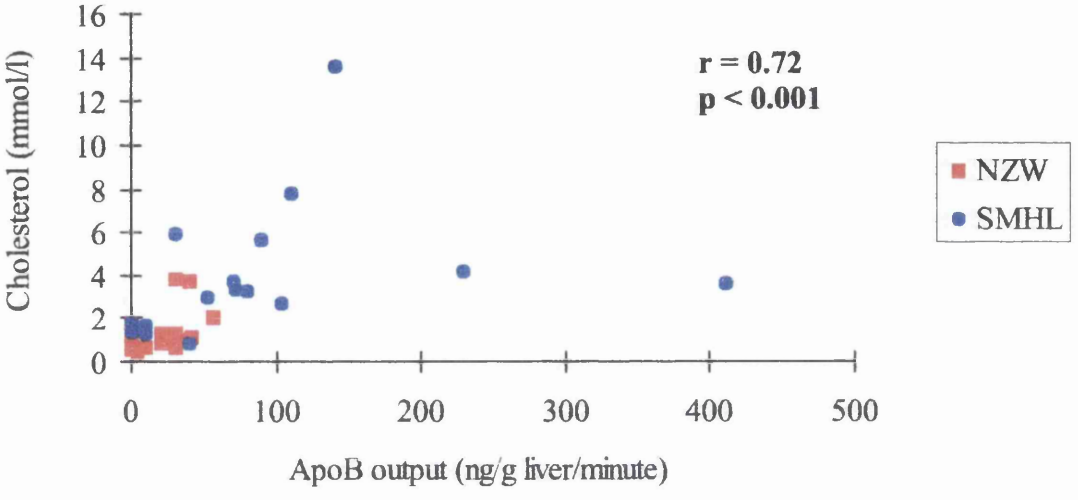
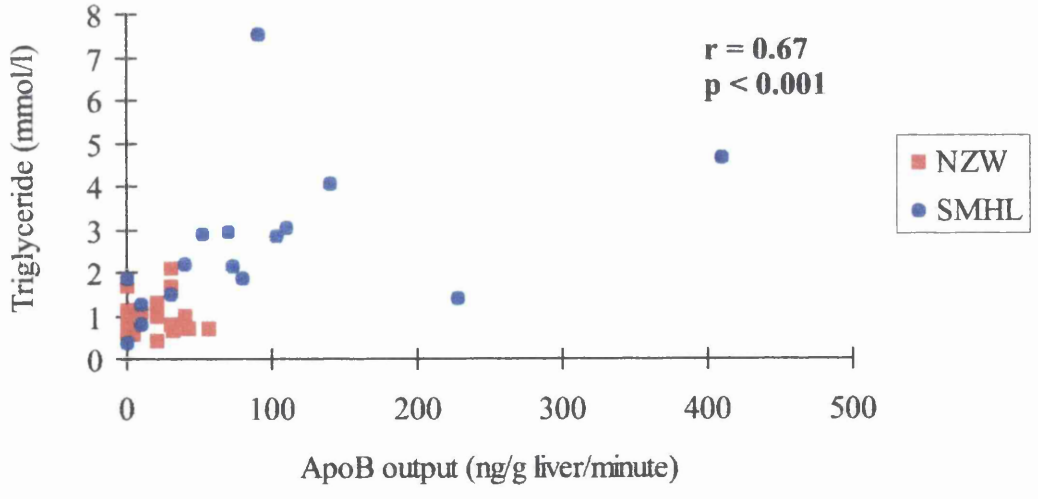


Figure 7.4.B Correlation of plasma triglyceride and apoB output in all perfused rabbit livers



$VLDL_1$ or non $VLDL_1$ fractions. The most striking age difference was in the apoB output. In contrast to the lipid outputs which increased in mature NZW rabbits, $VLDL_1$ apoB output was significantly increased in both the young NZW and SMHL rabbits. There was no difference in non $VLDL_1$ apoB, but total apoB output was also increased in both the young NZW and SMHL rabbits. Therefore, as the NZW rabbits matured, the cholesterol and triglyceride output increased, but the apoB output decreased. In the SMHL rabbits, there was no change in lipid output, but again, apoB output decreased.

These experiments show that there is a common mechanism in both NZW and SMHL rabbits that downregulates apoB production with age. This appears not to be due to a decreased availablity of lipid, indeed certainly in NZW, there is more lipid in the hepatocyte in mature rabbits, and the lipid to apoB ratios show that particles appear to become larger. What causes this downregulation is unclear. However the problem in SMHL rabbits is not the lack of downregulation, but the fact that the lipoprotein output is set at a much higher rate in both young and mature SMHL rabbits compared to NZW rabbits.

In conclusion, mature rabbits (NZW and SMHL) secrete regular sized particles, but in lower numbers than young rabbits. However overall, SMHL rabbits, like individuals with FCH, secrete an increased number of relatively lipid poor lipoprotein particles across the density spectrum, providing a possible explanation for the inter- and intra-individual variablility of lipid phenotype in these groups.

Chapter 8. Investigation of potential genetic abberations in the SMHL rabbit

8.1. Introduction

In all eukaryotes, DNA contains the information that controls all cell functions although only 3% of eukaryotic DNA has been found to encode proteins. The synthesis of protein is a two-step process: transcription, where mRNA is synthesised by RNA polymerase using the DNA as a template, and translation, where mRNA is the template that directs the correct association of amino acids into protein, on ribosomes. Each of these processes is regulated intra- or inter-cellularly and can be tissue specific or even cell specific. Control over protein production can be exerted transcriptionally, translationally or post translationally.

The polymerase chain reaction (PCR) is a relatively recent (Mullis, Faloona, Scharf, *et al* 1986, Mullis and Faloona 1987) but widely used procedure for the amplification of specific DNA and RNA sequences into workable amounts, for example for sequencing or testing for polymorphisms. The use of the thermostable DNA polymerase from *Thermus Aquaticus* (Taq) (Chien, Edgar and Trela, 1976, Saiki and Gelfand, 1989) has enabled the automation of this procedure.

Reverse transcriptase (RT) PCR involves the synthesis and amplification of DNA complementary to mRNA (cDNA) and has been shown to be extremely sensitive, enabling the detection of rare mRNA species (Byrne, Li, Sninsky, *et al* 1988, Wang, Doyle and Mark, 1989). RT PCR is documented as being thousands of times more sensitive than other methods of RNA detection such as Northern Blot Hybridisation or ribonuclease (RNase) protection assays (Larrick 1992, Siebert and Larrick 1992).

Rea, DeMattos and Pape (1993) successfully used RNase protection assays to investigate relative abundances of various lipid regulating genes in different tissues in rabbits. Although RT-PCR can detect small quantities of mRNA, it is difficult to

precisely quantify. The efficiency of the PCR assay is easily affected by a change in the reaction conditions, for example by such a change as would occur in primer or deoxynucleoside triphosphate (dNTP) concentration as amplification proceeds (Dickover, Donovan, Goldstein, *et al*, 1990). Because the amplification by PCR is exponential, small variations in efficiency can have major effects on the final concentration of amplicon. The use of an internal standard facilitates quantification. Until recently the best method for quantification of mRNA has been slot hybridisation (White and Bancroft, 1982) where RNA is bound to a nitrocellulose filter, and probed with a radiolabelled DNA or RNA probe. Other common methods of quantitation include Primer Extension where PCR products are radiolabelled and counted when separated by gel electrophoresis (Noonan, Beck, Holzmayer, *et al*, 1990); or the probing of PCR products on a Southern blot. However these methods are time consuming and often require large amounts of RNA to start with.

A relatively new, accurate method of quantification known as '5' nuclease PCR" is now in use (Gibson, Heid and Williams, 1996, Heid, Stevens, Livak, *et al*, 1996, Wittwer, Herrmann, Moss, *et al* 1997, Luthra, McBride, Cabanillas, *et al*, 1998, reviewed in Lie and Petropoulos, 1998). This is available in automated form on the ABI PRISM 7700 Sequence Detector (Taqman™), with attached Sequence Detection software (Perkin Elmer Applied Biosystems) using AmpliTaq Gold DNA polymerase which is thermostable and has 5'-3' nuclease activity, but no 3'-5' exonuclease activity. The basis of the technique is the ability to measure in real time the accumulation of a PCR product, by the release of fluorescence from a probe that is displaced from its hybridisation point on the cDNA during each amplification cycle. Measurement occurs during the linear phase of the reaction, that is during the period of exponential growth.

Primers are designed to amplify up a short region of cDNA from the gene of interest. A probe is designed to a region of cDNA within the amplicon, but not overlapping with the primers. This probe is labelled at its 5' end with a fluorescent reporter dye, FAM (6-carboxy-fluorescein), and at the 3' end, with a quencher, TAMRA (6-carboxy-tetramethyl-rhodamine). When the quencher is in proximity to the reporter, the release of fluorescence is inhibited. During the annealing phase of the PCR

reaction, the primers and probe hybridise to the template strands (Figure 8.1.). As the primers are extended the probe is displaced from the template and is cleaved by the polymerase. This distances the reporter from the quencher, resulting in an increase in fluorescence of the reporter which is proportional to the amount of product accumulated. Fluorescence is measured every 7 seconds, and the cycle number at which fluorsescence becomes detectable is recorded, and is defined as the threshold cycle, C_t. The C_t value is inversely related to the amount of cDNA of the gene of interest that was present. Using a standard curve of C_t values from a known amount of cDNA, values can be determined for the unknowns. In some cases the use of internal standards is beneficial to control for the efficiency of PCR.

We wished to compare the relative amounts of mRNA in NZW and SMHL rabbits for a range of genes that could contribute to the phenotype of cholesterol and apoB overproduction that we have characterised for the SMHL rabbits. In addition to apoB, we investigated the expression of MTP and PDI as they are important transport proteins involved in the lipidation of apoB (Figure 8.2.) (Jamil, Dickson, Chu, *et al,* 1995, Gordon, Jamil, Gregg, *et al,* 1996, Patel and Grundy, 1996, Gordon, 1997). We also investigated VLDL and LDL receptor mRNA, as indices of catabolism, CETP as transfer activity was significantly increased in the SMHL rabbits and cholesterol 7 alpha-hydroxylase as the rate limiting enzyme in the conversion of cholesterol to bile acids (Figure 8.2.) (Angelin 1995). In addition, we measured the expression of three constitutively expressed housekeeping genes, glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (an important enzyme in glycolysis), hypoxanthine-guanine phosphoribosyltransferase (HPRT) (an enzyme involved in the salvage pathway of purine bases) and beta actin (a structural protein). By correcting the C_t values of the genes of interest to the expression of a housekeeping gene, we hoped to eliminate inter-animal variability due to environmental factors or cell cycle position.

Figure 8.1. Principle of TaqMan 5' nuclease PCR assay

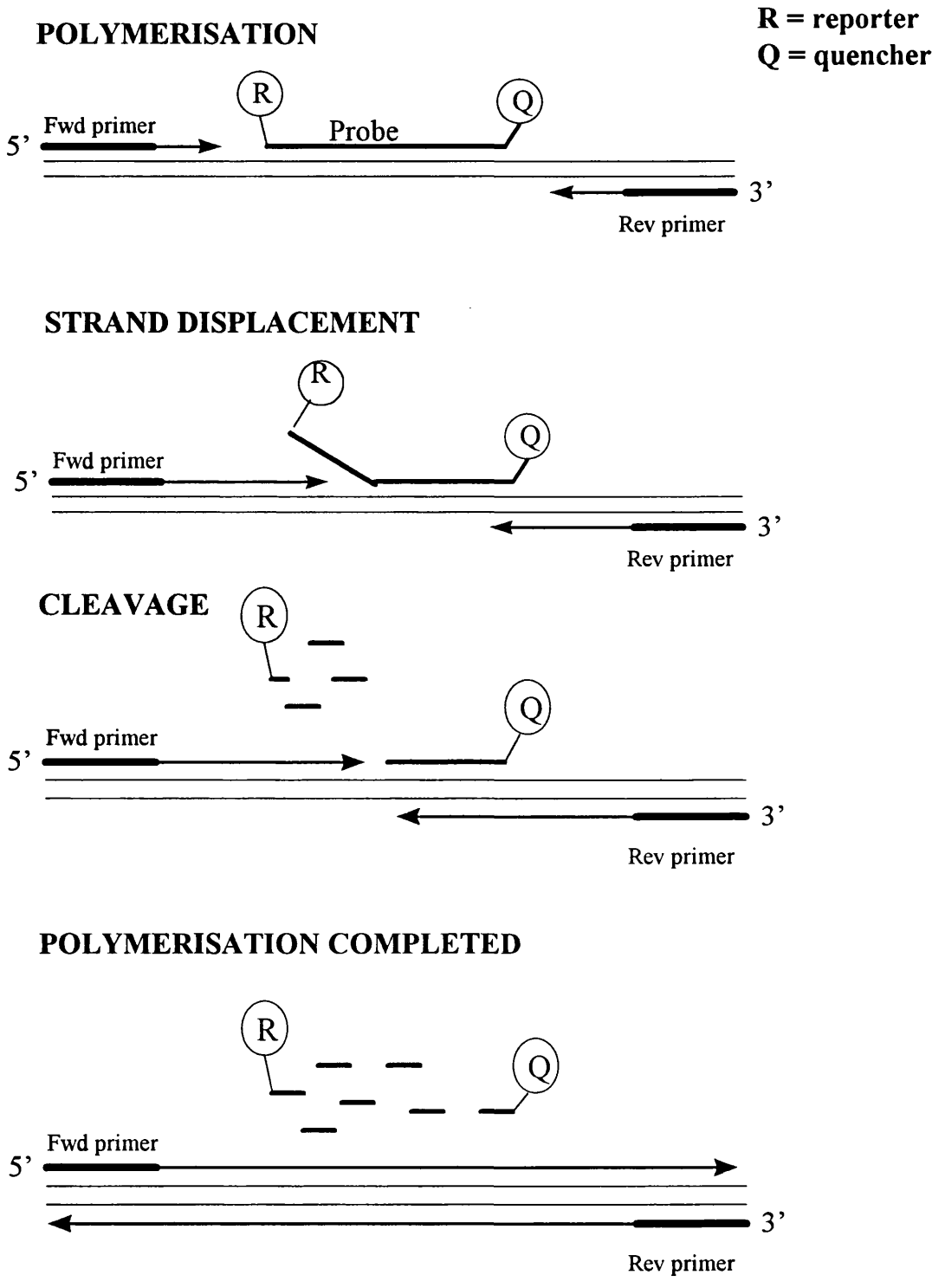
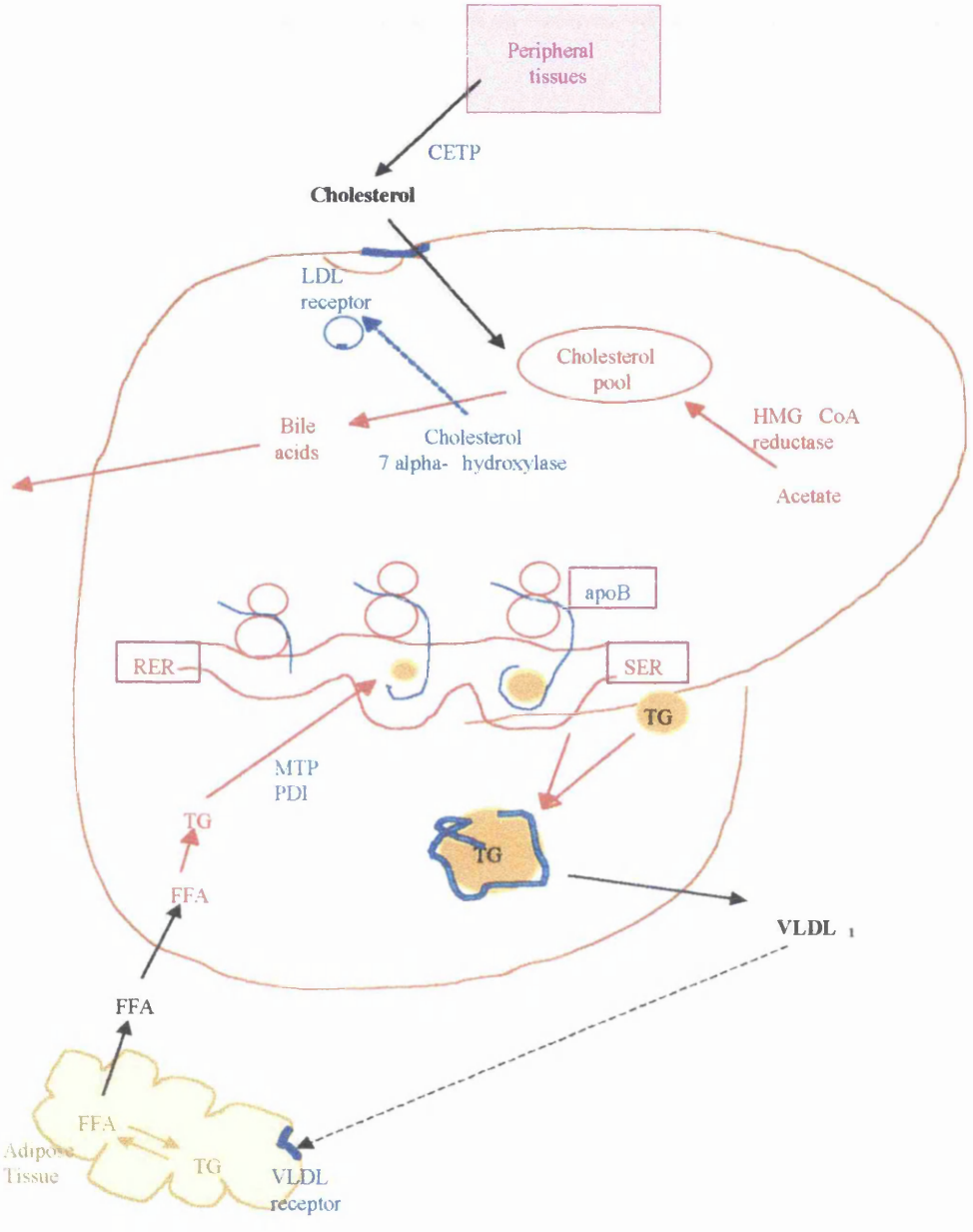


Figure 8.2. Site of action of candidate genes



The sites of action of the genes for which we measured mRNA levels are shown. ApoB is transcribed in the liver, MTP and PDI provide the lipid for the production of lipoprotein particles. The VLDL and LDL receptors are involved in the uptake of lipid, cholesterol 7 alpha-hydroxylase is involved in clearing cholesterol from the liver as bile, and CETP is involved in delivering cholesterol to the liver.

8.2. Methods

8.2.1 Liver samples

Immediately post-mortem, 1 - 5 gram pieces of liver (11 NZW and 17 SMHL) were removed, wrapped in tin foil and snap frozen in liquid nitrogen. These samples were stored at –70°C until required.

8.2.2 Isolation of RNA from liver tissue

RNA was extracted from liver using TRIzol™ Reagent (GIBCO BRL, Life Technologies) following the manufacturers instructions by a modification of the method of Chomczynski and Sacchi (1987). TRIzol™ Reagent contains phenol and guanidine isothiocyanate to maintain the integrity of the RNA and dissolve cell components while samples are homogenised to break down the tissue. All tubes and disposable items used were treated (by baking at 120°C or with 10% hydrogen peroxide) so as to be RNAase free, and dH_2O was treated with diethyl pyrocarbonate (DEPC) (0.1%).

Approximately 500 mg of frozen liver tissue was taken and homogenised in 5 ml TRIzol™ Reagent in polypropylene tubes. Samples were maintained on ice. The samples were incubated at room temperature for 5 minutes and then centrifuged (12,000 *g*, 4°C, 10 minutes) to pellet insoluble material such as extracellular membranes, polysaccharides and high molecular weight DNA. The supernatant was collected, and 1 ml chloroform was added to isolate RNA. Samples were shaken vigorously (15 seconds), incubated at room temperature (10 minutes), centrifuged (12,000 *g*, 4°C, 20 minutes), and the aqueous phase (containing RNA) was transferred to fresh tubes. The RNA was precipitated by the addition of 2.5ml isopropanol. Samples were mixed and incubated at –20°C (60 minutes), then centrifuged (12,000 *g*, 4°C, 20 minutes) to pellet the RNA. The supernatant was removed and the pellet washed with 5 ml 75% ethanol. The pellet was resuspended by mixing and was re-centrifuged (12,000 *g*, 4°C, 20 minutes). The wash procedure

was repeated with 1 ml 75% ethanol, the supernatant removed and the pellet air dried before being resuspended in 300 µl DEPC treated water.

A 1.2% agarose gel containing 18% formaldehyde and ethidium bromide, was prepared in 1 x MAE buffer (5 x MAE consists of: 0.1 mol/l 3-(N-morpholino)propanesulfonic acid (MOPS), 40 mmol/l sodium acetate, 5 mmol/l EDTA). Two µg RNA from each liver preparation was loaded on to the gel in 2 x volume sample buffer (SIGMA R-4268). Samples had been previously denatured at 65°C for 10 minutes and immediately cooled on ice. The gel was run at 100 volts for 75 minutes. In order to check the integrity of the RNA, the gel was placed on an ultra violet light box to visualise the ethidium bromide stained RNA, and was then photographed.

The optical density at 260 nm of each RNA sample was measured. An absorbance of 1.0 was taken to be equivalent to 40 µg RNA/ml.

8.2.3 Reverse transcription

One µg RNA was reverse transcribed to form cDNA. An additional 1 µg RNA was prepared in the same manner, with the reverse transcriptase enzyme omitted (replaced with an appropriate volume of distilled H_2O) to act as a negative control.

Samples were initially treated with deoxyribonuclease (DNase) to digest single and double stranded DNA. One µg RNA was incubated with DNase reaction buffer (20 mmol/l Tris-HCl, 2 mmol/l $MgCl_2$, 50 mmol/l KCl), 1 unit of DNase I (both from Gibco BRL) and DEPC treated water (15 minutes, room temperature). The DNase I was inactivated by the addition of 2.5 mmol/l EDTA (10 minutes, 65°C). Five hundred ng random primers (Promega) were added (10 minutes, 25°C) followed by First Strand Buffer (50 mmol/l Tris-HCl, Gibco BRL), 0.01µmol/l dithiothreitol and 0.5 nmol/l dNTP mix (2 minutes, 42°C). Two hundred units of SUPERSCRIPT II (Gibco BRL) were added and incubated for 50 minutes at 42°C,

followed by 15 minutes at 70°C to destroy the Superscript II. cDNA was stored at -20°C.

8.2.4 Amplification and sequencing of a region of rabbit MTP cDNA

cDNA was reverse transcribed from 1 μg of rabbit liver RNA as described previously (section 8.2.3). PCR was carried out in 50 μl reaction volumes containing 5 μl of 5 x diluted cDNA, 1.5 mmol/l $MgCl_2$, 0.5 μmol/l of each primer, 0.2 mmol/l of each dNTP and 5 units of Taq polymerase (Gibco BRL). The oligonucleotide primers were obtained from Cruachem Ltd. and were designed according to a region of human MTP which showed high homology with hamster MTP. The primer pair used was 5' AATGACCGGCTGTACAAGCTCAC 3' (forward) and 5' CCTTTGAAGATGCTCTTCTCTCC 3' (reverse) amplifying a 226 base pair (bp) product (NIH genetic sequence database GenBANK Accession number X59657, Sharp, Blinderman, Combs, *et al*, 1993). The amplification profile consisted of 30 cycles of 94°C for 1 minute, annealing at 55°C for 1 minute and extension for 30 seconds at 72°C, and one final cycle of extension at 72°C for 5 minutes.

An aliquot of the RT PCR and an amplified RT 'no template' blank were subjected to agarose gel electrophoresis together with Sigma PCR molecular weight standards. The correct size fragments were excised from the gel and purified using a QIAquick gel extraction kit according to the manufacturer's protocol (Quiagen). PCR fragments were kindly sequenced by Gene Expression Sciences at SmithKline Beecham Pharmaceuticals using the PCR primer pair described above on an ABI377 automated sequencer (PE Applied Biosystems). A consensus sequence was obtained using Lasergene software (DNASTAR Inc.).

8.2.5 Design of primers and probes

Rabbit sequences of apoB (Sudarickov and Surguchov, 1988), PDI (Fliegel, Newton, Burns, *et al*, 1990), VLDL receptor (Takahashi *et al*, 1992), LDL receptor (Yamamoto, Bishop, Brown, *et al*, 1986), CETP (Nagashima, McLean and Lawn, 1988), cholesterol 7 alpha-hydroxylase (Poorman, Buck, Smith, *et al*, 1993), GAPDH

(Applequist, Keyna, Calvin, *et al*, 1995), and beta-actin (Carter, Umenishi, Matthay, *et al*, 1997) were obtained from the GenEMBL database (Accession numbers X07480, J05602, D11100, M11501, M27486, S67315, L23961 and AF000313 respectively). A human sequence was found for HPRT (Lightfoot, Joshi, Nuki, *et al*, 1992) (Accession number L29383) which cross reacted with rabbit HPRT cDNA. Primers and probes were designed using the Primer Express programme (PE Applied Biosystems), which selects probes and primers specifically for the TaqMan assay. Several criteria must be met when chosing a probe and primer set.

1. The primers and probe must have at least 50% G + C content
2. The primers and probe must not be complimentary to each other, nor capable of forming secondary structures, nor must there be more than 4 consecutive identical residues
3. The probe and primers must not overlap, but should be in close proximity to each other
4. The melting temperature (T_m) of both primers should be identical, and the T_m of the probe should be 10°C greater
5. The amplicon should be 70 - 100 bp long
6. The probe must have more C residues than G residues and should be 20 - 30 bp long
7. There must not be a G residue at the 5' end of the probe

Primers and probes were synthesised by PE-applied biosystems UK. Sequences are given in appendix 2.

8.2.6 Quantitative PCR

cDNA equivalent to 50 ng RNA was incubated for 40 cycles (95°C, 15 seconds, 60°C 1 minute; preceded by 2 minutes at 50°C and 10 minutes at 95°C) with Taqman Universal mix containing AmpliTaq Gold DNA polymerase (Perkin Elmer), 100 nmol/l probe, 300 nmol/l forward primer and 300 nmol/l reverse primer, made up to 25 µl with dH_2O.

Samples and negative controls were run for each gene, and 'no template' controls were run to ensure background was minimal.

8.2.7 Calculation of results

For each gene of interest, C_t values from unknown samples were compared to a standard curve of C_t values generated from 4 fold dilutions of known concentrations of cDNA. From this, values for the unknown cDNAs could be calculated. To control for inter-individual variation, results were expressed relative to a housekeeping gene which was shown not to be different between the two rabbit groups. Values were expressed on a relative scale rather than as absolute values.

8.2.8 Statistics

Variables were tested for normal distribution. Distribution was normal after the logarithm of the values was taken, and two sample t-tests were performed.

8.3. Results

8.3.1 Population data

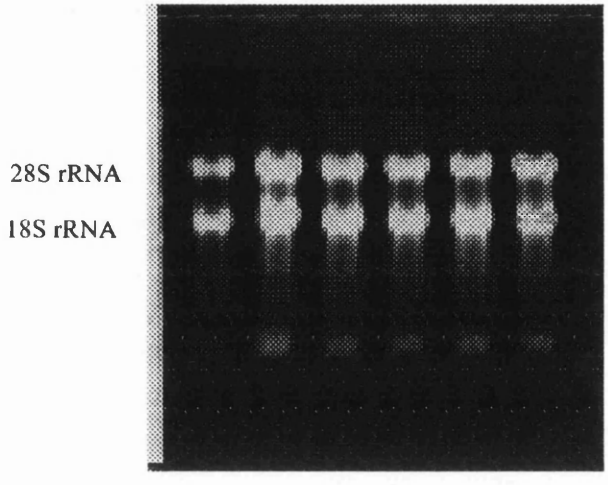
Plasma lipids

In the animals selected for this study, plasma cholesterol and triglyceride levels were significantly elevated in the SMHL when compared to the NZW group (Table 8.1.).

RNA yield

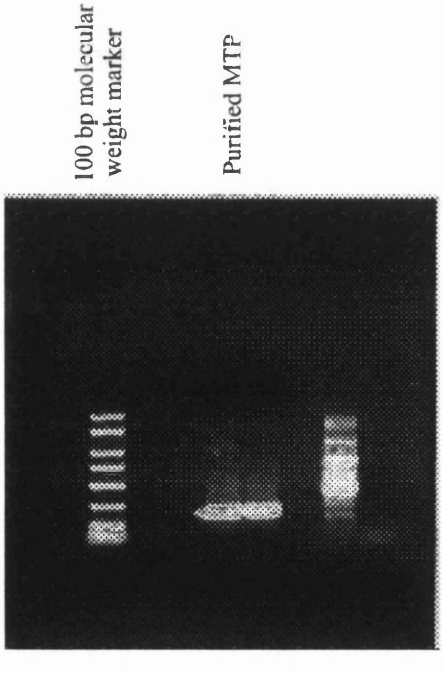
The RNA extracted from the liver samples from these rabbits was generally of good quality (Figure 8.3.) showing intact 28S and 18S ribosomal RNA bands.

Figure 8.3. Purified RNA



This gel picture clearly shows the 28s, 18s and tRNA bands produced on purification of RNA

Figure 8.4. Purification of the amplified region of the MTP gene



The amplified rabbit MTP insert is shown to be pure, and of the correct size (226 bp)

Table 8.1. Plasma lipids (mmol/l)

	NZW	SMHL	p value
Cholesterol	2.2 ± 0.3	6.9 ± 0.8	0.0001
Triglyceride	1.0 ± 0.2	3.0 ± 0.4	0.001

8.3.2 Sequence of rabbit MTP

The size of the band of rabbit MTP cDNA amplified by the human primers was estimated by agarose gel electrophoresis (section 8.2.4, Figure 8.4.) and was found to be consistent with the known human cDNA size, 226 bp. This was purified, and the sequence of the amplicon of rabbit MTP cDNA was determined (Figure 8.5.).

Figure 8.5. Consensus sequence of rabbit MTP (corresponding to bp 176 - 391 of human MTP)

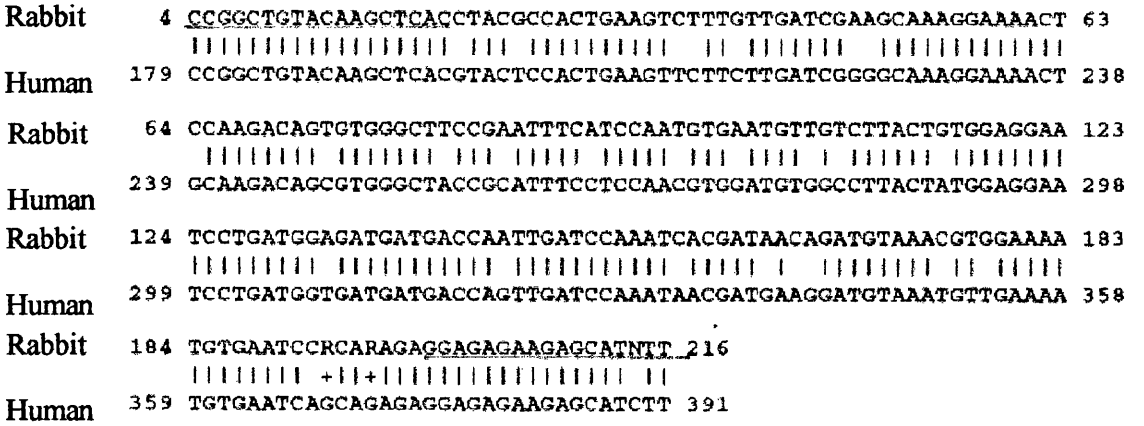
ATGCCGGCTG TACAAGCTCA CCTACGCCAC TGAAGTCTTT
GTTGATCGAA GCAAAGGAAA ACTCCAAGAC AGTGTGGGCT
TCCGAATTTC ATCCAATGTG AATGTTGTCT TACTGTGGAG
GAATCCTGAT GGAGATGATG ACCAATTGAT CCAAATCACG
ATAACAGATG TAAACGTGGA AAATGTGAAT CCRCARAGAG
GAGAGAAGAG CATNTT

This consensus sequence of rabbit MTP was found to be 87% homologous with that of human (Figure 8.6.).

8.3.3 Housekeeping genes

Three housekeeping genes were tested for use as an internal reference. GAPDH and HPRT were found to show no difference between NZW and SMHL rabbits (GAPDH 59.0 ± 5.3 vs 52.7 ± 4.0, HPRT 60.2 ± 7.1 vs 60.4 ± 7.1, NZW vs SMHL, NS, arbitrary values). Beta-actin values were significantly increased in SMHL rabbits

Figure 8.6. Alignment of rabbit MTP sequence with human MTP



___ indicates the forward and reverse primers used to amplify up the region of rabbit MTP. Homology between the two species is 87%.

(95.7 ± 10.7 vs 174.1 ± 21.4, NZW vs SMHL, p = 0.009) for unknown reasons. Due to the fact that the HPRT sequences were of human derivation, we decided to take the commonly used GAPDH as our reference gene.

8.3.4 Standard curves

A standard curve was constructed for each gene, using 4 fold dilutions of a NZW cDNA (expressed as equivalent ng of RNA) (Table 8.2.). A representative standard curve and amplification profile are shown in Figure 8.7.

Table 8.2. Standard curve C_t values for genes of interest

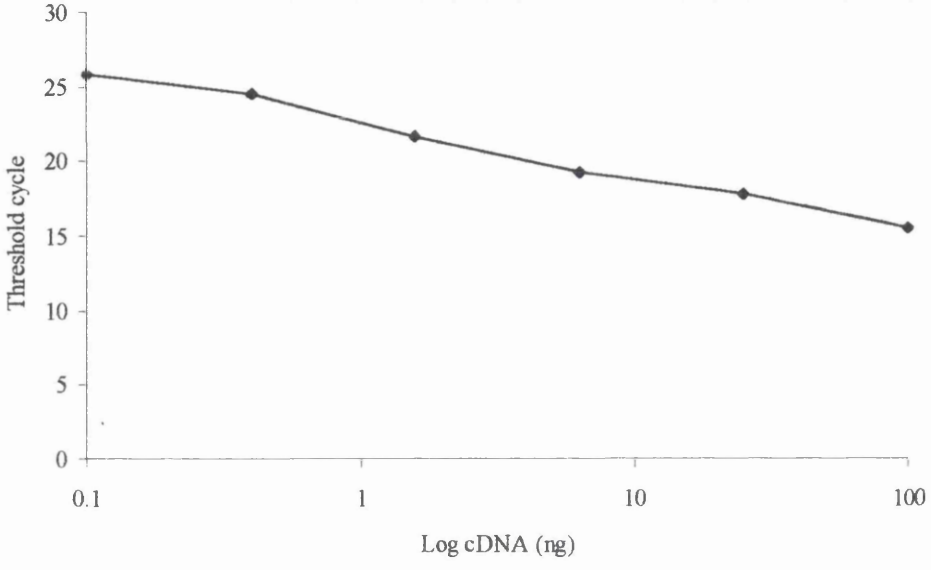
	C_t values					
Genes	**100 ng**	**25 ng**	**6.25 ng**	**1.56 ng**	**0.4 ng**	**0.1 ng**
GAPDH	16.07	19.41	22.46	24.58	27.14	29.02
ApoB	15.51	17.87	19.28	21.71	24.52	25.86
VLDL-R	26.30	28.01	29.45		33.66	35.14
LDL-R	25.04	26.93	29.75	32.24	33.97	
CETP	22.24	24.93	27.78	30.02	31.04	33.64
MTP		22.32	24.41	26.32	28.14	30.11
PDI	17.94	19.69	21.32	23.94	25.67	28.15
7-AH	24.17	25.94	28.52	30.15	32.30	34.39

VLDL receptor (VLDL-R), LDL receptor (LDL-R), cholesterol 7 alpha-hydroxylase (7-AH)

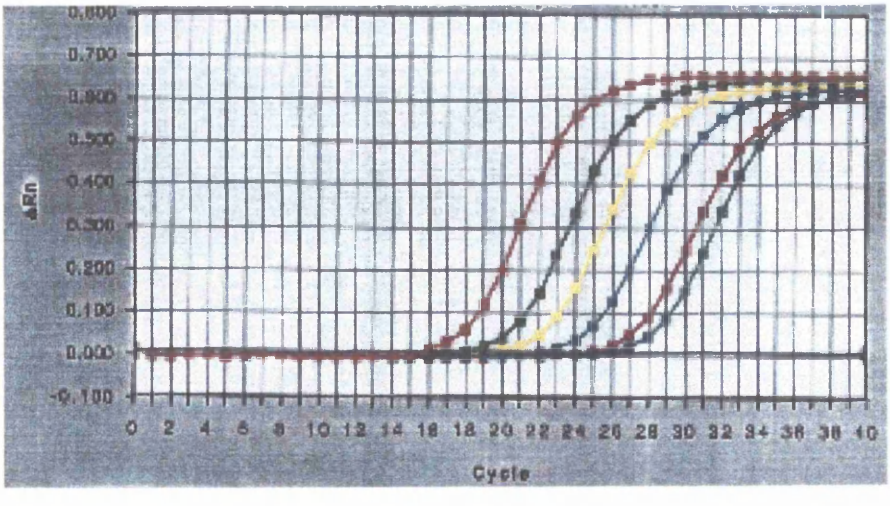
Standard curves were linear with correlation coefficients of greater than 0.99. A 4 fold increase in cDNA concentration causes a shift in C_t value of approximately 2. As a general rule, this procedure is sensitive enough to measure a 2 fold difference in cDNA concentration.

These data show that apoB, GAPDH and PDI were relatively abundant in the liver mRNA population, as they were present in detectable amounts at an early cycle

Figure 8.7.A Standard curve and amplification curves of apoB

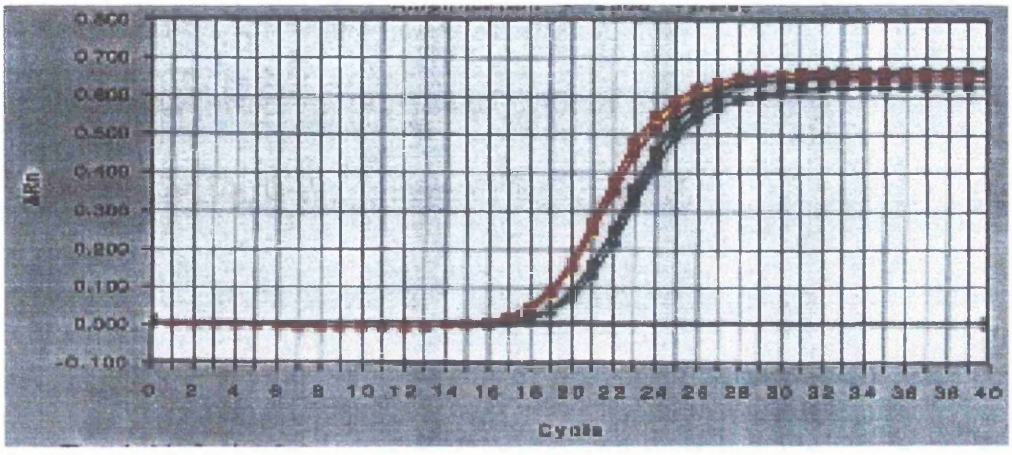


Graph of threshold cycle at which amplified apoB cDNA is measurable from a range of quantities of NZW rabbit cDNA



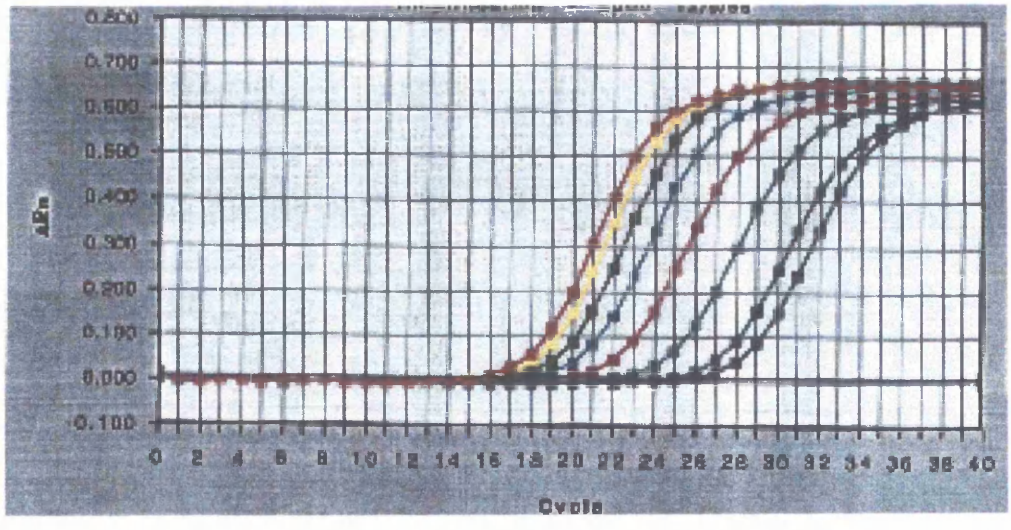
Graphical representation of the amplification of apoB cDNA over time

Figure 8.7.B Representative apoB amplification curves of 2 NZW and 2 SMHL rabbits



This figure clearly demonstrates that the SMHL rabbit apoB cDNA (,) is amplified above threshold at an earlier cycle number than the NZW rabbit apoB cDNA (,).

Figure 8.7.C ApoB amplification curves of 1 NZW and 1 SMHL rabbit overlaid on the apoB standard curve



This figure shows how the amplification curves of the SMHL ( ) and NZW ( ) rabbits are clearly differentiable on the apoB standard curve.

number, as compared to the VLDL receptor, LDL receptor and cholesterol 7-alpha hydroxylase mRNAs which did not amplify to detectable levels until a further 10 exponential increases in message had occurred. MTP and CETP mRNA were of moderate abundance.

8.3.5 Expression levels of genes of interest

To obtain an estimate of the amount of cDNA encoding for the amplified gene that was present in each sample, the C_t values from the housekeeping genes and the genes of interest from each sample were compared to the standard curves for each gene (Sequence Detection Analysis software, Perkin Elmer). The amout of cDNA of the gene of interest was divided by the amount of cDNA of GAPDH in each sample, to obtain a relative value for the expression of the gene of interest in each sample (Table 8.3.). Full values are given in appendix 3.

Expression of apoB mRNA was significantly increased in SMHL rabbits when compared to NZW (1.3 ± 0.16 vs 0.82 ± 0.08, $p = 0.005$). VLDL receptor mRNA was also significantly increased in SMHL (1.4 ± 0.26 vs 0.67 ± 0.08, $p = 0.02$), as was CETP mRNA (3.2 ± 0.34 vs 1.6 ± 0.3, $p = 0.002$). There were no differences in LDL receptor, MTP, PDI or cholesterol 7 alpha-hydroxylase mRNA between the two rabbit groups.

When observed values were related to the other housekeeping gene, HPRT, the results were very similar. ApoB mRNA was significantly elevated in SMHL rabbits compared to NZW (1.3 ± 0.60 vs 0.85 ± 0.32, $p = 0.01$, SMHL vs NZW), and VLDL-R mRNA was elevated, but this did not quite reach significance (2.5 ± 4.7 vs 0.74 ± 0.45, $p = 0.06$, SMHL vs NZW). We did not relate CETP to HPRT as the CETP assay was performed at a later date, and we also did not look at any of the ratios to beta actin as this gene was differentially expressed between the two groups of rabbits and would have skewed the results.

Table 8.3. Relative expression (to GAPDH) of mRNA of genes of interest

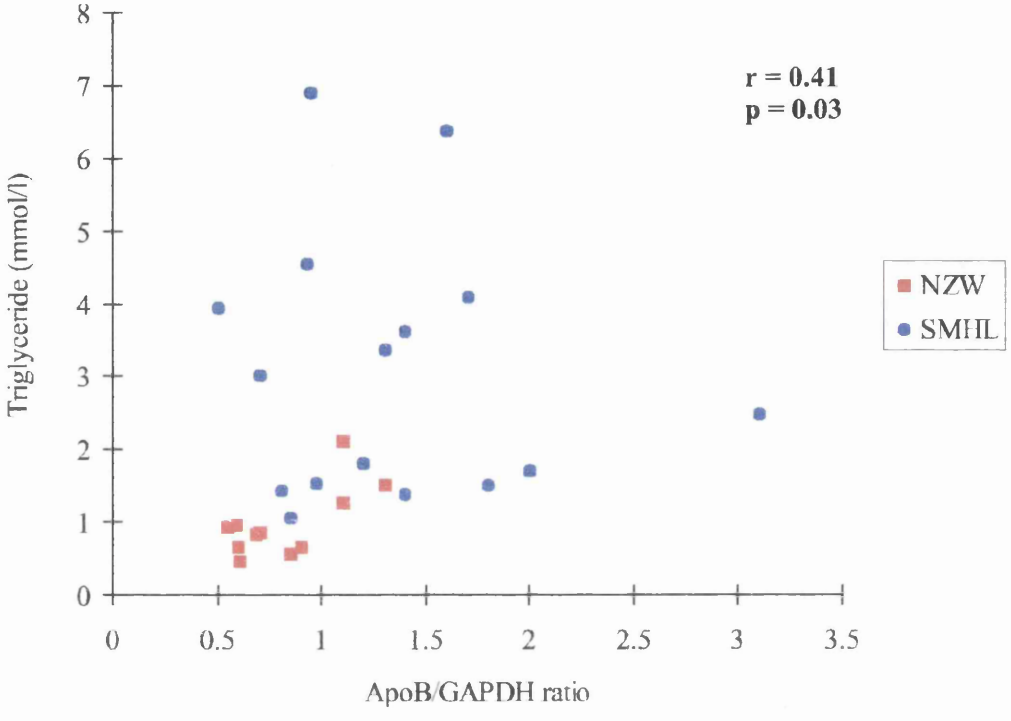
Genes		NZW	SMHL	p value
apoB	**mean**	0.82 ± 0.08	1.3 ± 0.16	0.005
	range	0.55 - 1.3	0.53 - 3.1	
VLDL-R	**mean**	0.67 ± 0.08	1.4 ± 0.26	0.02
	range	0.22 - 1.24	0.40 - 4.78	
LDL-R	**mean**	1.8 ± 0.32	2.2 ± 0.38	NS
	range	0.51 - 3.71	0.47 - 7.0	
CETP	**mean**	1.6 ± 0.30	3.2 ± 0.34	0.002
	range	0.52 - 3.50	0.77 - 5.5	
MTP	**mean**	0.71 ± 0.08	0.88 ± 0.11	NS
	range	0.32 - 1.18	0.23 - 2.24	
PDI	**mean**	1.1 ± 0.11	1.4 ± 0.18	NS
	range	0.46 - 1.6	0.59 - 3.1	
7-AH	**mean**	13.3 ± 4.2	10.7 ± 2.5	NS
	range	1.1 - 40.8	0.64 - 36.8	

Values given are mean ± SEM, and the range.

8.3.6 Correlation of plasma triglyceride with apoB mRNA levels

There was a significant positive correlation between plasma triglyceride levels in the animals selected for study and the ratio of apoB:GAPDH measure of mRNA abundance (Figure 8.8.), suggesting that the increased availability of apoB mRNA could contribute to the elevation of plasma triglyceride levels.

Figure 8.8. Correlation between plasma triglyceride levels and apoB mRNA levels



8.4. Discussion

The SMHL rabbits included in this study were moderately hyperlipidaemic. The quality of RNA isolated from liver tissue from both the NZW and SMHL rabbits was variable, but satisfactory, as evidenced by the presence of clear 18S and 28S ribosomal RNA bands on agarose gel electrophoresis (Figure 8.3.).

Although only a small portion of the rabbit MTP was amplified, the consensus sequence from forward and reverse sequencing matched the corresponding hamster and human sequences sufficiently for us to believe that the sequence was indeed rabbit MTP. It was fortunate that this small amplicon contained suitable sequences against which to construct a primer pair and a probe.

Housekeeping genes encode proteins vital for the survival of the cell and these are believed to be constitutively expressed. Unfortunately the levels of mRNA of these genes are not always constant as they have been shown to be affected by the differentiation of the cell, or by certain chemicals or growth factors (Siebert and Fukuda, 1985, Elder, French, Subramaniam, *et al*, 1988). Therefore we felt it was important to choose a housekeeping gene to use as an internal reference that did not show any differences between strains. Both the data normalised to GAPDH and to HPRT expression showed the same trends, but we chose GAPDH as it is more commonly used, and the probe and primers were species specific.

These results provide further evidence that this automated quantitative PCR technique is of superior sensitivity to any other method. We were able not only to detect hepatic VLDL receptor mRNA, but also to find a significantly different expression of this gene in the two strains, in a tissue where previous investigators have sometimes failed to find any expression (Takahashi *et al*, 1992, Webb, Patel, Jones, *et al*, 1994). It was surprising to see such a striking 109% upregulation in VLDL receptor mRNA. However, whether this is translated into active receptor molecules is unknown. Our perfusion studies (Chapter 7) provided evidence that the major disorder present in the SMHL rabbits was an overproduction of apoB, with perhaps a secondary defect in catabolism as suggested by the identical triglyceride output, but increased plasma

triglyceride concentration in the SMHL rabbits. If the increased VLDL receptor mRNA in the liver were to be translated into active protein, with a resultant increase in catabolism of VLDL particles, this would have a confounding effect on the liver perfusion experiments. The increase in CETP mRNA seen in the SMHL rabbits provides additional evidence for the increase in CETP transfer activity seen in these rabbits (Chapter 3, section 3.3.8), and may be in response to the hyperlipidaemic state of these rabbits.

Whilst several models of lipoprotein assembly have been proposed (Borén, Wettesten, Rustaeus, *et al*, 1993, Hamilton, Wong, Cham, *et al*, 1998) they all support the widespread belief that apoB secretion is regulated post translationally in the RER of hepatocytes or enterocytes. Post translational control is believed to be exerted by lipid availability (Atzel and Wetterau, 1993, Dixon and Ginsberg, 1993, Thompson, Naoumova and Watts, 1996) and both cholesteryl ester (Fungwe, Cagen, Wilcox, *et al*, 1992, Sniderman and Cianflone, 1993) and triglyceride (Wu, Sakata, Lui, *et al*, 1994, Ginsberg, 1995) have been cited as the driving factor.

ApoB mRNA levels have been shown to remain constant during conditions that affect apoB production. Sparks, Zolfaghari, Sparks, *et al* (1992) showed no change in mRNA levels when apoB was decreased in experimental diabetes in rats; likewise Pullinger, North, Teng, *et al* (1989) and Moberly, Cole, Alpers, *et al* (1990) showed an increased apoB secretion due to increased delivery of FFA to HepG2 cells, but no change in mRNA levels. However, others have seen a change in apoB mRNA abundance. Burnett, Wilcox, Telford, *et al* (1997) reported a decrease in apoB mRNA in response to atorvastatin therapy, and Sparks *et al* (1992) showed an increase of apoB mRNA in the liver of diabetic rats treated with insulin. When HepG2 cells are treated with 25-OH cholesterol (Dashti 1992), small (< 2 fold) increases in apoB mRNA levels in HepG2 cells have been reported, and a decrease in extracellular amino acid concentration has been shown to have the same effect (Zhang, Sniderman, Kalant, *et al*, 1993). Despite these reports, apoB synthesis is generally believed to be in excess of that secreted, however an association between apoB mRNA abundance and apoB secretion has been reported (Selby and Yao, 1995).

In this study, apoB mRNA was significantly increased by 59% in the SMHL rabbits. There were significant positive correlations between plasma cholesterol or triglyceride concentrations and apoB: HPRT levels. Correlations with apoB: GAPDH levels were not so strong, however there was a significant positive correlation with plasma triglyceride (Figure 8.8.) That such a correlation exists is surprising given the number of steps involved between the synthesis of apoB and the balance between the output and catabolism of lipoproteins giving rise to the plasma cholesterol and triglyceride concentration. The increase in apoB mRNA levels in SMHL rabbits is consistent with the increased production of apoB protein seen in the perfusion studies (Chapter 7).

The Taqman™ procedure showed no difference in expression of LDL receptor mRNA between the NZW and SMHL rabbits. This confirms preliminary data (La Ville *et al*, 1987) which showed there was no difference in catabolism of LDL by the LDL receptor in these rabbits. There was no difference in the expression of MTP and PDI mRNA. MTP has previously been shown to be partially regulated at the level of transcription (Pease and Leiper, 1996). However, here there was no difference in MTP expression, although both MTP and PDI correlated strongly with apoB mRNA levels, suggesting that lipid availability is not the rate limiting step, and the upregulation of apoB mRNA is driving the apoB overproduction.

We found cholesterol 7 alpha-hydroxylase activity to be very variable. Some values were extremely large, but this was probably due to the fact that the rabbit sample used for the standard curve was a low expressor. There were no significant differences between NZW and SMHL rabbits in our study, but we would have preferred to repeat the experiment using a different standard curve. HMG CoA reductase and cholesterol 7 alpha-hydroxylase control the balance of cholesterol leaving the liver as lipoproteins or in bile, and arriving in the liver via lipoprotein receptors. Indeed, cholesterol 7 alpha-hydroxylase has a role in regulating LDL receptor activity (Dueland, Trawich, Nenseter, *et al*, 1992).

Therefore the automated quantitative PCR technique has proved to be a sensitive tool for the investigation of gene expression. It has provided us with novel ideas on the

regulation of gene expression, that perhaps have been missed in earlier studies due to the less sensitive techniques employed. One limitation of the technique is that due to its sensitivity, it is necessary to have the gene sequence for the correct species available, as even 1 or 2 bp difference can cause the reaction to fail.

These studies have provided good evidence for the role of transcriptional control of apoB, and should provide impetus for the more detailed study of the regulation of the apoB promoter region. Thus the major defect in the SMHL rabbits, an overproduction of apoB, is at least partially driven by an increase in apoB mRNA levels. Whether there is a common *cis* or *trans* acting factor in the SMHL rabbits that upregulates the transcription of apoB, CETP and VLDL receptor mRNA is speculative, but would explain the overproduction of all of these genes. Alternatively upregulation of apoB may have the subsequent effect of upregulating CETP and the VLDL receptor. We have also shown that MTP and PDI mRNAs are not different between the NZW and SMHL rabbits, suggesting that lipid delivery by the MTP-PDI complex is not rate limiting in the production of apoB containing particles.

Chapter 9. General discussion and future prospects

Hazel felt sure there was some natural explanation, though he had no idea what it could be exactly. 'Holly, are we much nearer to solving our problem?' he asked.

Richard Adams, Watership Down

The main aim of this thesis was to characterise the lipoprotein abnormalities present in SMHL rabbits, assess the suitability of this strain as an animal model for the human disorder FCH and investigate the genetic defect involved. At the outset these were ambitious objectives, but we have been able to make progress on each point - the thesis contains a detailed description of the dyslipidaemia present in the animals, additional evidence for the similarity between the hyperlipidaemic phenotype exhibited by the SMHL rabbits and FCH, and proposes 3 potential gene candidates which may contribute to the disorder.

9.1. Characterisation of the lipoprotein abnormality in the SMHL rabbits

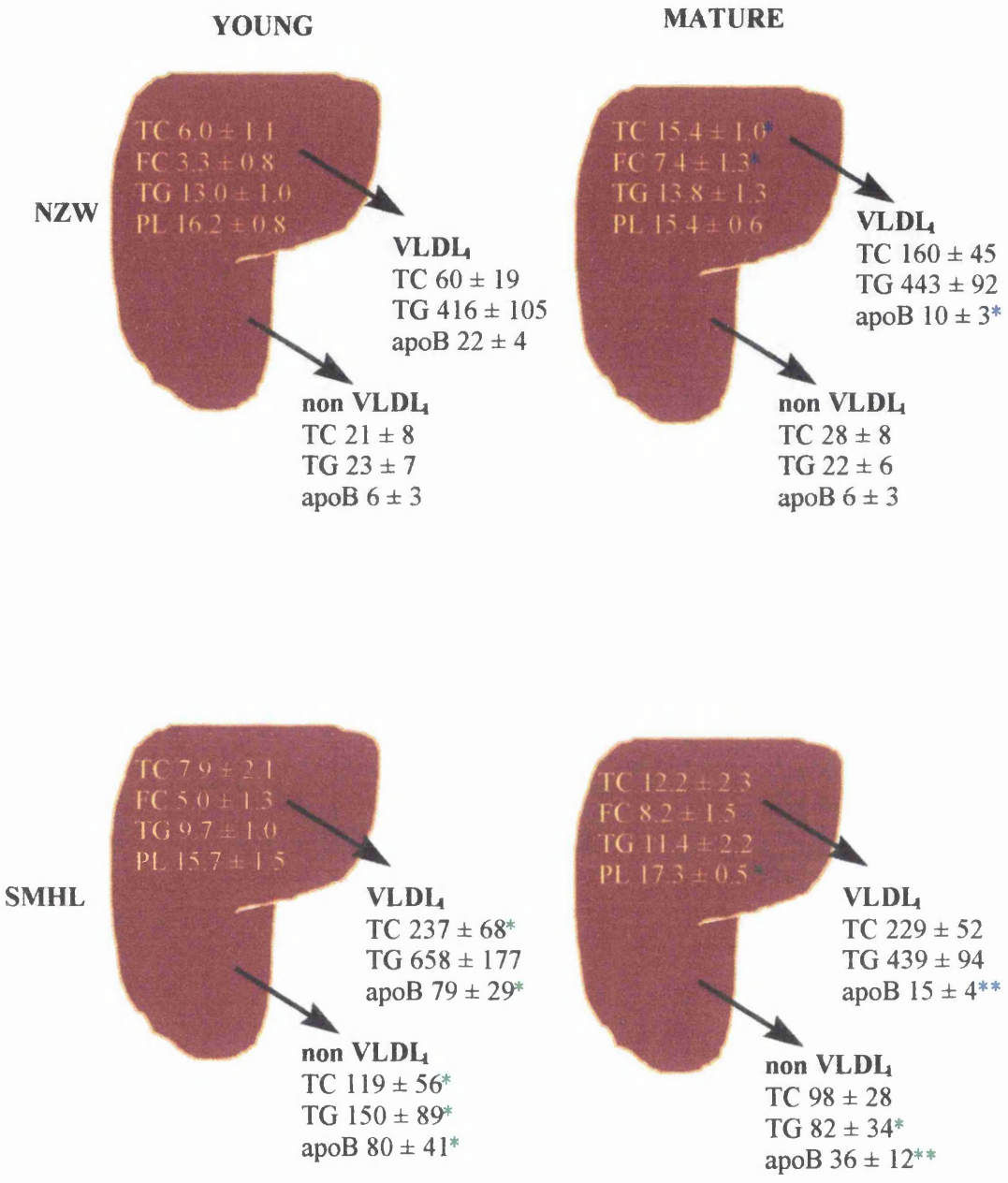
In her seminal paper, based on observations of plasma lipid and lipoprotein levels and radioactive kinetic studies, La Ville proposed that the disorder exhibited by the St Thomas hospital rabbits showed characteristics of FCH, in particular an overproduction of apoB containing lipoproteins (La Ville *et al*, 1987). To investigate the disorder present in the SMHL rabbits further, we developed a method for the perfusion of rabbit liver. We tested this method thoroughly, and found that for control animals, our results agreed with those previously published (Chapter 5). Therefore we performed a series of liver perfusion experiments to investigate the direct output of lipoproteins from the liver of both young and mature SMHL and NZW control rabbits (Chapters 6,7). Figure 9.1. describes the main findings of these experiments. In both young and old rabbits, apoB containing lipoproteins were overproduced by the SMHL rabbits. In particular, in young SMHL rabbits, $VLDL_1$ cholesterol and $VLDL_1$ apoB were overproduced (Chapter 7). In the non $VLDL_1$ fraction of the SMHL rabbits, output of cholesterol, triglyceride and apoB were all increased compared to NZW controls. These differences between the strains were not

driven by gross hepatic lipid concentration as there was little difference between hepatic lipid levels, and indeed, hepatic triglyceride levels were significantly decreased in the young SMHL rabbits compared to the young NZW rabbits. In the mature rabbits, there were fewer differences between the strains, partly due to large variability in the data (Chapter 6). There were no significant differences between cholesterol, triglyceride or apoB in the $VLDL_1$ fraction, but significant increases in output of triglyceride and apoB were seen again in the non $VLDL_1$ fraction of mature SMHL rabbits. Again there was no difference in hepatic stores of cholesterol or triglyceride in the mature NZW and SMHL rabbits. When all rabbits studied were grouped together there were significant positive correlations between plasma cholesterol and triglyceride concentrations and apoB output from the perfused livers, suggesting that the increased output of apoB may be driving the hyperlipidaemia seen in the SMHL rabbits.

When we looked at the effect of age within each strain, there were important differences in $VLDL_1$ apoB output, which was significantly decreased in both mature NZW and SMHL rabbits compared to their young counterparts. There was no accompanying decrease in $VLDL_1$ cholesterol or triglyceride outputs, implying that $VLDL_1$ particle size was larger in the mature rabbits compared to the young rabbits. Mature NZW rabbits also showed increased hepatic stores of total cholesterol and free cholesterol than young NZW, perhaps indicative of the cumulative effect of the cholesterol supplemented diet, while although mature SMHL rabbits showed trends towards increased hepatic cholesterol stores, these did not reach significance.

Thus in the SMHL rabbits there is clearly an increased output of apoB containing lipoprotein particles. In young rabbits this is evident in both the $VLDL_1$ and non $VLDL_1$ fractions, while in mature rabbits, this is seen only in the non $VLDL_1$ fraction. The $VLDL_1$ particles are apparently smaller, relatively lipid poor in young rabbits, and larger, more lipid rich in mature rabbits. Therefore there may be some regulatory mechanism which prevents the secretion of apoB particles before they are fully lipidated in mature rabbits but not in young rabbits.

Figure 9.1. Mean $VLDL_1$ and non $VLDL_1$ cholesterol, triglyceride and apoB outputs from the livers of young and mature NZW and SMHL rabbits



*Cholesterol, triglyceride and apoB output is measured in ng/g liver/minute, liver lipid content is measured as mg/g wet weight liver. * indicates significant differences between young and mature rabbits, $p < 0.05$, ** $p < 0.01$, * indicates significant differences between NZW and SMHL rabbits, $p < 0.05$, ** $p = 0.01$*

The overproduction of apoB in the SMHL rabbits corroborates the findings of La Ville *et al* from their radioiodinated kinetic studies. They demonstrated an overproduction of VLDL and LDL in the St Thomas' hospital rabbits. Their experiments also showed a decreased LDL FCR, which they attributed to saturation of receptor-mediated catabolism. The perfusion studies described in this thesis do not provide any information about catabolism, and indeed, as the catabolic state is unknown, this must be borne in mind when interpreting the results. Our own stable isotope kinetic studies, while they did not show any significant differences in kinetic parameters, showed trends towards impaired catabolism from IDL and LDL, as demonstrated by La Ville (Chapter 4), providing a stimulus for further investigation.

9.2. The SMHL rabbits as a model for FCH

As yet there are no generally accepted animal models for FCH. The literature describes a strain of swine which exhibits a 'marked resemblance' to FCH, but recently little has been heard of this model (Hasler-Rapacz, Nichols, Griggs *et al*, 1994). Based on the results of the wide variety of experiments described in this thesis, the SMHL rabbit continues to demonstrate characteristics analagous to those of the human disorder FCH (Table 9.1.). FCH was first described in 1973 as a disorder where 'affected family members characteristically had elevated levels of both cholesterol and triglyceride. However, increased cholesterol or increased triglyceride levels alone were also frequently observed' (Goldstein *et al*, 1973). This phenotype was evident in our SMHL rabbit population (Chapter 3), with the majority of animals expressing hypercholesterolaemia and hypertriglyceridaemia. The hypercholesterolaemia in these animals has previously been shown not to be associated with inactive LDL receptors (La Ville *et al*, 1987).

Table 9.1. Is the SMHL rabbit an appropriate model for FCH?

Phenotype	FCH	SMHL rabbits
Multiple lipid phenotype	↑ TC ↑ TG ↑ TC and TG (Goldstein *et al*, 1973)	↑ TC ↑ TG ↑ TC and TG
ApoB production	↑ in VLDL and/or LDL (Janus *et al*, 1980, Venkatesan *et al*, 1993)	↑ in $VLDL_1$ and/or non $VLDL_1$
ApoB catabolism	↓ in a subset (Aguilar Salinas *et al*, 1997)	Tendency to undercatabolism seen in kinetic studies
Lipoprotein size	↓ (Brunzell *et al*, 1983)	↓ (not significant)
Insulin resistance	Present in hypertriglyceridaemic individuals (Castro Cabezas *et al*, 1993)	Hyperinsulinaemic response to an oral glucose load
CETP levels	↑ concentration (McPherson *et al*, 1991)	↑ activity and mRNA
LPL levels	↓ in a subset (Babirak *et al*, 1992)	=

Kinetic studies have demonstrated that the common feature of FCH is an overproduction of apoB from the liver, in either the VLDL or LDL density classes or both, and this is what contributes to the multiple phenotypes seen (Janus *et al*, 1980, Venkatesan *et al*, 1993). Our own stable isotope kinetic experiments did not demonstrate any significant differences in apoB PR, however for these experiments, the SMHL rabbit group were not hyperlipidaemic compared to the control NZW rabbits (Chapter 4). The two SMHL rabbits that were relatively hyperlipidaemic did

exhibit greater PRs than the rest of that group or the NZW rabbits, suggesting an association between an increased $VLDL_1$ apoB PR and hyperlipidaemia. This association is strengthened by the findings of La Ville *et al* (1987) who demonstrated an increased VLDL PR in their group of St Thomas' hospital rabbits. The animals that she studied were more severely hyperlipidaemic than any of our SMHL rabbit groups. However, we established directly the major defect in the SMHL rabbits as an apoB overproduction by the perfusion experiments. ApoB output was significantly increased in young hyperlipidaemic SMHL rabbits in both $VLDL_1$ and non $VLDL_1$. In mature hyperlipidaemic SMHL rabbits only plasma cholesterol levels were significantly increased compared to NZW rabbits, and importantly apoB output was significantly increased in the non $VLDL_1$ fraction only.

An FCH kindred has been described that exhibits an impaired apoB catabolism (Aguilar-Salinas *et al*, 1997), but we have not demonstrated this in the SMHL rabbits. Our kinetic data showed a tendency towards decreased FDC, FTR and FCRs in the SMHL rabbits, although this may be in addition to an increased PR. Had we performed these kinetic studies on a larger number of more hyperlipidaemic SMHL rabbits, we may well have found significant differences.

Relatively small lipoprotein particles have been described as characteristic of FCH (Brunzell *et al*, 1983, Franceschini, Cassinotti, Vecchio, *et al*, 1994, Hokanson *et al*, 1995). When we calculated the ratio of cholesterol + triglyceride (in grams) to apoB (in grams) in our studies, we found that in the SMHL rabbits there was a decreased amount of lipid associated with each apoB molecule (i.e. each particle) as compared to the NZW rabbits, although this did not reach significance.

Insulin resistance has been associated with FCH (Hunt, Wu, Hopkins *et al*, 1989). Relatives of index patients with FCH with elevated triglyceride levels had increased fasting insulin concentrations and elevated FFA concentrations compared to normolipidaemic relatives (Castro Cabezas *et al*, 1993). Additionally, hypertriglyceridaemic FCH individuals demonstrated an impaired postprandial FFA metabolism, and the resultant increase in plasma FFA levels may be responsible for the decreased lipolytic activity in FCH. Whether elevated insulin levels cause the increase

in FFA or vice versa is unclear. Further confirming their role as an animal model for FCH, the SMHL rabbits showed exaggerated insulin and FFA levels in response to an oral glucose load when compared to NZW rabbits, however the lack of a correlation between FFA levels and plasma triglyceride levels may indicate that the insulin resistance is not the driving force in the lipoprotein overproduction seen in these rabbits.

Insulin resistance can also result in decreased LPL activity and therefore a reduced clearance of VLDL. Insulin resistance is not necessarily present in each individual with FCH, or in each SMHL rabbit. Millar, Watson, Stewart *et al* (unpublished observations) showed two sub-groups of mixed hyperlipidaemic individuals with different $VLDL_1$ and $VLDL_2$ triglyceride kinetics. One group had increased $VLDL_1$ PR similar to diabetic individuals, while the second group had normal PR but decreased FCR. A potential mechanism for the difference in these individuals is the presence of insulin resistance in the diabetic-like group, but not in the other group of mixed hyperlipidaemics. The rabbit groups which we studied were too small to look at this association, but it is a potential explanation for the variablility both in kinetic parameters and insulin response to the oral glucose dose.

Increased CETP levels are characteristic of combined hyperlipidaemia (McPherson, Mann, Tall, *et al*, 1991). In combined hyperlipidaemics, cholesteryl ester transfer from HDL to VLDL is upregulated compared to normals due to an increased level of VLDL (acceptor particle), and a decreased affinity of LDL for cholesteryl ester mass transfer. (Guérin, Bruckert, Dolphin, *et al*, 1996). We demonstrated a significant increase in plasma CETP activity in SMHL rabbits (Chapter 3, Table 3.5.) compared to NZW controls, but did not perform any investigation of cholesteryl ester mass transfer. It is possible that the increased CETP activity in the SMHL rabbits was due to the presence of an increased amount of acceptor particles (VLDL) although this would not explain the mRNA results.

A subset of FCH individuals exhibit decreased LPL levels (Babirak *et al*, 1992). However severe defects in LPL have not been found, and it has been suggested that this decrease in LPL levels may merely magnify the phenotype, rather than being a

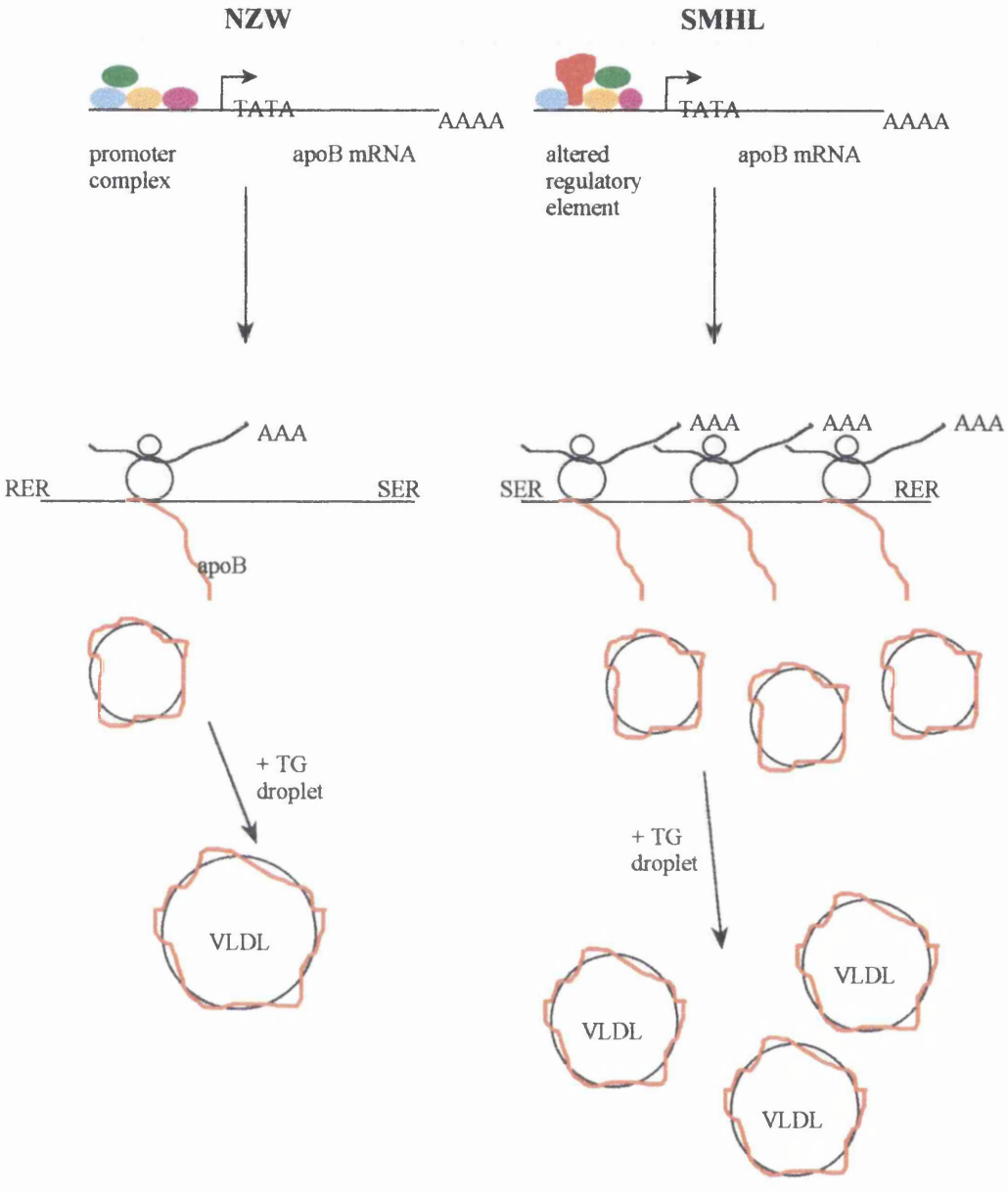
primary cause. We did not find any significant difference between LPL levels in SMHL and NZW rabbits.

Therefore in conclusion, as can be seen from Table 9.1., the SMHL rabbits exhibit characteristics entirely consistent with human FCH, and based on the results contained within this thesis, can be recommended as an animal model for the disease.

9.3. Genetic defects within the SMHL rabbits

The genetic basis of FCH is as yet unclear, and has been the subject of much speculation. FCH was first described in 1973 as a disorder with a major gene effect on plasma triglyceride levels, and a secondary effect on plasma cholesterol levels. Since then apoB has been the principal gene candidate, however no known mutations have been found to co-segregate with FCH. LPL and the apoAI-CIII-AIV gene cluster have also been implicated, but no consistent association has been found (Wijsman, Motulsky, Guo, *et al*, 1992, Gagné, Genest, Zhang, *et al*, 1994, Yang, Nevin, Peng, *et al*, 1995, Dallinga-Thie, Bu, van Linde-Sibenius Trip, *et al*, 1996). Therefore our data describing a significant increase in the levels of mRNA for the VLDL receptor, CETP and apoB in the SMHL rabbits are exciting new targets for study. We also investigated the mRNA levels of the LDL receptor, MTP, PDI and cholesterol 7 alpha-hydroxylase, but there was no difference in expression of these between NZW and SMHL rabbits. We therefore propose that there is either differential expression of a common regulatory element, similar to the sterol response element binding protein (Wang, Du, Martin, *et al*, 1997), or a mutation in the promoter region of the apoB, CETP and/or VLDL receptor genes in the SMHL rabbits that causes binding of an additional promoter, or prevents binding of a repressor protein, and therefore upregulates transcription of these genes. It is possible that the major gene effect is on the apoB gene, and that the upregulation of the VLDL receptor and CETP is an attempt to clear the increased amount of apoB containing lipoproteins in the circulation. However it must be borne in mind that there is no guarantee that an increase in mRNA means an increase in active protein.

Figure 9.2. Secretion of lipoprotein particles from NZW and SMHL rabbits



Based on the evidence contained within this thesis, it is possible that the defect in the SMHL rabbits that causes the FCH phenotype is the presence (or absence) of an additional regulatory element that causes the upregulation of apoB transcription. This same element may upregulate CETP and VLDL receptor transcription, or the presence of an increased amount of apoB may upregulate these genes.

We hypothesise that apoB is transcribed and translated at an increased rate in the SMHL rabbits. This results in the initiation of a greater number of lipoprotein particles in the RER (see Figure 1.2. for normal synthesis and secretion of lipoprotein particles). Each new particle has to associate with a minimum amount of lipid to allow the apoB to take on a viable formation, and this new lipoprotein particle is then secreted (Figure 9.2.). The increased number of particle initiations in SMHL rabbits leads to secretion of a greater number of smaller particles. This would explain the increased output of apoB seen in the perfusion studies even when lipid output was not significantly changed (as in mature rabbits). Indeed, there are significant positive correlations seen between cholesterol and triglyceride concentrations and output rates of apoB from the perfused livers, and between triglyceride concentrations and apoB mRNA levels (Figure 8.8.) in the TaqMan studies. It is interesting to speculate that there could be a correlation between apoB mRNA levels and apoB output rate from the liver, however these experiments would need to be performed in the same rabbit group.

9.4. Future prospects

Further characterisation of the SMHL rabbits is necessary. It would be interesting to investigate the effect of the 0.08% cholesterol diet on the mRNA levels of apoB, CETP and the VLDL receptor, to see if their upregulation is in response to the increased cholesterol, or if it is constitutive. Detailed investigation of the promoter region of the apoB gene in the SMHL rabbit may provide some information on the reason for the upregulation of the gene, either by looking at the DNA sequence or by identifying transcription factors by gel-shift (or other) assays. Culture of hepatic cells would allow the manipulation of the cells for example by drug (statin) treatment, or by stimulating VLDL synthesis by loading with FFA. An important question is whether the upregulation of apoB output in the whole SMHL rabbit is limited to apoB100, or whether apoB48 is also upregulated; mRNA levels in the intestine could be studied to investigate this. It would be of interest to find out if other genes are upregulated like apoB, CETP and VLDL receptor, for example ACAT, LCAT or HMG CoA reductase. Further studies to investigate the catabolism of apoB

containing lipoproteins would be of interest and would aid the interpretation of the perfusion results.

9.5. Conclusions

In conclusion, the SMHL rabbit is currently the most likely animal model for FCH. We have found no major inconsistencies with the phenotype reported in humans, and envisage that in the near future, this strain of rabbit will be widely used to provide more information on, and test new drugs for, FCH.

Appendix 1. Manufacturers addresses

Altec
Unit 4, Riverwey Industrial Park
ALTON, GU34 2QG
UK

Amersham International plc
AMERSHAM
UK

Beckman Instruments (UK) Ltd
Analytical Sales and Service Operation
Progress Road
HIGH WYCOMBE, HP12 4JL

BDH Laboratory Supplies
McQuilkin and Co
21 Polmadie Avenue
GLASGOW, G5 0BB

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

Boehringer Mannheim UK (Diagnostics and Biochemicals) Ltd
Bell Lane
LEWES, BN7 1LG

Chromacol Ltd
3 Little Mundells
WELWYN GARDEN CITY, AL7 1EW

Cruachem Ltd.
GLASGOW

DNASTAR Inc.
MADISON
Wisconsin, USA

Ethicon Ltd.
EDINBURGH
UK

Fisons Instruments
Crewe Road
Wythenshaw
MANCHESTER, M23 9BE

Froxfield Farm UK Ltd
Unit 3, King Lane
FROXFIELD, Nr PETERSFIELD, GU32 1DR

Gibco BRL
Life Technologies Ltd.
3 Fountain Drive, Inchinnan Business Park
PAISLEY, PA4 9RF
UK

Harlan Interfauna Ltd.
Abbots Ripton Road
Wyton
HUNTINGDON, PE17 2DT

V. A. Howe & Co. Ltd.
Beaumont Close
BANBURY, OX16 7RG

Isotec Inc
A Matheson USA Company
3858 Benner Road
MIAMISBURG
OH 45342, USA

Janke & Kunkel
STAUFEN IM BREISGAU
Germany

Jenway UK,
DUNMOW, UK

Leo Laboratories Ltd.
PRINCES RISBOROUGH, UK

Linco Research, Inc.
14 Research Park Drive
ST CHARLES
MO 63304, USA

Minitab Inc.
3081 Enterprise Drive,
STATE COLLEGE
PA 16801-3008, USA

OsteoTec Limited
9 Silver Business Park,
Airfield Way
CHRISTCHURCH, BH23 3TA, UK

Paar Scientific Ltd.
594 Kingston Road
Raynes Park
LONDON, SW20 8DN

Perkin Elmer Applied Biosystems
FOSTER CITY
CA 94404, USA

Perkin Elmer Applied Biosystems UK
WARRINGTON, UK

Pharmacia Biotech Ltd.
23 Grosvenor Road
ST ALBANS
AL1 3AW

Portex Ltd.
HYTHE, UK

Promega
MADISON
W1, USA

Quiagen
CRAWLEY, West Sussex

Randox Laboratories Ltd.
CRUMLIN, Co. Antrim, UK

Rhône Mérieux Ltd.
HARLOW, UK

Roar Biomedical, Inc.
2840 Broadway, M/S 271
NEW YORK, NY 10025, USA

SAAM Institute
University of Washington
SEATTLE, WA 98195, USA

Scottish Antibody Production Unit
Law Hospital
CARLUKE, ML8 5ES

Sigma Chemical Company
Fancy Road
POOLE, BH17 7TG

Special Diet Service
WITHAM, UK

Techne
Scotlab
Kirkshaw Road
COATBRIDGE, ML5 8AD

Technicon
MIT Service Inc
10065 Mesa Ridge Court
SAN DIEGO
California 92121, USA

Vygon UK Ltd.
CIRENCESTER, UK

Wako Chamicals GmbH
Nissanstraße 2
D-41468, NEUSS
Germany

Watson Marlow Ltd.
FALMOUTH
Cornwall, TR11 4RU, UK

Whatman International Ltd.
St. Leonards Road, 20/20
MAIDSTONE, ME16 0LS

Appendix 2. TaqMan primer and probe sequences

Rabbit GAPDH (base pairs 1051 - 1130)

TGGTCCACAT GGCCTCCAAG GAGTAAGAGC CCTCAAACCA
CCGGCCCCAG CGAGAGCACC AGAGGAGGAC GAGAGGCCCT

Rabbit beta actin (base pairs 1 - 70)

GTCCTTCCTG GGCATGGAGT CCTGCGGCAT CCACGAGACC
ACCTTCAACT CGATCATGAA GTGCGACGTG

Human HPRT (base pairs 581 - 700)

AAAAGGACCC CACGAAGTGT TGGATATAAG CCAGACTTTG
TTGGATTTGA AATTCCAGAC AAGTTTGTTG TAGGATATGC
CCTTGACTAT AATGAATACT TCAGGGATTT GAATCATGTT

Rabbit apoB (base pairs 201 - 280)

CAGTGCAACA ACCAGCTTGA GGTACAGTCC CCTGATGCTG
GAGAATGAGC TGAACGCAGA GCTTGCCCTT TCTGGGGCAT

Rabbit VLDL receptor (base pairs 2531 - 2610)

CTTGAAGACC ACGGAAGAGG ACCTCTCCAT TGACATCGGC
AGACACAGTG CTTCTGTTGG ACACACGTAC CCAGCAATAT

Rabbit LDL receptor (base pairs 2371 - 2450)

GGGCGTGCAT GGCGTTTTAG CTTTGCACCT CGCAAGCCGC
GCGAGTCTGT GACGACATTT GCACTTTGTG CGTTTCTGGA

Rabbit CETP (base pairs 1311 - 1390)

GGGCATCCCG GAGGTCATGT CTCGGCTCGA GGTGGCGTTC
ACAGCCCTCA TGAACAGCAA AGGCCTGGAC CTCTTCGAAA

Rabbit MTP (base pairs 1 - 80)

ATGCCGGCTG TACAAGCTCA CCTACGCCAC TGAAGTCTTT
GTTGATCGAA GCAAAGGAAA ACTCCAAGAC AGTGTGGGCT

Rabbit PDI (base pairs 1281 - 1360)

GCAAGCAGCT GGCTCCCATC TGGGACAAGC TGGGCGAGAC
GTACAAGGAG CACCAGGACA TCGTCATCGC CAAGATGGAC

Rabbit 7 alpha-hydroxylase (base pairs 341 - 410)

GCTAGCTGAG GGCTTGAAGC ATGACAACCT CCGAACCAGG
GACCACATCT CAGAACTGAT CCGCCTGCGC ATGTTTCTGA

Sequences in red are the primers, sequences in blue are the probes.

Appendix 3. TaqMan gene quantification

Ratios of abundance of mRNA of genes of interest to GAPDH. NZW rabbits.

Rabbit	ApoB	VLDLR	LDL-R	CETP	MTP	PDI	7-AH
1	0.69	0.57	0.51	0.52	0.43	0.81	2.4
2	0.55	0.86	2.0		0.72	1.4	2.5
3	0.70	0.54	0.60	0.94	0.55	1.0	5.3
4	0.60	1.2	1.2	0.98	0.72	1.6	7.2
5	0.61	0.64	2.3	1.63	0.82	1.5	1.1
6	0.59	0.22	1.2	2.3	0.45	0.55	4.7
7	0.85	0.53	1.8	0.90	0.32	0.45	5.8
8	0.90	0.88	3.7	2.6	0.97	0.87	33.7
9	1.1	0.39	3.6	1.8	1.1	1.4	26.1
10	1.1	0.84	1.3	3.5	0.86	1.1	16.3
11	1.3	0.67	1.9	1.1	0.76	0.98	40.8
Mean ± SEM	0.82 ± 0.08	0.67 ± 0.08	1.8 ± 0.32	1.62 ± 0.3	0.71 ± 0.08	1.1 ± 0.11	14.0 ± 4.2

Ratios of abundance of mRNA of genes of interest to GAPDH. SMHL rabbits.

	ApoB	VLDLR	LDL-R	CETP	MTP	PDI	7-AH
1	1.7	0.85	1.7	3.9	0.95	2.5	3.9
2	0.53	4.8	0.50	3.6	0.23	0.60	1.6
3	1.3	0.90	2.3	4.7	0.84	2.2	0.64
4	1.4	2.5	1.1	5.5	0.72	1.7	5.0
5	0.73	2.1	0.47	2.4	0.33	0.94	4.9
6	1.6	1.4	2.7	2.1	1.4	1.8	16.2
7	2.0	0.90	3.2	3.8	1.2	1.7	19.5
8	3.1	1.4	7.0	1.4	2.2	3.1	12.9
9	1.8	1.5	2.8	3.8	0.88	1.6	3.6
10				1.6			
11	1.4	0.80	3.5	4.4	0.88	1.2	2.5
12	0.93	0.97	1.8	4.4	0.65	0.96	17.1
13	0.81	0.59	1.4	4.4	0.58	0.58	16.7
14	0.95	1.4	1.4	1.4	0.84	0.84	3.3
15	0.85	0.70	1.5	2.4	0.66	0.79	20.6
16	0.97	0.40	2.0	4.0	0.88	0.99	6.4
17	1.2	0.93	2.2	0.77	0.87	1.5	36.8
Mean ± SEM	1.3 ± 0.16	1.4 ± 0.27	2.2 ± 0.39	3.2 ± 0.34	0.88 ± 0.11	1.4 ± 0.18	10.7 ± 2.5
p value	0.005	0.02	NS	0.002	NS	NS	NS

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ANIMAL MODELS

1.P.92 Effect of hypercholesterolemic diet and D2 vitamin on the atherosclerosis development in obese and prediabetic Sand Rats (Psammomys obesus)

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The Sand Rat (P.O) is known as a good model to study obesity and diabetes abnormalities, when submitted to hypercaloric diet instead of hypocaloric and halophiles plants usually eaten in the biotope. Limited studies have focused on the fundamental role of these disorders in the induction of lipid metabolic troubles, and of atherosclerosic lesions in these species which are a good model for vascular pathology studies, despite their athero-resistance. We have experimentally reproduced atherogenic lesions in 32 Sand Rats fed, during two months, with hypercholesterolemic diet enriched with cholesterol, some of these animals receiving orally high proportions of D2 vitamin. During the treatment the early development of obesity and prediabetes (DNID), was confirmed by glucose moderated intolerance, and hypercholesterolemia, hypertriglyceridemia, with a huge increase of LDLC without any change of HDLC. Histological analysis undertaken before and after two months of treatment showed hypertrophy and hyperplasy of pancreatic Langerhans islets which proved the diabetic state of these animals, but without skin microangiopathy or lipidic liver deposits. Regarding the macroangiopathy, only arterial preatheromateous lesions but no heart lesions were observed. The addition of a high oral dose of D2 vitamin, produced more important atherosclerotic lesions at various stages: lipidic, calcified, or ulcerus in aortic, coronary and renal arteries with consequentially ischemic effects represented by myocardial infarctions.

We conclude that Sand Rat living in Morocco, could be selected as model for the study of vascular pathology which could allow therapeutic assays for new antiatherogenic agents.

1.P.93 Effects on plasma lipids levels in cholesterol feeding male/female heterozygous KHC rabbits

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High serum cholesterol is a major risk factor for coronary heart diseases. For a comparison between male and female rabbits; we observed the responsive to dietary hypercholesterolemia as regards plasma lipids levels in KHC rabbits, which is LDL receptor deficiency.

Male and female Japanese White (JW) or heterozygous KHC rabbits were given a standard milled rabbit diet or containing 0.1% cholesterol (CH). All male and female JW and KHC rabbits showed no increase in their plasma CH levels after being fed a standard milled rabbit diet for 24 weeks. The plasma CH levels in male JW rabbits did not increased, but for heterozygous KHC rabbits the transitorily increased for 4 to 8 weeks (about 300 mg/dl) and gradually decreased after being subjected to the CH diet. On the other hand, as regards female JW rabbits, the plasma CH levels were increased for 4 to 24 weeks (about 300 mg/dl) and in heterozygous KHC rabbits gradually increased (about 700 mg/dl after 24 weeks) after being fed the CH diet. Plasma phospholipid levels tend to increase the CH levels in each experiment and plasma triglyceride levels were not changed in any of the experiments.

These results indicate that female rabbits were high responders for the cholesterol diet than the male rabbits and the responsiveness to dietary hypercholesterolemia was predominated as regards the genetic factor.

1.P.94 Characterisation of lipoprotein metabolism in the Froxfield mixed hyperlipidaemic rabbit

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The Froxfield mixed Hyperlipidaemic rabbit (FMHL) colony was derived from the St Thomas rabbit. We investigated the nature of the defect in these rabbits who exhibit a lipoprotein phenotype similar to Familial Combined Hyperlipidaemia. Ten FMHL and 10 control New Zealand White (NZW) rabbits were fed a low cholesterol (0.085% w/w) diet. Median plasma cholesterol was significantly higher in FMHL than NZW (108 vs 54 mg/dl; $p < 0.01$). Median plasma triglyceride (TG) showed no significant differences between groups, but was elevated and highly variable in FMHL (range FMHL 31–403, NZW 31–62 mg/dl). Lipoprotein subfractions, very low density lipoprotein 1 (VLDL1) Sf 60–400, VLDL2 Sf 20–60, intermediate density lipoprotein (IDL) Sf 12–20 and low density lipoprotein (LDL) Sf 0–12, were separated by density gradient ultracentrifugation. Median VLDL1, VLDL2, IDL and LDL masses were significantly higher in FMHL than NZW (33.2 vs 12.5, 71.7 vs 17.9, 82.3 vs 25.2, 36.6 vs 10.8 mg/dl; all $p < 0.01$). Apolipoprotein (apo) B kinetics were investigated by injecting tri-deuterated (d_3) leucine (10 mg/kg) into 4 rabbits from each group and the enrichment of d_3 leucine in apoB from VLDL1, VLDL2, IDL and LDL was followed by gas chromatography-mass spectrometry. Data was fitted to a multicompartmental model. Preliminary analysis shows a positive correlation between TG levels and VLDL1-apoB synthesis ($r = 0.92$; $p < 0.005$). FMHL, even those without raised VLDL1-apoB synthesis rates, have a higher VLDL1 TG:apoB ratio (18.1 vs 12.2). FMHL have elevated VLDL2 masses due to lower fractional catabolic rates (FCR) than NZW (14.6 vs 33.8 pools/day). Cholesterol levels showed an inverse correlation with IDL- and LDL-apoB FCR ($r = 0.83$, $p = 0.01$; $r = 0.72$, $p < 0.05$). We conclude that in FMHL hypertriglyceridaemia is due to increased VLDL1-apoB synthesis and hypercholesterolaemia is a consequence of decreased LDL-apoB FCR.

1.P.95 Effects of Δ5-olefinic acids from *Pinus pinaster* seed oil on lipoprotein metabolism in apoE deficient mice

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The aim of the present study was to assess the lipid-lowering potential of oil extracted from *Pinus pinaster* seeds. This oil contains Δs-olefinic fatty acids: all *cis*--5,9,12–18:3 and all *cis*-5,11,14–20:3 acids. The dose response effect of Δs-olefinic oil supplementation at 0%, 5% and 10% wt/wt on lipid and lipoprotein levels was evaluated on apoE KO mice. Diets were completed to 10% wt/wt of fat with lard. Compared to control (10% wt/wt of lard), the 5% and 10% *P. pinaster* regimen were associated to lower levels of total cholesterol (C) (−25%, $p < 0.02$; −38%, $p < 0.001$ respectively), phospholipids (−17%, $p < 0.05$; −28%, $p < 0.01$) and VLDL-C (–26%, $p < 0.05$; −38%, $p < 0.01$). In contrast, total triglycerides (TG) and VLDL-TG increased in a dose dependent manner in the *P. pinaster* supplemented mice resulting in a significant decrease in the VLDL-C to VLDL-TG ratio ($p < 0.0001$). This suggests that compared to lard *P. pinaster* oil decrease VLDL remnant levels in apoE KO mice. Gel filtration chromatography confirmed these results. Finally, apo AI and apo AII concentrations were not modified by *P. pinaster* regimen. In conclusion, we have demonstrated that hypercholesterolemia can be reduced by Δ5-olefinic oil supplementation in apo E deficient mice.

1.P.96 Plasma lipids and atherosclerosis in Froxfield mixed hyperlipidaemic (previously St Thomas Hospital) rabbits

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Froxfield mixed hyperlipidaemic (FMHL) rabbits are derived from the remaining colonies of St Thomas Hospital rabbits[1]. The present study was designed to characterise the changes in plasma lipids and atherosclerosis in FMHL rabbits that were fed a mildly atherogenic diet until they were 6 months old.

Nine week old male and female FMHL rabbits (n = 12/sex) were selected from larger groups on the basis of their elevated plasma cholesterol (>3.6 mM) and triglyceride (>2.6 mM) concentrations after having been fed a standard rabbit diet supplemented with 0.075% w/w cholesterol (0.08% total cholesterol) and 2% w/w corn oil from weaning. The rabbits continued to receive the diet until they were 6 months old. Plasma cholesterol and triglyceride concentrations were measured throughout this period. At the end of the study the rabbits were killed and their hearts and aortas were perfuse-fixed *in situ*. The aortas were removed, cleaned of extravascular fat and stained with sudan IV. The percentage area of atherosclerotic plaque was measured *en face* using colour video image analysis.

Plasma cholesterol and triglyceride concentrations were similar for nine week old male and female rabbits (7.3 vs 8.8 mM and 4.5 vs 4.3 mM respectively). However, at 6 months of age plasma cholesterol in female FMHL rabbits had risen to 22 ± 8 mM whilst in the males it had fallen to 2.5 ± 2.2 mM. Plasma triglyceride concentrations fell to approximately 2.7 mM in both

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Apolipoprotein B is overproduced in the froxfield mixed hyperlipidaemic rabbit

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The Froxfield Mixed Hyperlipidaemic rabbit (FMHL) is a putative model of familial combined hyperlipidaemia (FCHL). To investigate the pathogenesis of the disease exhibited by these animals, we studied apolipoprotein (apo) B secretion in perfused livers. Four FMHL (>l year old) and 5 age matched New Zealand White (NZW) fed a 0.075% cholesterol diet were euthanised (Euthatal), and their livers were perfused for 3 hours with recirculating oxygenated Krebs-Henseleit buffer, pH 7.4, maintained at 37°C. Samples were taken at time 0, and every 30 minutes throughout the experiment. Cholesterol, triglyceride (TG), glycerol, urea, albumin, AST and GGT were measured at each time point. Bile flow was recorded. Very low density lipoprotein (VLDL) 1 (Sf 60-200), VLDL2 (Sf 20–60), intermediate density lipoprotein (IDL) (Sf 12–20) and low density lipoprotein (LDL) (Sf 0–12) were separated by density gradient ultracentrifugation from the 0, 1, 2 and 3 hour samples. Cholesterol, TG and apoB were measured in each subfraction, and results were calculated for VLDL1 and VLDL2+IDL+LDL. Liver function tests indicated that the livers were normal. Total TG production rates (mean ± SE) were of the same magnitude in both groups (43±20 vs 40±15 mg/liver/min). FMHL rabbits had a moderately raised apoB production rate compared to NZW (4.1±0.8 vs 2.2±0.9 μg/liver/min, p=0.15). VLDL1apoB production was higher in FMHL than NZW (2.6±0.5 vs 1.2±0.4 μg/liver/min, p=0.05), whereas VLDL2+IDL+LDL apoB production was similar in both groups (1.5±0.6 vs 0.9±0.5 μg/liver/min, NS). Young NZW (3–4 months old) fed standard chow were studied in a similar manner. Older rabbits produced equal amounts of apoB as VLDL1 and VLDL2+IDL+LDL, whereas young NZW produced 85% of their apoB as VLDL1 . In conclusion, as in FCHL, FMHL constitutively overproduce apoB containing lipoproteins, particularly in the VLDL1 density range.

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Hypertriglyceridemia in transgenic mice overexpressing human APO A-II is due to defective catabolism by lipoprotein lipase

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The metabolic role of apolipoprotein (apo) A-II, the second major apolipoprotein of HDL, was studied in transgenic mice expressing human apo A-II at 2 times, (line hAIItgδ) or 4 times (line hAIItgλ) the normal concentration. Hemizygous mice fed chow presented postprandially an apparent chylomicronemia, with plasma triglyceride (TG) concentrations ranging from 746 (line δ) to 1524 mg/dl (line λ), compared with 50 mg/dl in control C57BL/6 mice. Plasma TG returned to normal after an overnight fast in hAIItgδ mice but remained higher in hAIItgλ mice. Plasma HDL decreased 2–3-fold in both transgenic mice, and were negatively correlated with apo A-II expression. The postprandial accumulation of large VLDL suggested impaired TG hydrolysis by lipoprotein lipase (LPL), the first enzyme to act on VLDL upon secretion. Postheparin LPL activity was decreased by only 28%, while hepatic lipase (HL) activity decreased by 20% in the higher-expressing hAIItλ line. Adipose tissue and muscle LPL activities were unaffected in transgenic mice, indicating that LPL was normally produced in both tissues. On the contrary, VLDL-TG hydrolysis by commercial LPL proceeded at much slower rates in transgenic compared with control mice, the V_{max} decreasing from 96 nmol free fatty acids (FFA) / min in control VLDL to 46 and 23 nmol FFA/min in hAIItg δ and λ VLDL, respectively. Unexpectedly appreciable amounts of human dimeric apo A-II were present in all apo Bcontaining lipoproteins of transgenic mice, whereas normally apo A-II is absent from VLDL. Our transgenic mice represent a model of postprandial hypertriglyceridemia characterized by accumulation of TG-rich lipoproteins — due to defective catabolism by LPL — and concomitantly decreased HDL, thus resembling the proatherogenic profile of familial combined hypertriglyceridemia.

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Fasting and postpradial lipids in subjects with familial combined hyperlipidemi treated with HMG-CoA reductase inhibitors and combination therapy with fibrat

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Patients with Familial Combined Hyperlipidemia (FCH) are characterized by overpr duction of hepatic apolipoprotein (apo) B and impaired postprandial lipemia. Treatme with HMGCoA-reductase inhibitors is usually not sufficient to normalize fasting plasm lipids. The effect of monotherapy with reductase inhibitors on fasting plasma lipids a postprandial lipemia was evaluated in 7 male, FCH subjects by standardized oral retin palmitate (RP)-fat loading tests. Fasting plasma triglycerides (5.6±1.3 mM, mean±SEM cholesterol (8.1±0.8 mM) and apo B (1.6 g/L) concentrations improved significantly aft therapy with Simvastatin (3.9±1.2 mM; 6.8±0.8 mM, and 1.3 g/L, respectively), howe er fasting HDL-C concentrations (0.60±0.1 mM) did not change significantly (0.63±0 mM). The clearance of chylomicrons (Sf>1000; chylo) and chylo remnants (Sf<100 was assessed by the areas under the respective RP curves. Single-drug therapy wi Simvastatin resulted in improved remnant clearance (24.4±4.1 versus 62.9±11.1 h.mg/l P<0.05) without changes in chylomicron clearance (52.8±12.9 versus 51.8±13.4 h.mg/L In 3 FCH subjects combination therapy with gemfibrozil resulted in more pronounce improvements in fasting plasma triglycerides (64% reduction) and apo B (23% reductio concentrations and significant improvement of chylomicron clearance (chyloRP-AU 30.2±1.8 versus 63.4±29.4 h.mg/L; 41% improvement) and remnant clearan (remnantRP-AUC 39.8±11.3 versus 88.0±22.3 h.mg/L; 51% improvement). Howeve HDL-C concentrations increased only by 23% (from 0.51±0.04 mmol/L to 0.62±0.0 mmol/L). In conclusion, single drug therapy with reductase inhibitors in FCH results improved fasting plasma lipids and remnant clearance without changes in HDL-C co centrations. Combination therapy with fibrates results in more pronounced improvemen in fasting lipids and significantly improves both chylomicron and remnant clearan which is reflected by a moderate increase of HDL-C concentrations.

24 POSTER PRESENTATIONS

Gene-gene interaction between apo E and apo B in determining plasma levels of ap B-containing lipoproteins

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A common apo B gene variant consists of an insertion/deletion polymophism in the sig nal peptide, which has been shown to influence apo B-containing lipoprotein levels i several, partly contradicting studies. The apo E gene polymorphism gives rise to thre common isoforms (E2, E3 and E4) associated with the low density lipoprotein (LDL) cho lesterol concentration.

In order to study the influence of the apo B signal peptide variants and the apo E poly morphism on apo B-containing lipoprotein levels, 249 healthy, 50-year-old men wer recruited and genotyped for the respective gene variants.

The apo B signal peptide polymorphism influenced total plasma cholesterol and LDL cho lesterol (ins: 3.45 vs. del: 3.68 mmol/1, p<0.05) whereas no effect was seen on VLD triglycerides. The influence of the signal peptide polymorphism was further investigate according to apo E genotype. Subjects with the apo E2/3 genotype (n=33) exhibited sig nificantly lower LDL cholesterol with the ins variant compared with the del variant (ins 2.84 vs. del: 3.50 mmol/1 p<0.05). In contrast, apo E3/3 subjects (n=136) showed a sig nificant influence by the signal peptide variants on VLDL triglyceride levels (ins: 1.19 vs del: 0.84 mmol/1, p<0.05), but no difference in LDL cholesterol (ins: 3.59 vs. del: 3.7 mmol/1, ns). Due to the triglyceride phenotype in subjects with the apo E3/3 genotype, fa loading tests were performed in a subset (n=103) of the whole group. The baseline dif ference in plasma triglyceride concentration was maintained during the test and wa attributed to an increased abundance of large VLDL (Sf 60-400) in subjects with the in variant. The signal peptide polymorphism did not influence apo B48 containing lipopro tein levels. The fasting and postprandial ins/del triglyceride differences did however no remain significant after adjustment for BMI.

This investigation shows a gene-gene interaction between apo E and apo B genes in deter mining plasma levels of apo B-containing lipoproteins and argues for a role of the apo signal peptide variants in regulation of atherogenic lipoproteins.

APOLIPOPROTEIN B OVERPRODUCTION BY THE PERFUSED LIVER OF THE ST THOMAS' MIXED HYPERLIPIDAEMIC (SMHL) RABBIT.

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Short title: ApoB overproduction in the SMHL rabbit.

Abbreviations: apoB, apolipoprotein B; VLDL, very low density lipoprotein; IDL, intermediate density lipoprotein; LDL, low density lipoprotein; FCH, familial combined hyperlipidaemia; SMHL, St Thomas' Mixed Hyperlipidaemic rabbit; NZW, New Zealand white rabbit

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Abstract

The St Thomas' Mixed Hyperlipidaemic (SMHL) rabbit (previously St Thomas' Hospital rabbit) is a putative model of familial combined hyperlipidaemia (FCH). When fed a low (0.08%) cholesterol diet, it exhibits elevations in both plasma cholesterol and triglyceride compared to New Zealand White (NZW) controls. To determine the mechanism for this hyperlipidaemia we studied the secretion of apolipoprotein B (apoB) containing lipoproteins from perfused livers of both young and mature rabbits. During a 3 hour perfusion we measured the total cholesterol and triglyceride content of the medium and the cholesterol, triglyceride and apoB content of very low density lipoprotein $(VLDL)_1$ (Sf 60 - 400), $VLDL_2$ (Sf 20 - 60), intermediate (Sf 12 - 20) and low (Sf 0 - 12) density lipoproteins (IDL, LDL). Lipoprotein concentrations increased linearly throughout the perfusion period. The rate of cholesterol output was 3-fold higher (459 vs 137 ng/g liver/min, $p = 0.003$) in SMHL vs NZW rabbits whilst that of triglyceride was similar (841 vs 662 ng/g liver/min, NS). $VLDL_1$ cholesterol output was elevated 2-fold (232 vs 123 ng/g liver/min, $p < 0.05$) and $VLDL_2$ + IDL + LDL cholesterol output, 4.5-fold (106 vs 23 ng/g liver/min, $p < 0.005$) in SMHL vs NZW rabbits. ApoB output in $VLDL_1$ was 38 ng/g liver/min in SMHL and 14 ng/g liver/min in NZW (NS). In SMHL $VLDL_2$ + IDL + LDL apoB was increased 9-fold at 53 vs 6 ng/g liver/min in NZW ($p < 0.001$). We conclude that the SMHL rabbit overproduces apoB-containing lipoproteins particularly in the $VLDL_2$ + IDL + LDL fraction, a characteristic consistent with its use as a model of FCH.

Key words: Liver perfusion, lipoprotein production, VLDL, IDL, LDL, apoB production, Froxfield Mixed Hyperlipidaemic rabbit, St Thomas Hospital rabbit.

Introduction

Familial combined hyperlipidaemia (FCH) was first described in 1973 [1,2,3] as a monogenic disorder distinct from familial hypercholesterolaemia and familial hypertriglyceridaemia. Affected families show a range of phenotypes; elevated cholesterol, triglyceride or both, with an underlying increase in apoB concentration. The disorder is estimated to be associated with 1 in 10 premature myocardial infarctions and to affect 1-2% of the general population. Currently, diagnosis depends on a knowledge of family histories. However, if a mechanism for the disorder could be elucidated, this may facilitate a clearer description and improved detection of the condition.

Animal studies may help to identify the pathogenesis of the disease. The St. Thomas Hospital rabbit was first described in 1987 as exhibiting increased though variable plasma lipid levels [4]. Elevated very low density lipoprotein (VLDL, Sf 20 - 400), intermediate density lipoprotein (IDL, Sf 12 - 20) and low density lipoprotein (LDL, Sf 0 - 12) cholesterol and LDL triglyceride levels were seen when compared to New Zealand White (NZW) controls. Both the LDL particles and LDL receptors of these rabbits were shown to be normal and on this basis it was proposed that these rabbits have a disorder similar to FCH. Little has appeared in the literature in recent years on this animal model except for a report from Johns Hopkins University on a strain of rabbit derived from St Thomas Hospital rabbits that, on investigation provided evidence for two distinct genetic mechanisms, one acting on triglyceride, and the other on apoB [5]. In 1993 the remaining rabbits were used to establish a new colony, whose members have now been renamed the St Thomas' Mixed Hyperlipidaemic (SMHL) rabbit. When fed a low cholesterol diet the rabbits from the colony developed mixed hyperlipidaemia. In preliminary studies performed on a group of

moderately hyperlipidaemic rabbits we found that the lipid elevation was due to an increased mass of $VLDL_1$ (S_f 60 – 400), $VLDL_2$ (S_f 20 – 60), IDL and LDL [6].

Having observed a reproducible phenotype in the SMHL animals we undertook a series of liver perfusion experiments to measure hepatic production of apoB-containing lipoproteins in these animals compared to NZW controls. Current concepts of the aetiology of FCH in man suggests that the underlying defect is an overproduction of apoB containing lipoproteins by the liver [7,8], possibly as a result of supranormal amounts of fatty acids being delivered to the organ from adipose tissue [9,10]. We therefore tested the hypothesis that SMHL rabbits have a metabolic abnormality of apoB overproduction by the liver.

Methods

Animals

The SMHL rabbits were maintained as an inbred colony at Froxfield Farms (Froxfield, Nr Petersfield, Hants., UK). Nine SMHL (5 female and 4 male) and 9 age- and sex-matched NZW controls were fed a chow diet (0.005% cholesterol wt/wt) (Stanrab diet, Special Diet Service, Witham, UK) for a minimum of 4 weeks and 10 SMHL and 10 age- and sex-matched NZW rabbits were fed a diet supplemented with a low (0.075% wt/wt) level of cholesterol (0.08% total) for 12 weeks in order to determine the effects of a diet with a slightly elevated cholesterol content on lipoprotein composition and mass.

Plasma cholesterol and triglyceride levels were measured in the inbred colony on 60 young

SMHL, 12 young NZW, 16 mature SMHL and 18 mature NZW male rabbits fed the 0.08% cholesterol diet. From these animals, 17 SMHL rabbits and 19 age-matched NZW controls were selected for liver perfusion experiments on the basis of their lipid levels. The selected rabbits had moderate mixed hyperlipidaemia; plasma cholesterol was 4.0 ± 0.8 vs 1.3 ± 0.2 mmol/l, $p < 0.005$ and plasma triglyceride was 2.6 ± 0.4 vs 1.0 ± 0.1 mmol/l, $p < 0.001$ (SMHL vs NZW). Six SMHL and 7 NZW rabbits were studied between 11 and 16 weeks of age ('young'), and the remainder were studied when more than 5 months old ('mature'). The animals were housed individually in cages under standard conditions and were fed *ad libitum* the diet containing 0.08% cholesterol.

The procedures involving animals in these studies were subject to UK Home Office regulations.

Surgical procedure

On the day of the perfusion experiment the animal was terminally anaesthetised by the injection of 1 ml Euthatal (Rhône Mérieux Ltd., Harlow, UK) per 1.4 kg body weight plus 1 ml heparin (1000 iU/ml) (Leo Laboratories Ltd., Princes Risborough, UK) into the marginal ear vein. A thoracotomy/laparotomy was performed along the line of the linea alba and the thoracic aorta was ligated using 2-0 silk (Davis and Geck, Cyanamid of Great Britain Ltd., Gosport, UK). The intestines were displaced to the left and the inferior vena cava (IVC) was cannulated superior to the diaphragm using plastic tubing (internal diameter 2 mm, external diameter 3.2 mm). The hepatic portal vein (HPV) was cannulated (14G IV catheter; Vygon UK Ltd., Cirencester, UK), the IVC was ligated between the renal and hepatic veins (polyamide 66 suture, Ethicon Ltd., Edinburgh, UK) and the common bile duct was

cannulated (translucent vinyl tubing, internal diameter 0.63 mm, external diameter 1.4 mm, Portex Ltd., Hythe, UK).

Perfusion

Livers were flushed with 750 ml oxygenated Krebs Henseleit buffer [11] (pH 7.4, 37°C) at 75 cm hydrostatic pressure prior to being connected to the perfusion apparatus. Perfusions were performed at 37°C in a thermostatically controlled room. The apparatus consisted of two peristaltic pumps (Watson Marlow MHRE and 503U), pumping buffer at 100 ml/minute with inflow via the HPV and outflow through the IVC. Oxygenation was by a 'Hamilton lung' [12] containing 5 metres of silastic tubing. The pH was constantly monitored (Jenway 3050 portable pH meter, Jenway, UK, Dunmow, UK) and maintained at 7.4 by the addition of 1 M $NaHCO_3$ when necessary. A 3.2 mm internal diameter, 6.4 mm external diameter tubing (AlteSil high strength tubing, Altec, Alton, UK) was used throughout the system. The total volume of recirculating perfusate was 120 ml. It was not necessary to have red blood cells in the perfusate as preliminary experiments showed no difference in liver function, viability, bile output or apoB production when erythrocytes were present or absent. Their presence caused an artificial increase in the cholesterol concentration in the perfusate, presumably as a consequence of erythrocyte degradation.

A 15 ml sample of perfusate was withdrawn at 0, 60, 120 and 180 minutes and 5 ml samples were removed at 30, 90 and 150 minutes. Sample volume was replaced with an equal volume of oxygenated, warmed Krebs Henseleit buffer. The liver and intestines were kept moist by covering with gauze soaked in buffer. The volume of bile produced was recorded. At the end of the experiment the livers were examined and found to be well perfused with no

necrotic or hypoxic areas. Pieces of liver were taken and stored at –70°C for analysis of their lipid content.

Analysis of perfusate

Samples of perfusate were spun (1780 g, 10 minutes) to pellet any cellular material. Cholesterol, cholesterol ester, triglyceride, phospholipid (Boehringer Mannheim GmbH, Lewes, UK) and glycerol (Randox Laboratories Ltd., Crumlin, UK) were measured with kits [13]. Urea, albumin, AST and GGT were determined on an Olympus A300 multichannel analyser using manufacturers reagents. Protein was measured by a modification [14] of the method of Lowry *et al* [15]. Lipoprotein subfractions were treated by the addition of an equal volume of isopropanol to precipitate apoB. The apoB content was calculated by subtracting isopropanol soluble protein from total protein [16].

Lipoprotein separation

Lipoproteins in the perfusate were concentrated 5 fold by centrifugation at a density of 1.065 g/ml (83150 g, 18 hours). The total apoB containing lipoprotein fraction so obtained was separated into $VLDL_1$, $VLDL_2$, IDL and LDL by centrifugation in a density gradient constructed of layers of d = 1.0988, 1.0860, 1.0790, 1.0722, 1.0641 and 1.0588 g/ml [17, 18]. Cholesterol, triglyceride, total protein and apoB were measured in each fraction. Concentrations were corrected back to that present in the original perfusate sample.

Extraction of lipid from liver samples

Immediately on thawing, samples of liver were taken and the wet weight was recorded (0.25 – 0.5 g). Following a modification [19] of the method of Folch [20], samples were homogenised (Janke & Kunkel KG homogeniser, Staufen I Breisgau, Germany) in small volumes of methanol (BDH) and the volume adjusted to 50 ml with methanol. After standing for 30 minutes with occasional mixing 100 ml chloroform (BDH) was added. After an overnight extraction at 15°C the samples were filtered (Whatmans No. 1 filter paper, Whatman International Ltd., Maidstone, UK) and protein precipitated by the addition of 50 ml 0.05% calcium chloride solution. Samples were again maintained overnight at 15°C and the chloroform (bottom) layer removed and dried on a centrifugal evaporator (Howe). The pellet was redissolved in isopropanol (2 ml) and total cholesterol, cholesteryl ester, triglyceride and phospholipid contents were measured as above.

Measurement of $VLDL_1$ loss during perfusion

Total VLDL was isolated from 50 ml rabbit plasma at d = 1.006 g/ml (62918 g, 18 hours). From this $VLDL_1$ was prepared by density gradient centrifugation as described above. $VLDL_1$ was then labelled with $Na[^{125}I]$ (Amersham International plc, Amersham, UK) as described previously [21].

Livers from 2 NZW rabbits (fed standard rabbit chow) were prepared as above. After taking a pre-treatment sample at time 0, 5 μCi of ^{125}I-labelled $VLDL_1$ was added to the circulating buffer. During the perfusion $VLDL_1$, $VLDL_2$, IDL and LDL were isolated as described above, apoB was isolated, resolubilised in 0.1 M NaOH and its specific activity was

measured.

^{3}H leucine experiments

To demonstrate that the accumulating lipoproteins in the recirculating perfusate represented newly synthesised products, the incorporation of radioactive leucine into apoB was measured during perfusion of livers from 2 mature NZW and 2 mature SMHL rabbits. 100 μCi ^{3}H leucine (Amersham International plc) (165 Ci/mmol) was added to the perfusate at time 0 and further doses of 100 μCi ^{3}H leucine were added at 30 minute intervals, just after each sample was removed for lipoprotein isolation. The specific activity of apoB isolated from $VLDL_1$, $VLDL_2$, IDL and LDL was then determined by radioactivity measurement in a β scintillation counter and assay of apoB protein as described above.

Statistical analyses

Variables were tested for normal distribution. Plasma cholesterol and triglyceride concentrations were transformed to a normal distribution by taking the logarithm of their values. Two sample t-tests were performed to determine significance. Where the distribution of a variable or series of variables could not consistently be converted to normal, as was the case with all perfusate sample measurements, a Mann-Whitney *U* test was performed. All statistical analyses were carried out using Minitab version 10. Data are expressed as mean ± standard error of the mean (SEM) unless otherwise stated.

Results

Effect of 0.08% cholesterol diet on plasma lipids and lipoproteins

To uncover the hyperlipidaemic phenotype in SMHL rabbits it was necessary to add a small amount of cholesterol to the diet (Table 1). Cholesterol supplementation had no significant effect on plasma lipid levels in NZW rabbits, however plasma cholesterol was significantly elevated in SMHL rabbits on the 0.08% cholesterol diet compared to SMHL on normal chow and NZW fed either normal or supplemented diet. There were no significant differences in plasma triglyceride concentrations between any of the groups. It was noted that despite having normal total lipid levels, chow fed SMHL rabbits were dyslipidaemic. $VLDL_2$ and IDL mass levels were elevated compared to NZW rabbits fed the same diet ($p = 0.08$, $p = 0.01$ respectively). When fed the cholesterol supplemented diet all four apoB containing lipoproteins were increased in SMHL compared to NZW rabbits fed either normal or supplemented diet.

Population lipids and lipoproteins

In the male rabbits in the colony screened for plasma lipid levels prior to selection of animals for perfusion experiments, plasma cholesterol levels of both young and mature SMHL rabbits were significantly elevated (though variable) compared to NZW rabbits (5.97 ± 0.37 vs 1.79 ± 0.14 mmol/l, $p < 0.001$ and 3.11 ± 0.95 vs 1.32 ± 0.23 mmol/l, $p = 0.04$, in young and mature rabbits respectively (Figure 1A)). Plasma triglyceride levels were significantly

elevated in young SMHL rabbits compared to NZW controls (3.31 ± 0.25 vs 1.14 ± 0.14 mmol/l, $p < 0.001$ (Figure 1B)) but not in mature SMHL vs NZW animals. SMHL rabbits showed a decrease with age in plasma lipid concentrations, $p = 0.006$ for young vs mature cholesterol levels and $p < 0.001$ for young vs mature triglyceride levels (Figure 1) but NZW rabbits did not.

Perfusion experiments

Liver perfusion experiments were performed on young and mature SMHL and NZW rabbits that had been fed the 0.08% cholesterol diet from weaning. Liver function tests were normal throughout the perfusions. The livers remained patent and produced bile at 27, 17, 29 and 15 µl/liver/minute in young NZW, young SMHL, mature NZW and mature SMHL respectively (none of these was significantly different). Examination of the livers at the end of the experiment revealed no necrotic or hypoxic areas. Mean age at sacrifice for young NZW vs SMHL rabbits was 3.0 vs 2.8 months, mean body weight was 2.5 vs 2.0 kg and mean liver weight was 71 vs 74 g, and mean age at sacrifice for the mature NZW vs SMHL rabbits was 9.8 vs 10.2 months, mean body weight was 4.0 vs 3.6 kg and mean liver weight was 103 vs 92 g.

In those rabbits selected for perfusion studies the mean plasma cholesterol and triglyceride levels were elevated in SMHL compared to NZW controls and similar to the mean values seen for the whole colony. Thus in young animals used for perfusion studies plasma cholesterol was 3.7 ± 0.4 mmol/l in SMHL and 1.0 ± 0.2 mmol/l in NZW ($p = 0.001$), while plasma triglyceride was 3.6 ± 0.9 mmol/l in SMHL and 0.7 ± 0.07 mmol/l in NZW ($p = 0.005$). In mature rabbits plasma cholesterol was 4.1 ± 1.3 vs 1.4 ± 0.3 mmol/l ($p < 0.05$)

and plasma triglyceride was 2.0 ± 0.4 vs 1.2 ± 0.1 mmol/l (NS) (SMHL vs NZW respectively).

Lipid levels during perfusion

Mean perfusate cholesterol and triglyceride levels rose linearly in all groups of rabbits over the 180 minutes of the experiment (Figure 2). Only data up to 150 minutes were available from the perfusate experiments with the young SMHL rabbits. In young rabbits, cholesterol content in the perfusate was barely measurable in the NZW group rising to 2.0 ± 0.5 μg/g liver after 180 minutes, however in the perfusate from the SMHL rabbits the cholesterol content rose from 5 ± 5 to 58 ± 31 μg/g liver at 150 minutes, and was significantly higher when compared to that from NZW rabbits at all time points from 60 minutes until the end of the experiment. In young rabbits the calculated cholesterol output was 380 ± 170 vs 10 ± 6 ng/g liver/minute (SMHL vs NZW, $p < 0.01$). Perfusate cholesterol levels in the mature rabbits rose from 7 ± 5 to 41 ± 11 μg/g liver in NZW rabbits during the experiment and from 6 ± 4 to 96 ± 21 μg/g liver in SMHL rabbits. The cholesterol content of the perfusate was significantly increased in SMHL rabbits in samples taken at 60, 90 and 150 minutes and the calculated cholesterol output was 510 ± 101 vs 220 ± 67 ng/g liver/minute in SMHL vs NZW rabbits (NS). Mature NZW rabbits had a significantly greater cholesterol output compared to young NZW of 220 ± 67 vs 10 ± 6 ng/g liver/minute ($p < 0.005$). SMHL rabbits on the other hand did not show a significant effect of age.

The mean triglyceride output was higher in young SMHL rabbits compared to young NZW but this did not reach significance (810 ± 290 vs 270 ± 100 ng/g liver/minute, $p = 0.12$). Perfusate triglyceride rose from 0 to 48 ± 24 μg/g liver in NZW rabbits and from 0 to 144 ±

63 μg/g liver in SMHL rabbits, reaching a significantly higher level when compared to NZW rabbits from 90 - 150 minutes. Mature rabbits in both groups showed a similar triglyceride output of 860 ± 130 vs 890 ± 246 ng/g liver/minute (SMHL vs NZW respectively, NS). Triglyceride perfusate levels rose from 5 ± 4 to 160 ± 50 and 0 to 149 ± 29 μg/g liver in the NZW and SMHL respectively. Neither NZW nor SMHL rabbits showed any significant age related differences in triglyceride output.

Lipoprotein production during perfusion

The lipoproteins in the perfusate were separated and their cholesterol and triglyceride contents were analysed. Data are shown in Table 2. In young rabbits, $VLDL_1$, $VLDL_2$ and IDL cholesterol output were significantly increased in SMHL compared to NZW rabbits but no difference was seen in LDL cholesterol output. $VLDL_2$ triglyceride output was significantly increased in SMHL rabbits. In mature SMHL rabbits mean IDL triglyceride was increased compared with NZW rabbits ($p < 0.05$, Table 2) but no other significant differences were observed in individual fractions. We then compared the output of triglyceride-rich and cholesterol-rich lipoproteins from the liver. The cholesterol and triglyceride contents of $VLDL_2$, IDL and LDL (i.e. non $VLDL_1$) were combined as these represent cholesterol-rich lipoproteins and the values obtained were compared with those for the triglyceride-rich $VLDL_1$ (Figure 3). In young rabbits perfusate levels of $VLDL_1$ cholesterol rose from 0 to 12.5 ± 4 μg/g liver in NZW rabbits and from 0.8 ± 0.3 to 37.8 ± 12.2 μg/g liver in SMHL rabbits (Figure 3A), being significantly higher in SMHL rabbits at all time points from 30 - 150 minutes. $VLDL_1$ cholesterol output was significantly increased in young SMHL rabbits compared to NZW rabbits (237 vs 60 ng/g liver/minute respectively, Table 2). Non $VLDL_1$ perfusate cholesterol levels rose from 0.6 ± 0.4 to 4.7 ± 1.7 and from

0.7 ± 0.7 to 18.8 ± 11 μg/g liver in NZW and SMHL rabbits respectively and were significantly higher in SMHL rabbits between 90 and 150 minutes. Non $VLDL_1$ cholesterol output rate was elevated in SMHL rabbits compared to NZW rabbits (118 ± 56 vs 21 ± 8 ng/g liver/minute respectively, $p = 0.01$).

In mature rabbits there was no significant difference in mean $VLDL_1$ cholesterol output between the two strains of rabbit (160 vs 229 ng/g liver/minute, NZW vs SMHL, Table 2). In NZW rabbits, $VLDL_1$ perfusate cholesterol levels rose from 0 to 25 ± 8 μg/g liver (Figure 3B), and in SMHL rabbits from 0 to 46 ± 11 μg/g liver over the three hours of the experiment. Although there was a 5-fold increase in SMHL rabbit non $VLDL_1$ cholesterol output when compared to NZW rabbits this was not significant (98 ± 28 vs 23 ± 4 ng/g liver/min). Non $VLDL_1$ cholesterol levels increased from 0 to 21 ± 7 and 1 ± 1 to 5 ± 2 μg/g liver in SMHL and NZW rabbits respectively during the three hour perfusion, reaching significance at 30, 90 and 150 minutes.

There were no significant differences in $VLDL_1$ triglyceride output rates in young NZW vs SMHL rabbits because of the large inter-animal variability (Table 2, Figure 3C). Likewise in mature animals $VLDL_1$ triglyceride output was similar in the two groups (Table 2, Figure 3D). However, $VLDL_1$ triglyceride concentration was higher in the perfusate from young SMHL vs NZW rabbits at 60, 90 and 150 minutes. Non $VLDL_1$ triglyceride output was higher in the perfused livers from young SMHL rabbits than from young NZW rabbits (153 ± 93 vs 20 ± 6 ng/g liver/minute respectively, $p < 0.05$), and non $VLDL_1$ triglyceride concentrations were significantly higher at all time points from 60 minutes to 150 minutes. In mature SMHL perfused livers non $VLDL_1$ triglyceride output rates tended to be higher

than in those from NZW rabbits although the difference was not significant (78 ± 38 vs 30 ± 11 ng/g liver/minute respectively). There was however a significant increase in the non $VLDL_1$ triglyceride concentration in the perfusate from the mature SMHL rabbits after 180 minutes.

ApoB output from perfused livers

Apolipoprotein B was measured in the individual lipoproteins secreted into the hepatic perfusate. The results are shown in Table 2 and Figure 4. ApoB as a percentage of total lipoprotein protein was similar in SMHL and NZW rabbits. In $VLDL_1$ it comprised 41% of the total protein in SMHL vs 38% in NZW rabbits, in $VLDL_2$ 60% vs 64%, in IDL 79% vs 82% and in LDL 75% vs 76%. Individual $VLDL_1$, $VLDL_2$ and IDL apoB outputs were significantly elevated in young SMHL rabbits compared to NZW controls (Table 2). In mature rabbits, mean $VLDL_1$ apoB output was not significantly different between the two strains but $VLDL_2$ and IDL apoB output were both significantly increased in SMHL compared to NZW rabbits. In livers from young rabbits the $VLDL_1$ apoB output was higher in SMHL than in NZW controls (79 vs 22 ng/g liver/minute respectively, Table 2). Non-$VLDL_1$ apoB output in SMHL rabbits was 80 ± 41 ng/g liver/minute vs 6 ± 3 ng/g liver/minute in NZW rabbits ($p < 0.005$) (Figure 4A). The total apoB output was significantly higher in livers from SMHL rabbits compared to NZW (159 ± 56 vs 28 ± 7 ng/g liver/minute, $p < 0.01$). In contrast to the situation in young animals, livers from mature NZW rabbits released $VLDL_1$ and non-$VLDL_1$ apoB at similar rates (Table 2). There were no significant differences in $VLDL_1$ apoB concentrations during the perfusion in SMHL and NZW rabbits but non-$VLDL_1$ apoB concentrations were significantly increased in SMHL rabbits at all time points from 30 minutes until the end of the experiment (Figure 4B). Non-

$VLDL_1$ apoB output was increased 6-fold in SMHL rabbits relative to NZW (36 ± 12 vs 6 ± 3 ng/g liver/minute respectively, $p = 0.01$). The non $VLDL_1$ apoB output accounted for 75% of the total apoB output. However the total apoB output in these mature rabbits was not significantly increased in the SMHL rabbit group (48 ± 14 vs 16 ± 4 ng/g liver/minute in SMHL and NZW, $p = 0.07$).

$VLDL_1$ apoB output was significantly increased in the young NZW compared to the mature NZW (22 vs 10 ng/g liver/minute respectively, $p = 0.04$, Table 2) and in young SMHL versus mature SMHL rabbits (79 vs 15 ng/g liver/minute respectively, $p < 0.01$, Table 2). No differences were found between the young and mature rabbits of either strain in non-$VLDL_1$ apoB output.

When both young and mature NZW and SMHL rabbits were grouped together, there was a highly significant correlation between the rate of apoB output by the perfused livers and plasma cholesterol levels at time of sacrifice ($r = 0.72$, $p < 0.001$) (Figure 5A). Plasma triglyceride was also significantly correlated with total apoB output rate ($r= 0.67$, $p < 0.001$) (Figure 5B).

To examine the extent to which $VLDL_1$ was converted to denser lipoproteins during the 3 hour perfusion, ^{125}I labelled $VLDL_1$ was added to the perfusate of 2 NZW rabbits 10 minutes prior to the beginning of the experiment. ApoB was isolated to follow the fate of the particles. At time 0, 93% of apoB radioactivity was found in the $VLDL_1$ fraction, with 6% in $VLDL_2$ and 1% in IDL. After 3 hours 77% of the counts remained in $VLDL_1$, 7.5% were found in $VLDL_2$, 2.5% in IDL and 1.5% in LDL. The lack of increase in $VLDL_2$, IDL and LDL radioactivity indicated that delipidation was limited in this system.

In order to show that the lipoproteins that accumulated were derived by *de novo* production of apoB, ^{3}H leucine was added to the perfusate of 2 mature NZW and 2 mature SMHL rabbits. By 3 hours, leucine radioactivity in $VLDL_1$ apoB rose by an average of 5-fold above the zero time value in NZW and 15-fold in SMHL rabbit livers, in $VLDL_2$ it rose 2 and 6-fold; in IDL, 2 and 2-fold and in LDL, 2 and 3-fold respectively showing that the liver was synthesising these lipoproteins.

Liver lipids

Hepatic lipid concentrations were measured after the perfusion experiments. Young and mature NZW and SMHL rabbits were found to have very similar concentrations of cholesterol, free cholesterol, triglyceride and phospholipids in their livers. Mature NZW rabbits stored more cholesterol (total and free) in their livers than did young NZW rabbits (total cholesterol 15.4 ± 1.9 vs 6.0 ± 1.1 mg/g wet weight liver respectively, $p = 0.015$ and free cholesterol 7.4 ± 1.3 vs 3.3 ± 0.8 mg/g wet weight liver respectively, $p = 0.017$). The lipids in the livers of SMHL rabbits did not differ with age and were not significantly different to those in NZW rabbits (total cholesterol 7.9 ± 2.1 vs 12.2 ± 2.3 mg/g wet weight liver and free cholesterol 5.0 ± 1.3 vs 8.2 ± 1.5 mg/g wet weight liver in young vs mature SMHL rabbits respectively). Triglyceride concentrations did not vary between the mature rabbits of either strain but young NZW and SMHL rabbits showed a difference (13.0 ± 0.97 vs 9.7 ± 0.96 mg/g wet weight of liver, NZW vs SMHL, $p = 0.05$). Phospholipid concentrations were significantly higher in mature SMHL rabbits than mature NZW rabbits (17.3 ± 0.5 vs 15.4 ± 0.6 mg/g wet weight liver respectively, $p = 0.046$). When young and mature rabbits were grouped together the only difference was a significant increase in

triglyceride concentrations in the NZW rabbits (13.4 ± 0.78 vs 10.6 ± 1.2 mg/g wet weight of liver, NZW vs SMHL, $p < 0.05$).

Discussion

Appearance of the hyperlipidaemic phenotype of SMHL rabbits is dependent on supplementing their diet with a small amount of cholesterol. NZW animals fed the same diet showed no change in plasma lipid levels. However, even on the chow diet, plasma $VLDL_2$ and IDL lipoprotein concentrations were elevated in SMHL rabbits indicating the presence of a lipoprotein disturbance. In the large series of animals studied from the SMHL rabbit colony the plasma lipid pattern was variable with increases in both cholesterol and triglyceride being the most common phenotype. More detailed examination showed that all four apoB containing lipoprotein species were elevated in SMHL rabbits fed the cholesterol supplemented diet. Therefore these animals derived from the original St Thomas Hospital stock express a consistent mixed hyperlipidaemia compatible with their potential role as an animal model for FCH. The colony is inbred and as yet no attempt has been made to investigate the genetics of the disorder. In examining the animals over a period of time it was clear that the hyperlipidaemia in male SMHL rabbits decreased with age (Figure 1) and we have recently reported [22] that plasma cholesterol and triglyceride levels in male SMHL animals decline steadily from 8 weeks of age reaching a plateau at 14-16 weeks. We therefore made the decision to study both young and mature rabbits in the perfusion experiments.

Rabbits used in the perfusion studies had plasma lipid levels close to the mean values of the groups from which they were selected. When all rabbits studied (young and mature) were grouped together, triglyceride, cholesterol and apoB accumulated in the recirculating medium

in an approximately linear manner over the 3 hour perfusion period. Output rates for triglyceride were similar to those previously published for perfused rabbit livers [23]. Comparing hepatic triglyceride output in the four apoB containing lipoproteins in SMHL and NZW animals revealed no difference in $VLDL_1$ despite the fact that this was the most abundant triglyceride carrying particle released from the liver. $VLDL_2$ and IDL triglyceride output rates were significantly increased in SMHL rabbits when both young and mature rabbits were combined, but there was no difference in LDL triglyceride output. Cholesterol output however was substantially higher from SMHL vs NZW livers in $VLDL_1$, $VLDL_2$ and IDL fractions (Table 2, Figure 3). The 5-fold higher output of cholesterol in $VLDL_2$+IDL+LDL from SMHL livers was accompanied by a 9-fold increase in apoB released in these denser lipoprotein particles. Given that 55% of apoB released from the livers of SMHL animals appeared in the non-$VLDL_1$ density range and the finding that only a trivial amount of denser lipoproteins are generated by delipidation of $VLDL_1$ during the perfusion (lipase is removed from the liver during the heparin flush and little activity reappeared during perfusion, data not shown) we surmised that these rabbits overproduced these denser lipoproteins. Thus the elevation in plasma levels of $VLDL_1$ $VLDL_2$, IDL and LDL seen in Table 1 is likely to be due to increased hepatic production of these species rather than decreased catabolism. In the original metabolic studies by La Ville *et al* [4] on St Thomas Hospital rabbits, no difference was seen in receptor mediated catabolism of LDL and in kinetic experiments with VLDL and LDL tracers in intact animals, an increase in production was suggested as the basis of the hyperlipidaemia. A lower fractional catabolic rate was seen for LDL but this was attributed to saturation of receptors by the expanded LDL pool [4]. The present perfusion experiments provide direct evidence for apoB overproduction as the underlying cause of the hyperlipidaemia in this animal model. The correlation of apoB output with plasma cholesterol and triglyceride levels provides strong

support that apoB output is a major determinant of circulatory cholesterol and triglyceride levels (Figure 5). Despite the differences in lipoprotein production between SMHL and NZW rabbits there was no gross difference in the liver content of cholesterol or triglyceride and it may be that lipid availability was not the basis of the variation in lipoprotein production rates, although critical lipid pools more closely associated with lipoprotein assembly were not measured.

These studies demonstrate again that apoB containing lipoproteins can be generated by the liver across a wide spectrum of composition and size. Previous *in vivo* tracer studies from this [24, 25] and other laboratories [26] showed that not all LDL derives from VLDL delipidation. Rather it is necessary to postulate that small VLDL, IDL and LDL can also be produced directly by the liver to explain satisfactorily the findings of kinetic studies. In earlier rabbit liver perfusion studies in control and Watanabe Heritable Hyperlipidaemic rabbits, virtually all apoB was released as VLDL, leading to the suggestion that 'direct' LDL synthesis was really due to very rapid lipolysis. In young NZW animals we can confirm these findings, almost all output of triglyceride and apoB is in the form of VLDL, particularly $VLDL_1$ (Table 2). We found little evidence for extensive delipidation in our perfusion system and demonstrated also linear ^3H leucine incorporation into the apoB of denser lipoproteins throughout the 3 hour period indicating that these species did not derive from wash out of the space of Disse as has been suggested previously [23]. The observation that up to half of the apoB output from the liver of mature NZW or young or mature SMHL rabbits was in the form of $VLDL_2$ or IDL leads us to conclude that direct production of these species does occur.

SMHL rabbits therefore have a combined hyperlipidaemic phenotype compatible with their

use as a potential animal model of FCH. The demonstration that apoB overproduction is the underlying metabolic defect increases the analogy with the human situation. Our finding that the lipoproteins released in this inherited hyperlipidaemia can range in size across the entire apoB containing lipoprotein spectrum, highlights a possible mechanism to explain the inter- and intra-individual variability of the lipid phenotype in FCH. An underlying overproduction of apoB containing lipoproteins could reveal itself as raised VLDL or raised LDL levels.

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Table 1

Effect of 0.08% cholesterol diet on plasma lipid and lipoprotein levels in SMHL and NZW rabbits

	Cholesterol diet		Chow diet	
	NZW	SMHL	NZW	SMHL
Number	10	10	9	9
Cholesterol (mmol/l)	1.6 ± 0.3	3.8 ± 0.7[a,b]	0.8 ± 0.1	1.1 ± 0.2
Triglyceride (mmol/l)	0.49 ± 0.04	1.3 ± 0.46	0.8 ± 0.2	1.2 ± 0.3
$VLDL_1$ mass (mg/dl)	16 ± 4	97 ± 35[a]	38 ± 11	43 ± 16
$VLDL_2$ mass (mg/dl)	21 ± 5	99 ± 22[a,b]	10 ± 4	20 ± 4
IDL mass (mg/dl)	31 ± 10	108 ± 24[a,b]	7 ± 1	18 ± 3
LDL mass (mg/dl)	15 ± 4	36 ± 6[a,b]	4 ± 1	7 ± 2

Plasma lipid and lipoprotein measurements in NZW and SMHL rabbits fed either normal (0.005% cholesterol) or 0.08% cholesterol diet (mean ± SEM). Significant differences $p < 0.05$ using the Mann Whitney *U* test [a] SMHL vs NZW on 0.08% cholesterol diet, [b] SMHL on 0.08% cholesterol diet vs SMHL on normal diet.

Table 2

$VLDL_1$, $VLDL_2$, IDL and LDL cholesterol, triglyceride and apoB output (ng/g liver/min) in young and mature NZW and SMHL rabbits fed a 0.08% cholesterol diet from weaning

	Cholesterol		Triglyceride		ApoB	
Young	NZW	SMHL	NZW	SMHL	NZW	SMHL
$VLDL_1$	60 ± 19	237 ± 68[a]	374 ± 93	658 ± 177	22 ± 4	79 ± 29[a]
$VLDL_2$	9 ± 5	73 ± 26[c]	17 ± 3	87 ± 44[a]	3 ± 1	36 ± 13[c]
IDL	3 ± 2	40 ± 26[a]	5 ± 2	55 ± 40	1 ± 1	37 ± 23[b]
LDL	2 ± 2	10 ± 7	5 ± 2	9 ± 7	2 ± 1	7 ± 5
Mature						
$VLDL_1$	160 ± 45	229 ± 52	408 ± 97	476 ± 84	10 ± 3	15 ± 4
$VLDL_2$	18 ± 6	65 ± 20	13 ± 3	56 ± 30	2 ± 1	14 ± 5[a]
IDL	8 ± 2	26 ± 10	5 ± 2	19 ± 6[a]	3 ± 1	16 ± 6[a]
LDL	2 ± 1	7 ± 3	4 ± 2	8 ± 3	0	6 ± 2

$VLDL_1$, $VLDL_2$, IDL and LDL cholesterol, triglyceride and apoB output (ng/g liver/minute) in young and mature NZW and SMHL rabbits (mean ± SEM). Significant differences [a] $p < 0.05$, [b] $p < 0.01$, [c] $p < 0.005$ between NZW and SMHL rabbits using the Mann Whitney *U* test.

Figure Legends

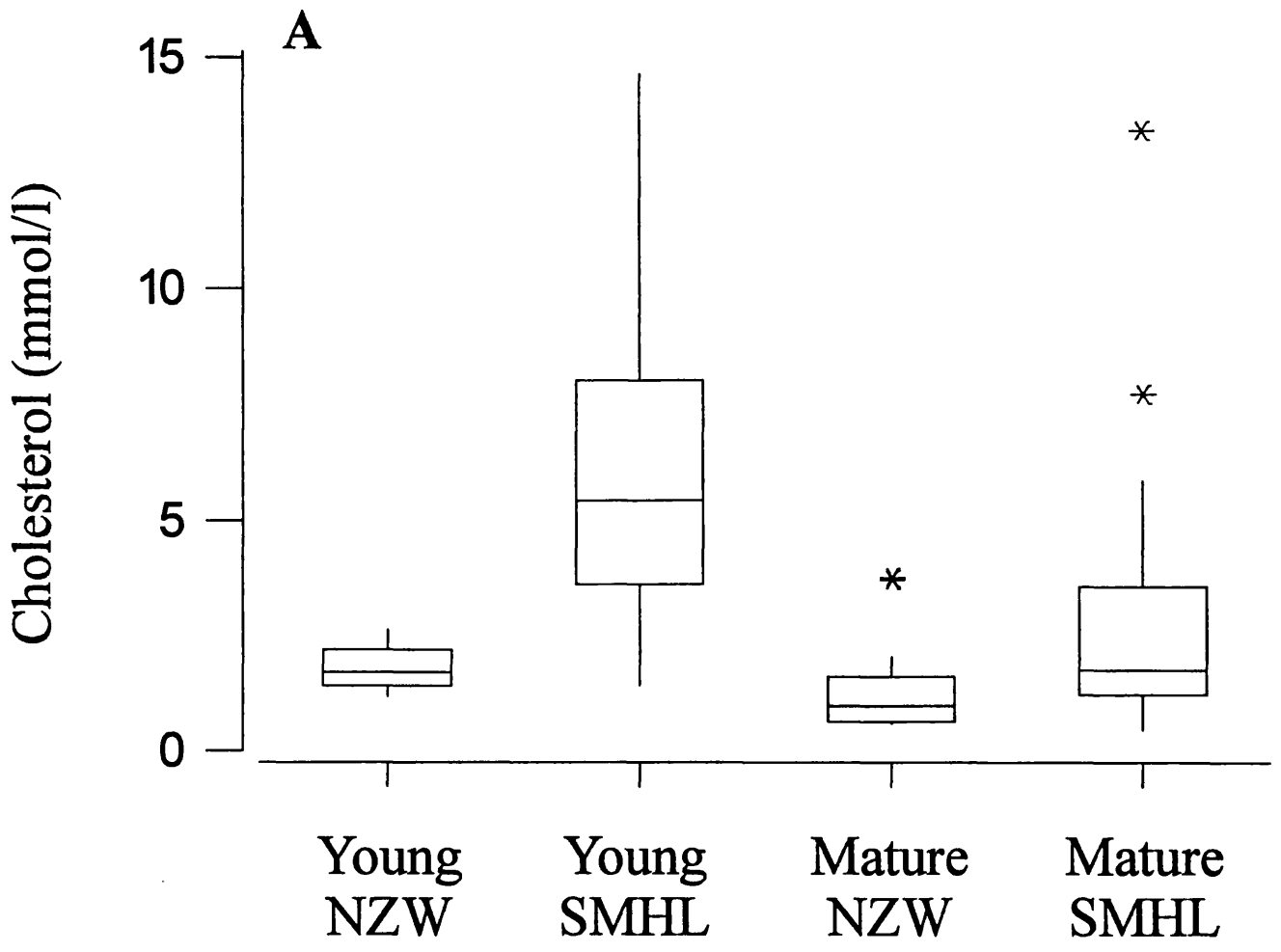
Figure 1. A. Distribution of plasma cholesterol concentrations in male NZW and SMHL rabbits. Levels were determined in young SMHL rabbits (n = 60), mature SMHL rabbits (n = 16), young NZW rabbits (n = 11) and mature NZW rabbits (n = 18). **B.** Distribution of plasma triglyceride concentrations in NZW and SMHL rabbits. The horizontal line represents the median, the upper and lower limits of the box are the 75 and 25 percentile, the vertical lines show the range and the asterisks represent outliers.

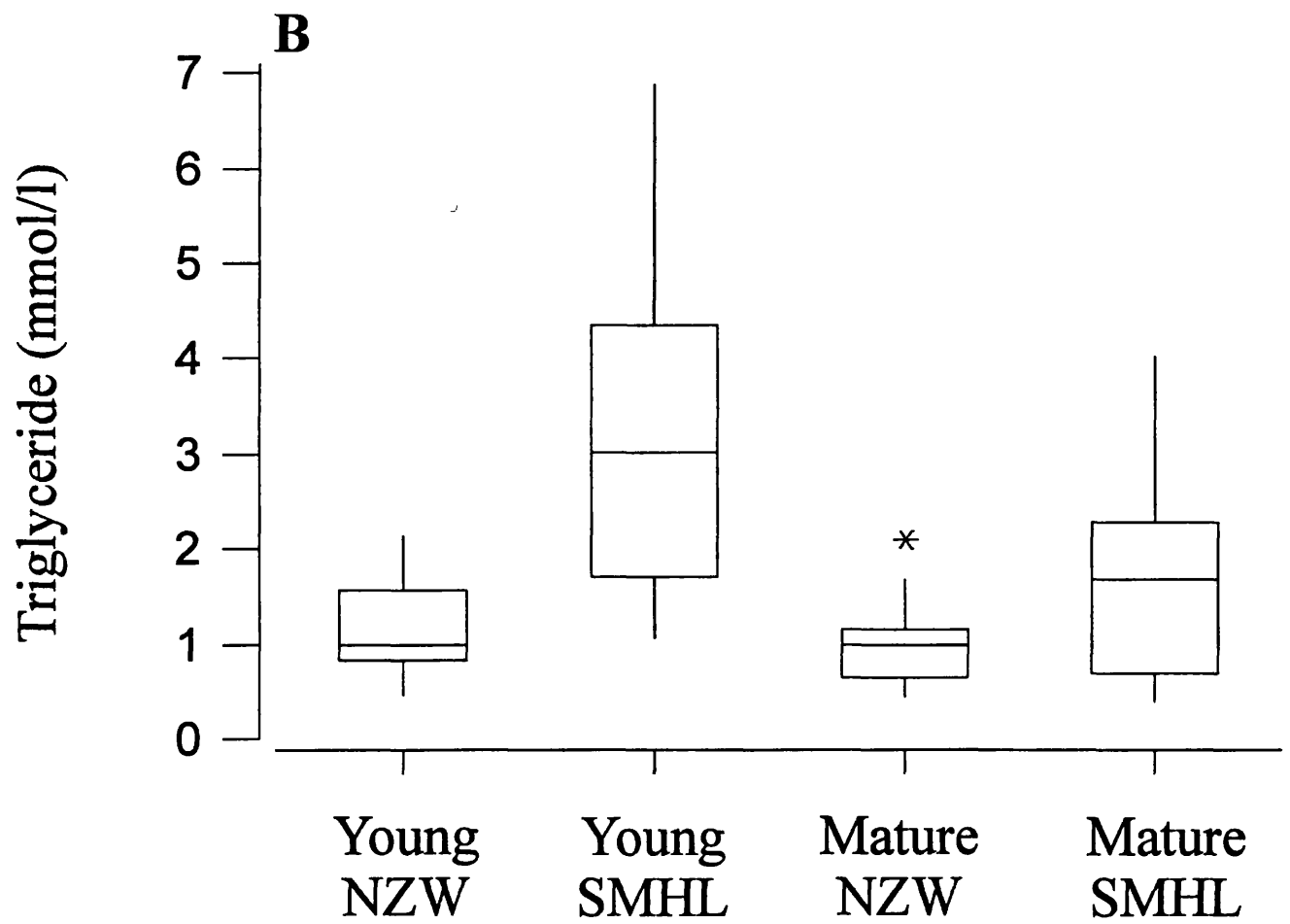
Figure 2. A. Mean cholesterol output from the liver of NZW and SMHL rabbits during the three hours of the perfusion study. The point missing at 180 minutes for young SMHL rabbits is due to insufficient data at that time. Young NZW (—▲—), mature NZW (—●—), young SMHL (--▲--), mature SMHL (--●--). Significant differences (young or mature NZW vs SMHL) * $p < 0.05$ by the Mann Whitney *U* test. **B.** Mean output of triglyceride from the livers of NZW and SMHL rabbits during the three hours of the perfusion study.

Figure 3. A. Mean $VLDL_1$ and $nonVLDL_1$ cholesterol output in young NZW and SMHL rabbits. NZW $VLDL_1$ (—▲—), NZW $nonVLDL_1$ (—●—), SMHL $VLDL_1$ (--▲--), SMHL $nonVLDL_1$ (--●--). Significant differences (NZW vs SMHL) * $p < 0.05$ by the Mann Whitney *U* test. **B.** Mean $VLDL_1$ and $nonVLDL_1$ cholesterol output in mature NZW and SMHL rabbits. **C.** Mean $VLDL_1$ and $nonVLDL_1$ triglyceride output in young NZW and SMHL rabbits. **D.** Mean $VLDL_1$ and $nonVLDL_1$ triglyceride output in mature NZW and SMHL rabbits.

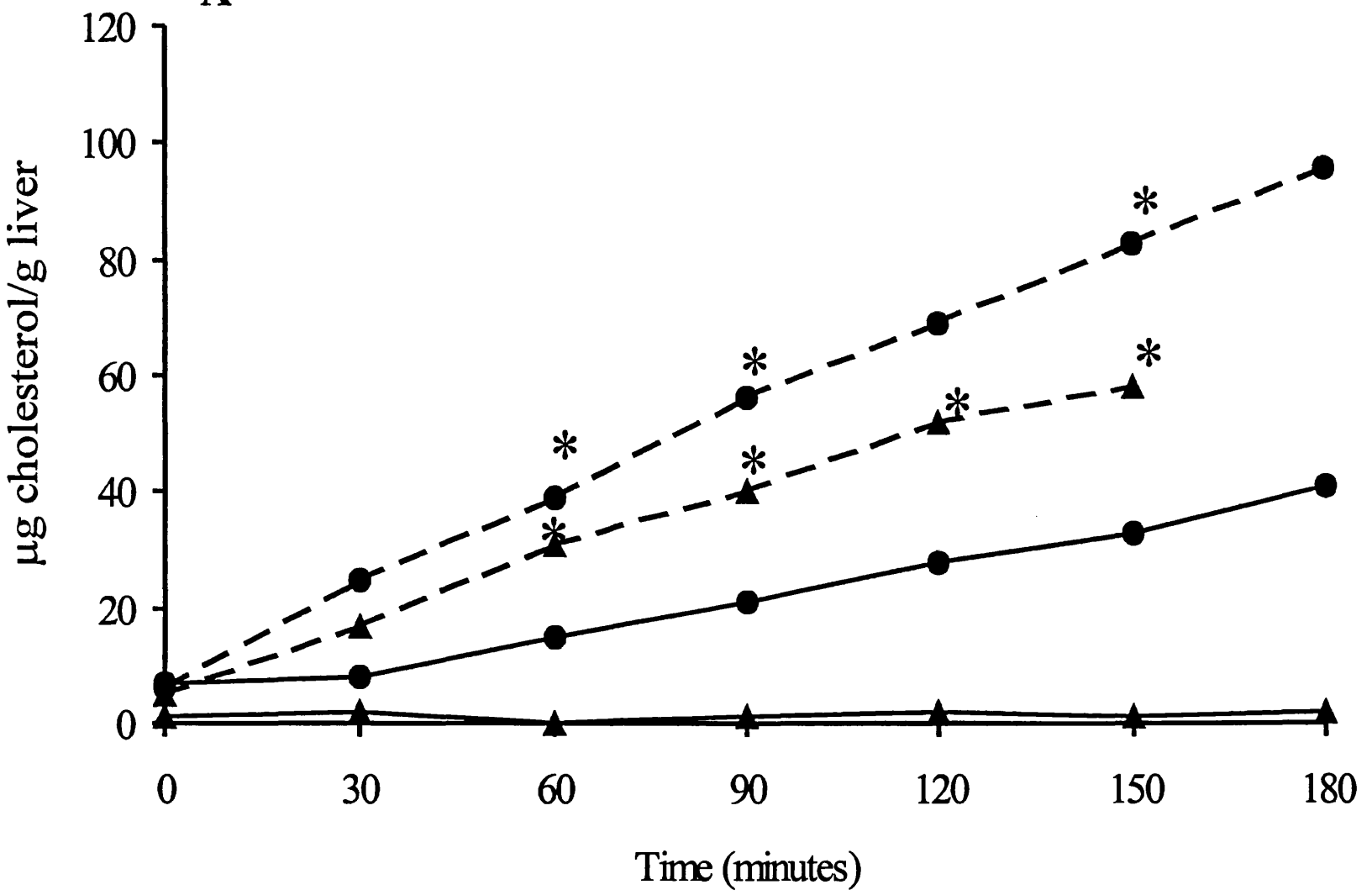
Figure 4. **A.** Mean $VLDL_1$ and $nonVLDL_1$ apoB output from young NZW and SMHL rabbits. NZW $VLDL_1$ (—▲—), NZW $nonVLDL_1$ (—●—), SMHL $VLDL_1$ (--▲--), SMHL $nonVLDL_1$ (--●--). Significant differences (NZW vs SMHL) * $p < 0.05$ by the Mann Whitney *U* test. **B.** Mean $VLDL_1$ and $nonVLDL_1$ apoB output from mature NZW and SMHL rabbits.

Figure 5. Correlation of apoB output rates with plasma cholesterol (**A**) and triglyceride (**B**) concentrations in NZW rabbits (▲) and SMHL rabbits (●) at the time of sacrifice.

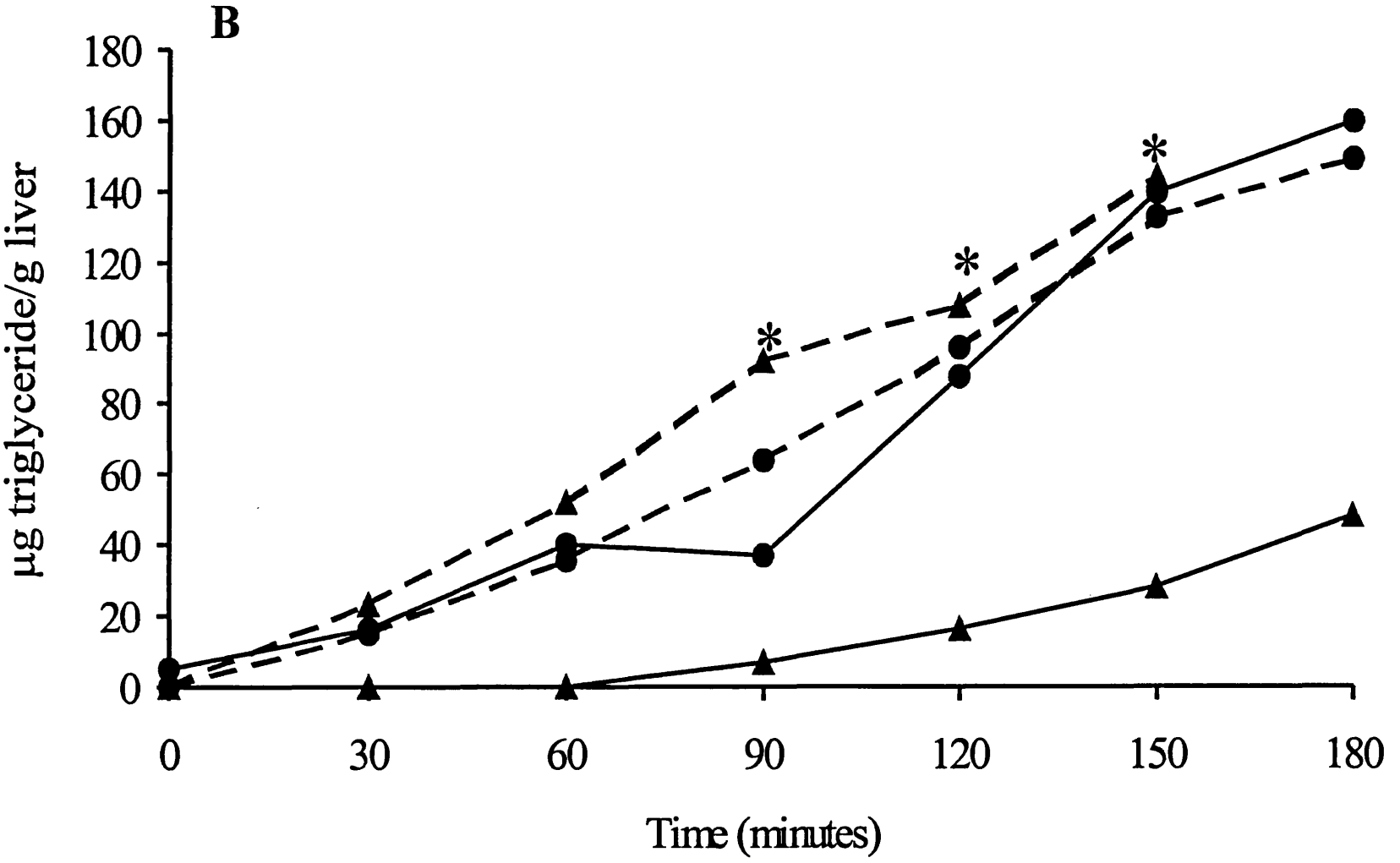

A
Cholesterol (mmol/l)
15
10
5
0
Young NZW
Young SMHL
Mature NZW
Mature SMHL

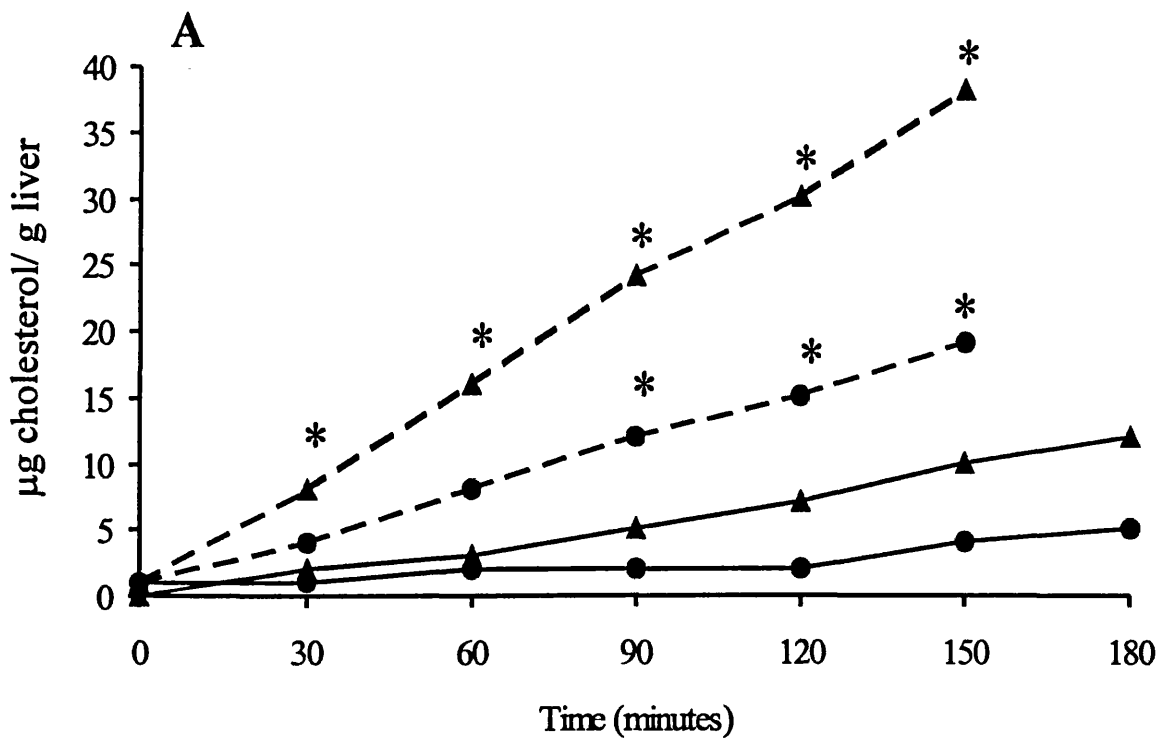

B
Triglyceride (mmol/l)
7
6
5
4
3
2
1
0
Young NZW
Young SMHL
Mature NZW
Mature SMHL

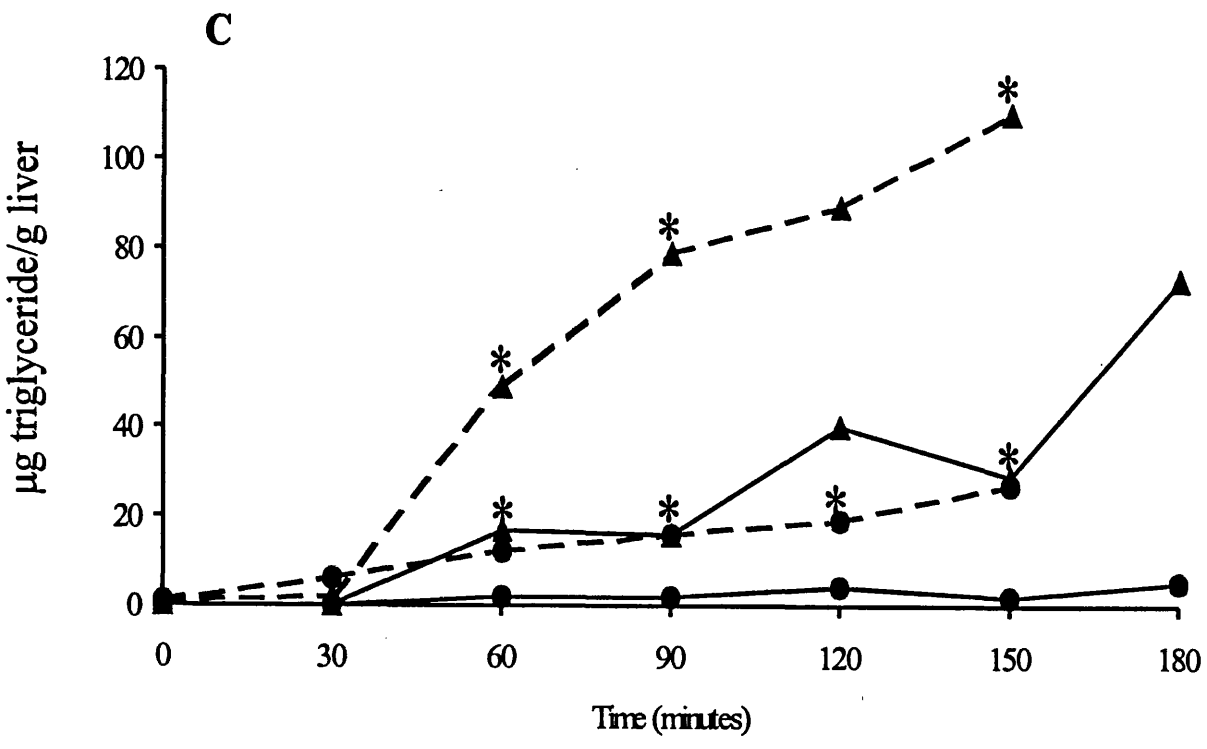
A

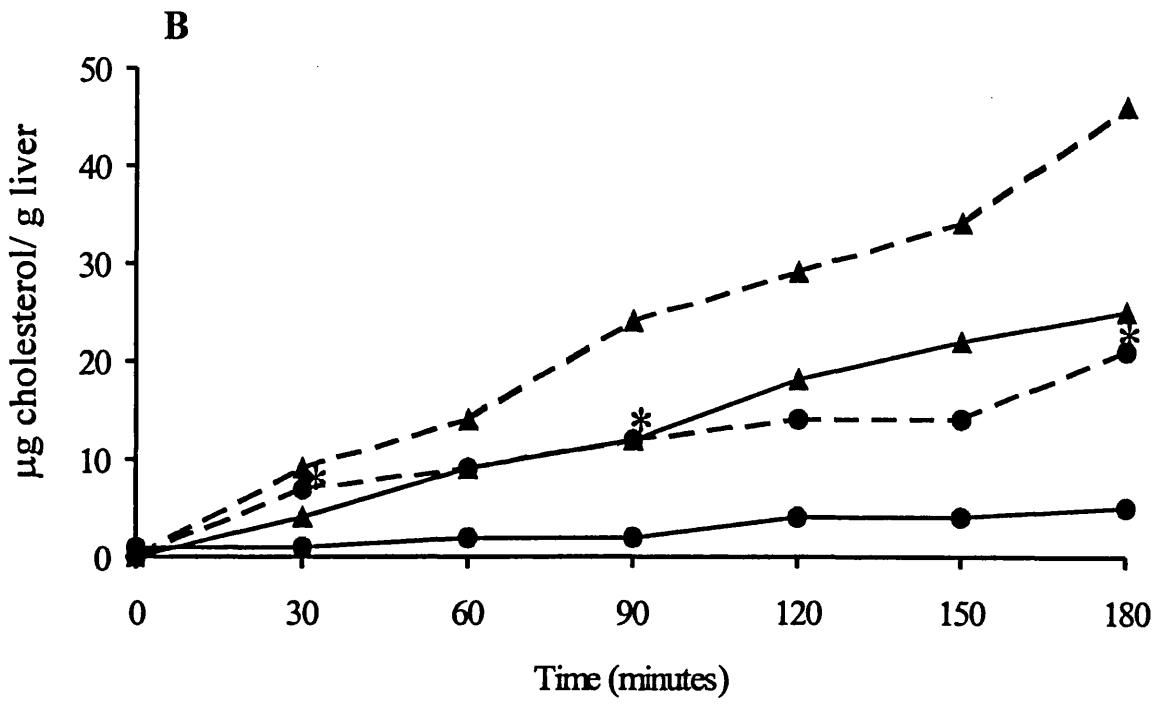

µg cholesterol/g liver
120
100
80
60
40
20
0
0
30
60
90
120
150
180
Time (minutes)

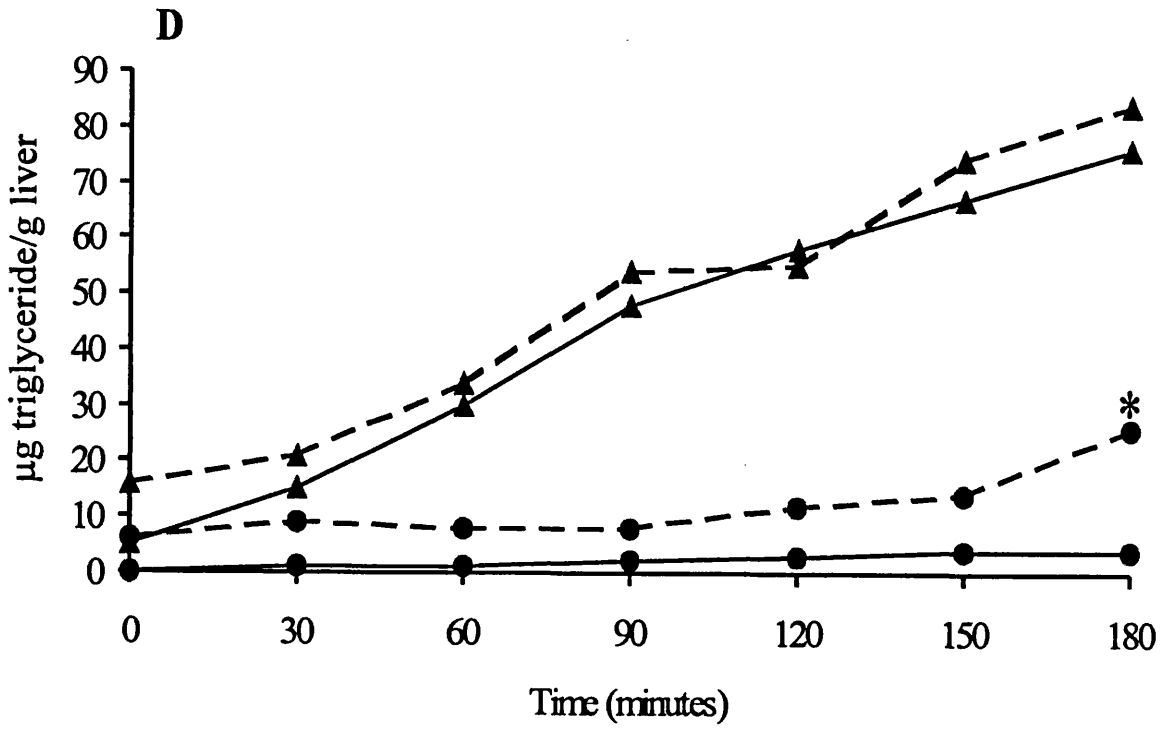
B

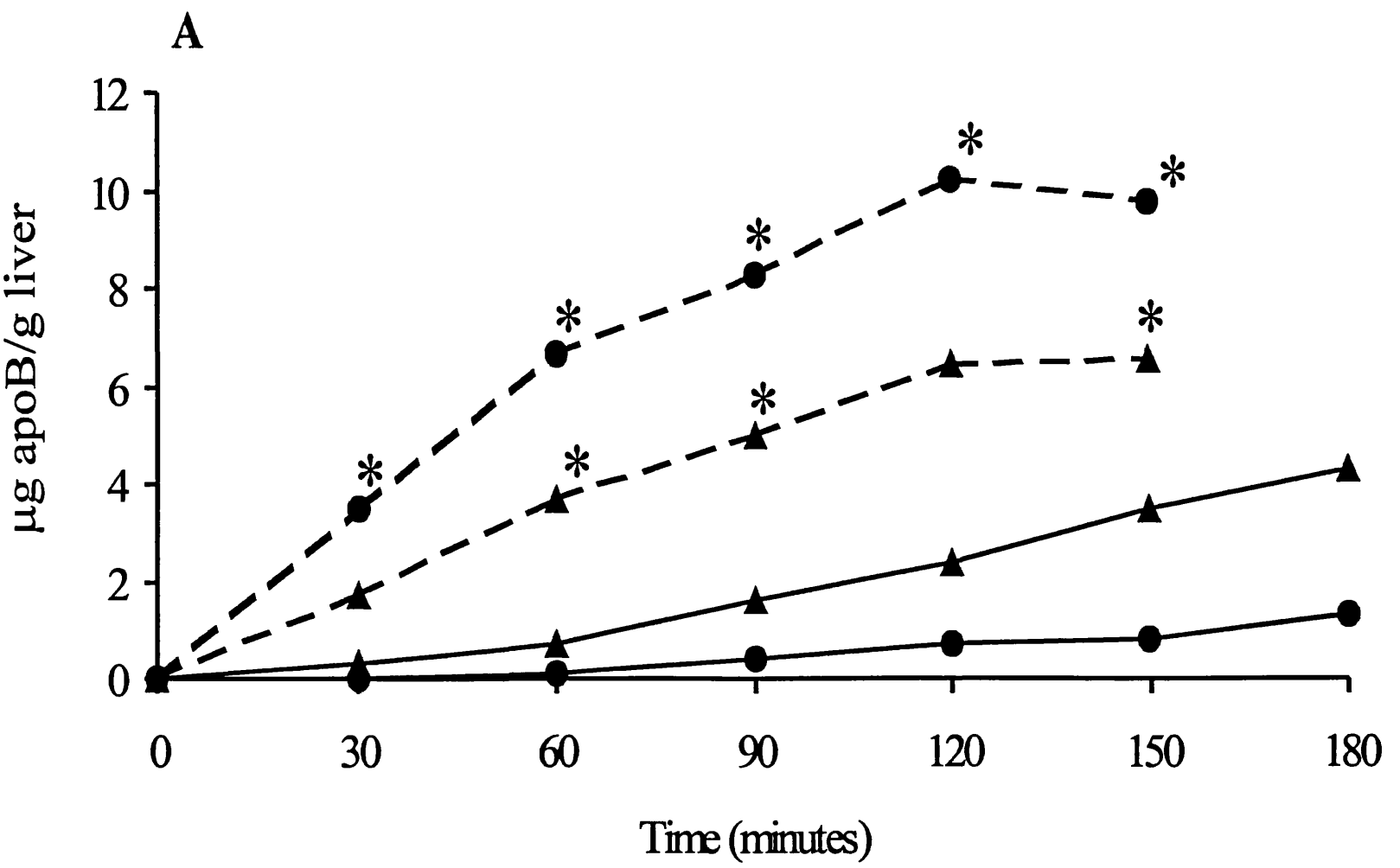

µg triglyceride/g liver
180
160
140
120
100
80
60
40
20
0
0
30
60
90
120
150
180
Time (minutes)

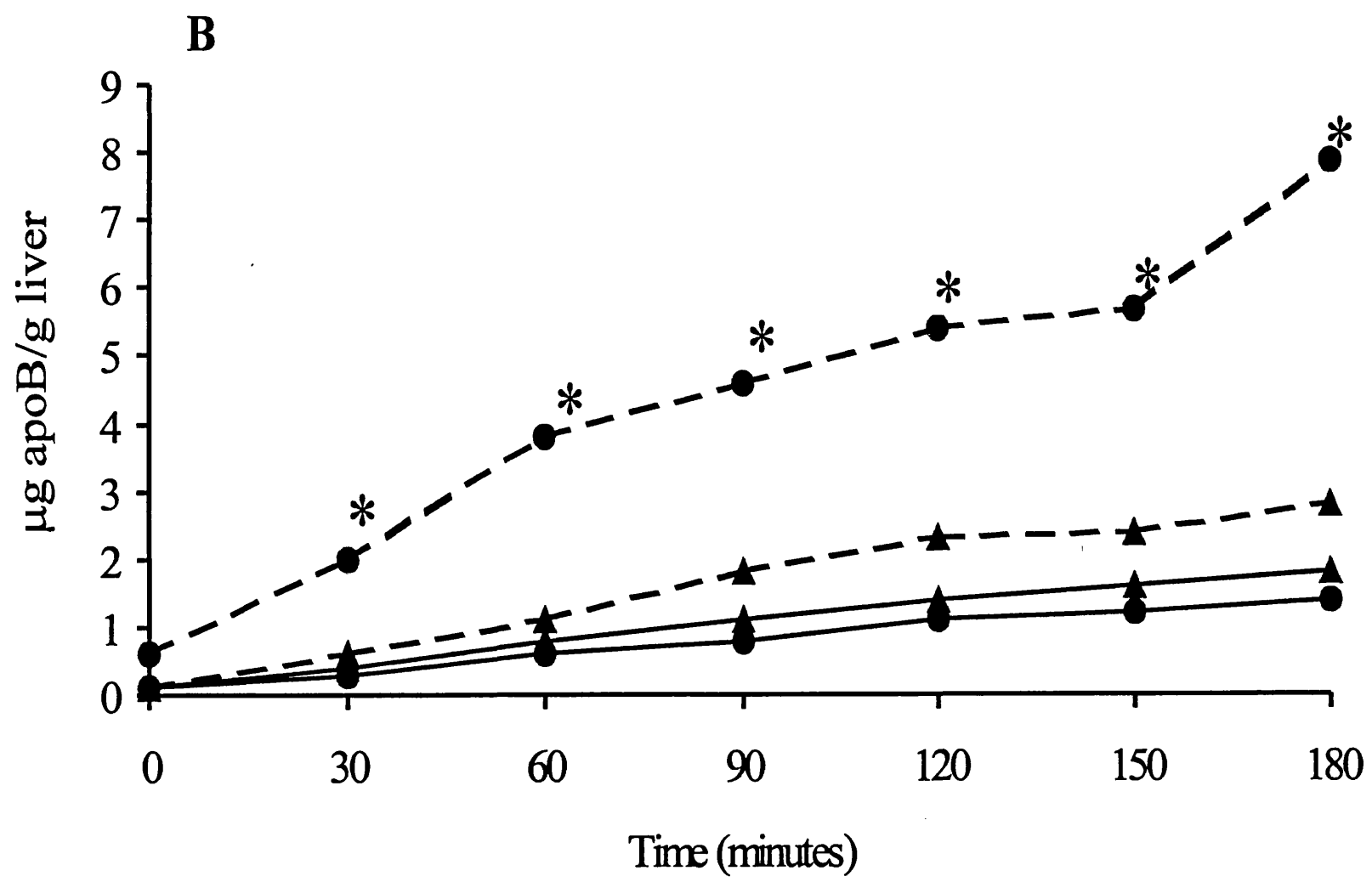

A
µg cholesterol/ g liver
0
5
10
15
20
25
30
35
40
0
30
60
90
120
150
180
Time (minutes)

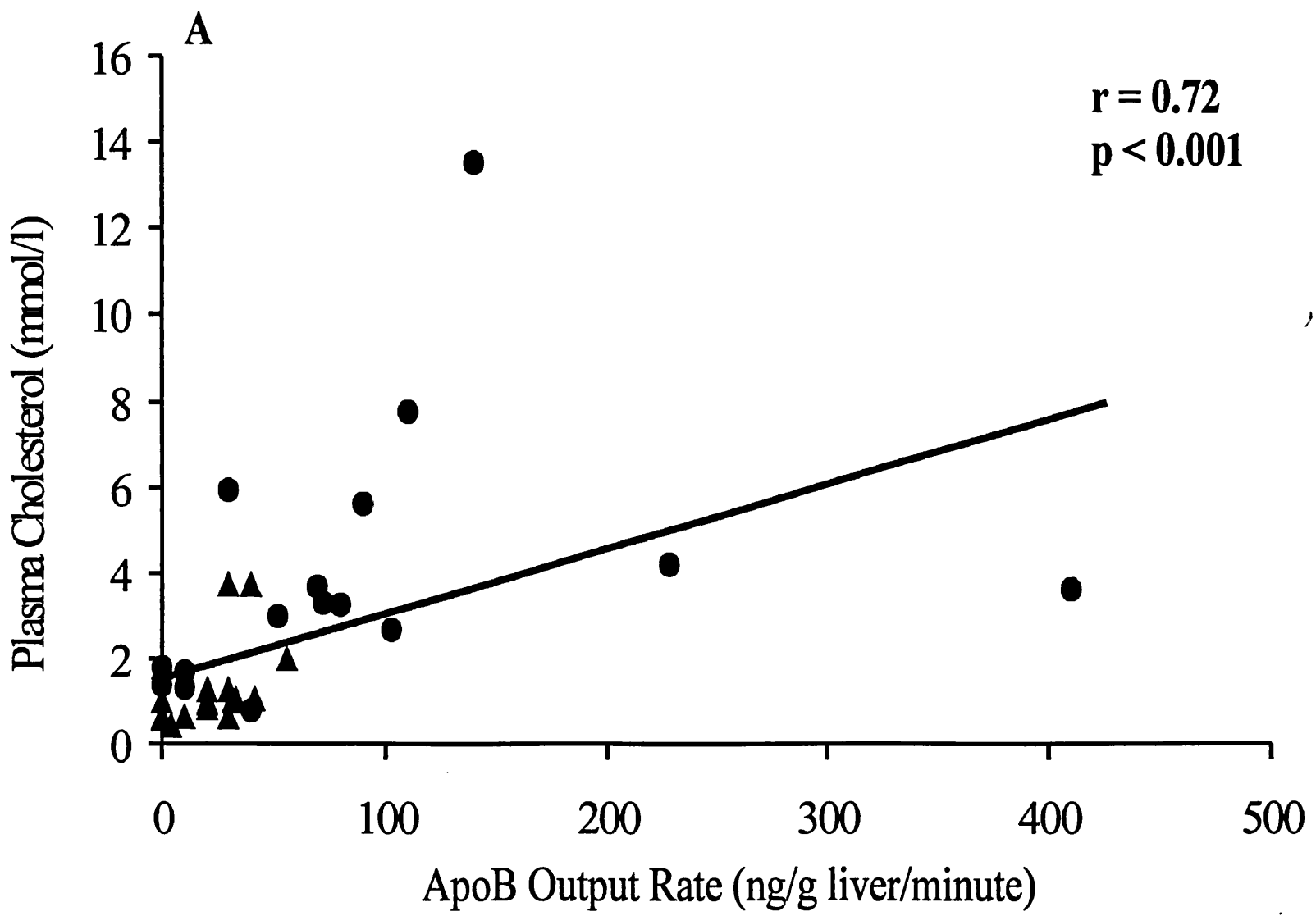

C
µg triglyceride/g liver
0
20
40
60
80
100
120
0
30
60
90
120
150
180
Time (minutes)

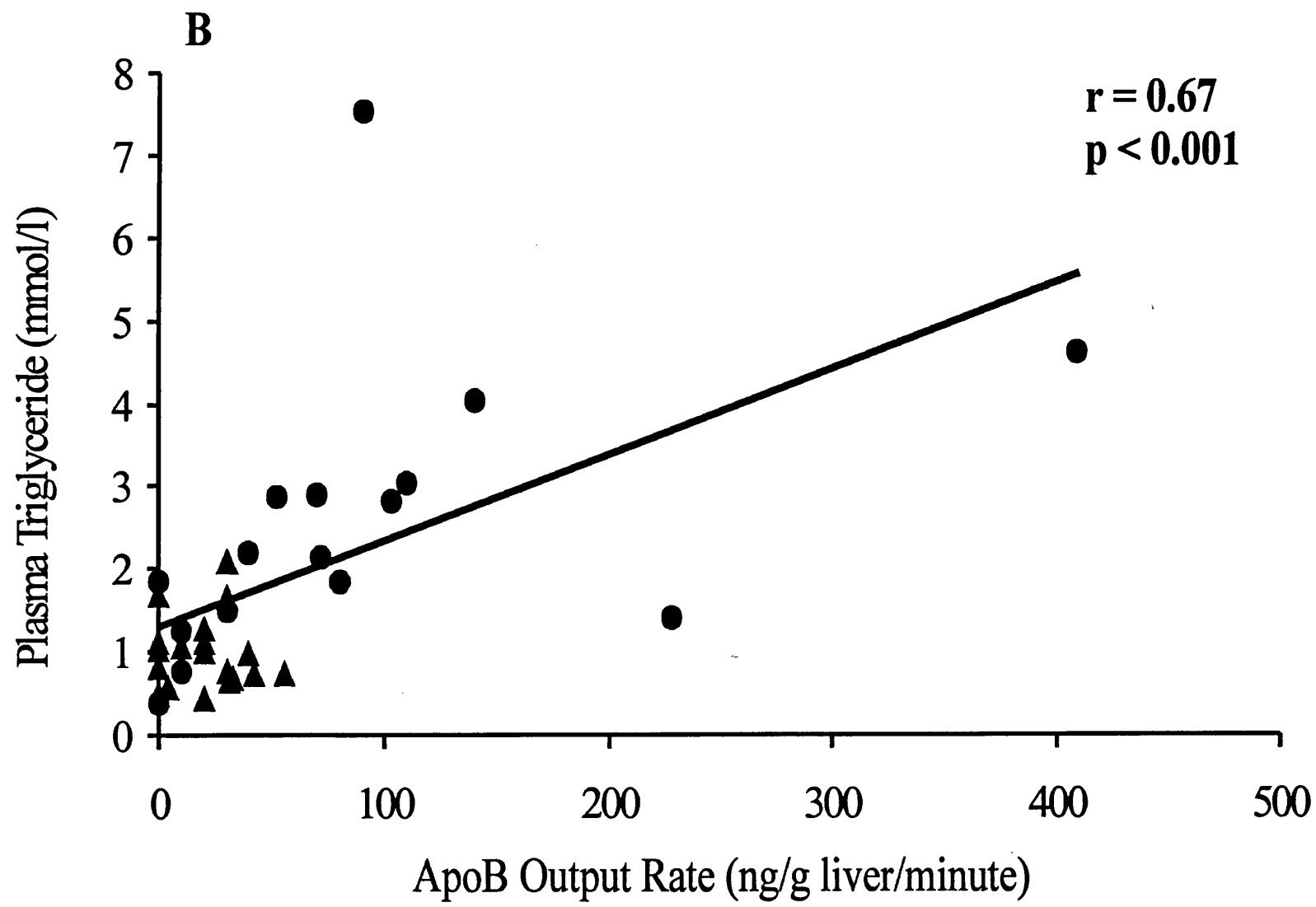

B
µg cholesterol/ g liver
0
10
20
30
40
50
0
30
60
90
120
150
180
Time (minutes)


D
µg triglyceride/g liver
0
10
20
30
40
50
60
70
80
90
0
30
60
90
120
150
180
Time (minutes)


A
µg apoB/g liver
0
2
4
6
8
10
12
0
30
60
90
120
150
180
Time (minutes)


B
µg apoB/g liver
0
1
2
3
4
5
6
7
8
9
0
30
60
90
120
150
180
Time (minutes)


A
r = 0.72
p < 0.001
Plasma Cholesterol (mmol/l)
0
2
4
6
8
10
12
14
16
0
100
200
300
400
500
ApoB Output Rate (ng/g liver/minute)


B
r = 0.67
p < 0.001
Plasma Triglyceride (mmol/l)
0
1
2
3
4
5
6
7
8
0
100
200
300
400
500
ApoB Output Rate (ng/g liver/minute)