THE EFFECTS OF PLASMA FACTORS ON THE REMODELLING AND METABOLISM OF HIGH DENSITY LIPOPROTEINS *IN VIVO*

A thesis submitted by
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Abstract of the thesis

Epidemiological studies have demonstrated an atheroprotective role of high density lipoproteins (HDL). This thesis is concerned with the remodelling and kinetics of HDL in plasma. Reconstituted HDL (rHDL) were prepared from their constituents and provided a mean of incorporating radiolabelled constituents into HDL for *in vivo* kinetic studies. When injected into wild-type rabbits, lipid-free ^{125}I-apoA-I or ^{125}I-apoA-I in pre-β-migrating discoidal rHDL rapidly appeared in α-migrating particles and decayed with the same fractional catabolic rate (FCR) as when they were injected as a component of spherical rHDL. Spherical rHDL did not change in size when they were injected into wild-type rabbits but were reduced in size in hepatic lipase transgenic rabbits. The FCR of apoA-I in hepatic lipase (HL) transgenic rabbits were double that in wild-type rabbits. Spherical rHDL containing radiolabelled cholesteryl esters ([^{3}H]CE) or unesterified cholesterol ([^{3}H]UC) were injected into rabbits with and without treatment with a cholesteryl ester transfer protein (CETP) inhibitor. The total loss of cholesteryl esters (CE) from plasma remained unchanged in the presence of CETP inhibition but the total loss of CE from LDL was markedly reduced and was compensated by an increase in the loss of CE from HDL. The loss of UC was rapid in both treated and untreated animals and only a small proportion of injected UC was converted into CE in HDL. While the FCR of HDL UC was similar in both the treated and untreated animals, an increase in plasma HDL cholesterol by CETP inhibition was translated into an increase in the total UC loss out of plasma in treated animals.

In conclusion, (1) lipid-free apoA-I rapidly incorporates into preexisting α-migrating particles, (2) pre-β-migrating discoidal HDL are rapidly converted into α-migrating HDL, (3) the FCR of apoA-I is independent of the form in which it is introduced into plasma, (4) HL reduces the size of α-migrating HDL and increases the rate of catabolism of apoA-I, (5)

CETP inhibition leads to an increase in CE clearance via HDL without affecting the overall CE flux out of plasma, (6) the in vivo esterification of UC to CE in HDL is slow, (7) CETP inhibition probably increases the overall UC flux out of plasma, and (8) the results suggest that UC in HDL may serve an important role in reverse cholesterol transport *in vivo*.

Declaration

This work contains no material which has been accepted for the award of any other degree or diploma in any university and that, to the best of the student's knowledge and belief, the thesis contains no material previously published or written by another person, except where due reference is made in the text.

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Patrick Kee

Date: 21st May 2004

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Presentations, awards and publications

Presentations

Nov 2000 Annual Scientific Meeting, Australian Atherosclerosis Society, Adelaide, Australia

Study of the metabolism of HDL subfractions using radioactively labelled reconstituted high density lipoproteins

May 2001: Cardiovascular Registrars Research Forum, Melbourne, Australia

A Study on the metabolism of HDL subpopulations using radioactive labelled reconstituted HDL

Aug 2001: Scientific Meeting, Gladstone Institute of Cardiovascular Research, San Francisco, USA

Apolipoprotein A-I metabolism in hepatic lipase transgenic rabbits

Nov 2001 Annual Scientific Meeting, Australian Atherosclerosis Society, Fremantle

Apoliporotein A-I metabolism in hepatic lipase transgenic rabbits

Nov 2002 Scientific Meeting, Pfizer Global Research, Groton, USA

Effects of hepatic lipase and CETP on the remodelling and metabolism of high density lipoproteins

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Effects of hepatic lipase on the remodelling and metabolism of high density lipoproteins

Awards

Nov 2000	AMRAD Young Investigator Award (Australian Atherosclerosis Society)
Nov 2001	Aventis Young Investigator Award (Australian Atherosclerosis Society)
2001-2002	Pfizer Cardiovascular Research Grants Study title: Effect of hepatic lipase on the remodeling of high density lipoprotein in vivo.
2002-2003	National Heart Foundation Research Grants Study title: Metabolism of apoA-I and cholesterol in HDL in the presence or absence of hepatic lipase and cholesteryl ester transfer protein

Publications

Kee P, Rye K-A, Taylor JL, Barrett PHR, Barter PJ. Metabolism of apoA-I as lipid-free protein or as component of discoidal and spherical reconstituted HDLs: studies in wild-type and hepatic lipase transgenic rabbits. Arterioscler Thromb Vasc Biol. 2002;22:1912-1917.

Abbreviations

ABCA1	ATP-binding cassette transporter A1
Apo	apolipoprotein
BSA	Bovine serum albumin
CE	Cholesteryl esters
CETP	Cholesteryl ester transfer protein
CHD	Coronary heart disease
DETAPAC	Diethylenetriamine pentaacetic acid
DTNB	5,5'-Dithio-bis(nitrobenzoic acid)
EDTA-Na_2	Ethylenediaminetetraacetic acid, disodium salt
EL	Endothelial lipase
FCR	Fractional catabolic rate
FPLC	Fast performance liquid chromatography
HDL	High density lipoproteins
HL	Hepatic lipase
HL	Hepatic lipase
HUVEC	Human umbilical vein endothelial cells
IDL	Intermediate density lipoproteins
LCAT	Lecithin:cholesterol acyltransferase
LDL	Low density lipoproteins

LPL	Lipoprotein lipase
LRP	LDL-related protein
LSR	Lipolysis-stimulated receptor
MBP	Membrane binding protein
PAPC	1-palmitoyl-2-arachidonyl-*sn*-glycero-3-phosphocholine
PLPC	1-palmitoyl-2-linoleoyl-*sn*-glycero-3-phosphocholine
PLTP	Phospholipid transfer protein
POPC	1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine
RCT	Reverse cholesterol transport
rHDL	Reconstituted high density lipoproteins
SDS	Sodium dodecyl sulphate
$sPLA_2$	Type 2 secretory phospholipase A_2
SR-BI	Scavenger receptor-BI
TBS	Tris-buffered saline
TLC	Thin layer chromatography
TNF	Tumour necrosis factor
UC	Unesterified cholesterol
VLDL	Very low density lipoproteins

1 Introduction

This thesis is concerned with the remodelling and the kinetics of high density lipoproteins (HDL) in plasma. The main reasons for studying HDL is to understand their role in the process of reverse cholesterol transport (RCT), the pathway that may protect against the development of atherosclerosis. However, before focussing on HDL, it is necessary to provide an overview of plasma lipoproteins and their role in the development of atherosclerosis.

1.1 Plasma lipoproteins and lipid transport

1.1.1 Overview

There are several classes of plasma lipoproteins. Collectively, they function to transport cholesterol and triglyceride between different tissues (Figure 1.1). Triglyceride and the esterified form of cholesterol (cholesteryl esters) are insoluble in an aqueous environment and occupy the hydrophobic core of the lipoprotein particle. Phospholipids and unesterified cholesterol (UC) cover the surface of the lipoprotein and serve as an interface between the aqueous environment on the surface and the hydrophobic core. The surface of the lipoprotein is covered by various apolipoproteins, which vary with the class of lipoprotein. Lipoproteins are classified according to their densities into chylomicrons, very low-density lipoproteins (VLDL), intermediate-density lipoproteins (IDLs), low-density lipoproteins (LDL), and high-density lipoproteins (HDL). The electrophoretic mobility, size, and apolipoprotein contents of these lipoproteins are summarised in Table 1.1 (Thompson 1983; Lund-Katz et al. 2003). The characteristics and functions of various apolipoproteins are summarised in Table 1.2.

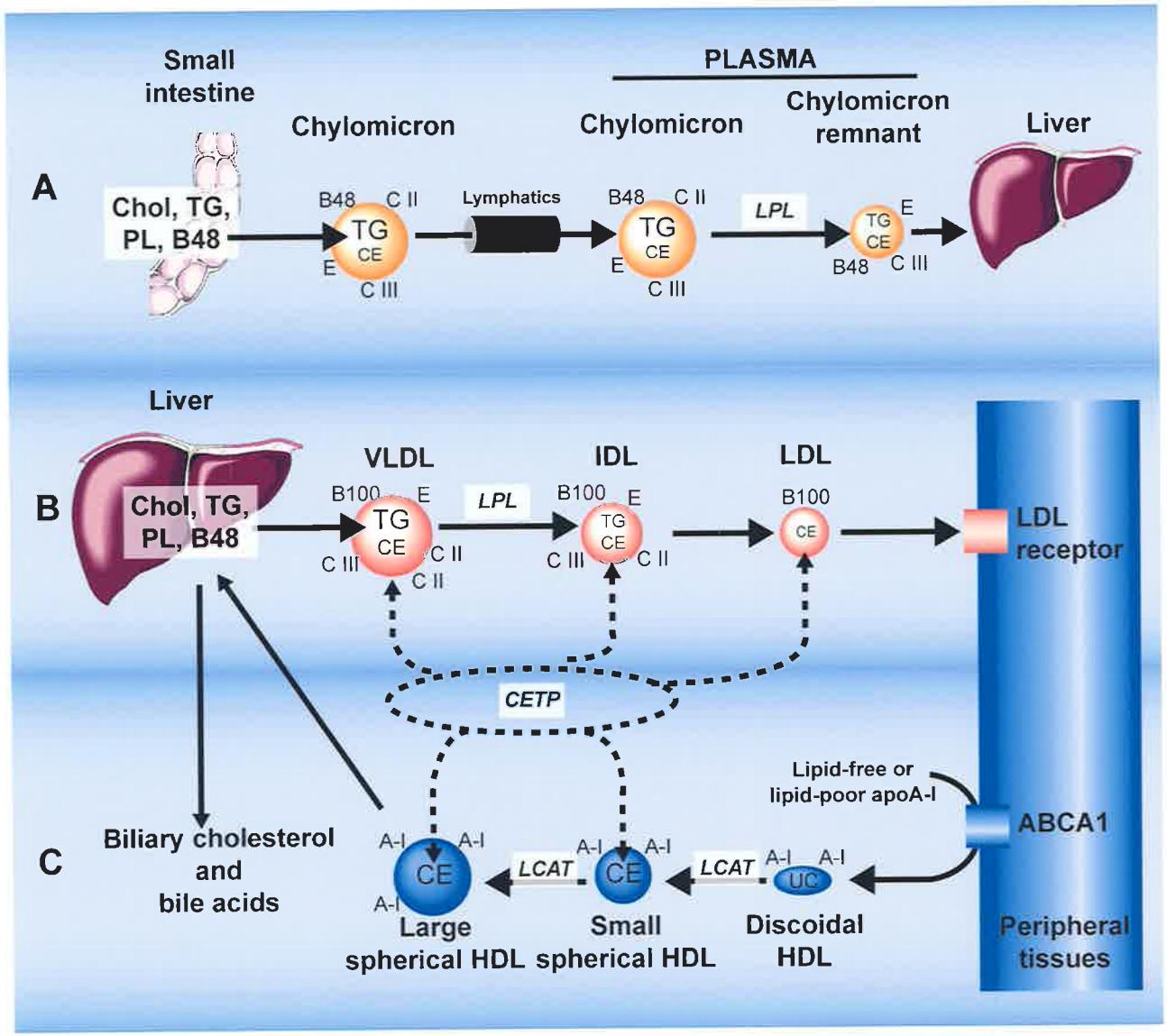

Figure 1.1. A. Transport of dietary lipids from the small intestine to the liver. B. Transport of endogenous hepatic lipids via VLDL, IDL and LDL to peripheral tissues ("forward" cholesterol transport). C. Transport of cell cholesterol from peripheral tissues to the liver via HDL (reverse cholesterol transport). Dotted lines denote the CETP-mediated redistribution of cholesteryl esters and triglyceride between HDL and apoB-containing lipoproteins. ABCA1=ATP binding cassette co-transporter A1; A-I=apoA-I; B48=apoB-48; B100=apoB-100; CETP=cholesteryl ester transfer protein; C II=apoC-II; C III=apoC-III; Chol=cholesterol; CE=cholesteryl esters; E=apoE; LCAT=lecithin:cholesterol acyltransferase; LPL=lipoprotein lipase; PL=phospholipids; TG=triglyceride;

Table 1.1 Physical-Chemical Characteristics of the Major Lipoprotein Classes (after Thompson, 1983 and Lund-Katz et al, 2003)

Lipoprotein	Density, g/dL	Molecular Mass, kDa	Diameter, nm	% of total lipids			% of total lipoprotein		
				TG	Chol	PL	UC	CE	Protein
Chylomicrons	0.95	400×10^3	75-1200	80-95	2-7	3-9	1-2	1-2	1-2
VLDL	0.95-1.006	$10\text{-}80 \times 10^3$	30-80	55-80	5-15	10-20	5-7	8-10	10
IDL	1.006-1.019	$5\text{-}10 \times 10^3$	25-35	20-50	20-40	15-27	13	20	18
LDL	1.019-1.063	2.3×10^3	18-25	5-15	40-60	20-28	15	30	25
HDL	1.063-1.210	$1.7\text{-}3.6 \times 10^3$	5-12	5-10	38-43	6-23	0.3-6	1.8-20	40-91
Pre-beta						6.6	0.3	1.8	91.3
HDL_2				4.2		31.3	5.8	17.6	40.2
HDL_3				3.4		22.7	2.8	14.7	55.5

Note: TG, triglyceride; Chol, the sum of unesterified cholesterol and cholesteryl ester; PL, phospholipid. The remaining percent composition is made up of the apoproteins. Percentages are expressed as percentage of weight of each lipoprotein subclass.

1.1.2 Chylomicrons

Chylomicrons transport dietary fat from the intestine to other tissues. After the ingestion of fat, pancreatic lipase digests triglyceride into fatty acids and glycerol in the small intestine. The fatty acids and glycerol are absorbed by the small intestinal enterocytes, reformed into triglyceride and packaged into chylomicrons for secretion into the circulation via the thoracic duct (Nilsson-Ehle et al. 1980; Goldberg 1996; Zechner 1997). The main apolipoprotein in chylomicrons is apoB-48 but chylomicrons also contain other apolipoproteins such as apoA-I and A-IV. In the circulation, chylomicrons acquire apoC-I, C-II, C-III and apoE from circulating HDL (Robinson and Quarfordt 1978; Mahley et al. 1984). ApoC-II activates lipoprotein lipase which promotes the hydrolysis of triglyceride into fatty acids and glycerol (Bengtsson and Olivecrona 1980). The hydrolysis of chylomicron triglyceride reduces the particle size and generates redundant surface remnants, which may serve as precursors of HDL (Redgrave and Small 1979; Tall et al. 1982). Chylomicrons are catabolized into chylomicron remnants, which are enriched in cholesteryl esters (CE). ApoE on chylomicron remnants facilitate their uptake by hepatic receptors such as LDL receptor (Brown and Goldstein 1986), LDL-related protein (LRP) (Beisiegel et al. 1991), VLDL receptor (Takahashi et al. 1992), lipolysis-stimulated receptor (LSR) (Yen et al. 1994), and membrane binding protein (MBP) 200 and 235 (Gianturco et al. 1994).

1.1.3 Very low density lipoproteins, intermediate density lipoproteins and low density lipoproteins

VLDL are secreted by the liver and carry triglyceride and cholesterol of hepatic origin into the circulation. VLDL contain apoB-100 as their main apolipoprotein but also contain a range of apoCs and apoE. Similar to chylomicrons, apoC-II in VLDL activates LPL and promotes

the hydrolysis of triglyceride into fatty acids and glycerol (Wang et al. 1993). The rate of hydrolysis of VLDL triglyceride is much slower than that of chylomicrons (Xiang et al. 1999), explaining the longer residence time of VLDL in the circulation (Havel 1995).

IDL are the catabolic product of VLDL and contain equal amount of triglyceride and cholesterol. The main apolipoproteins in IDL are apoB100 and apoE. IDL are normally present in very small amounts due to their rapid clearance from the circulation.

LDL are the major carriers of CE in man and are responsible for the delivery of cholesterol from plasma to both the liver and peripheral cells. The sole apolipoprotein on LDL is apoB-100, which mediates the binding of LDL to LDL receptors. This is followed by the internalisation of LDL into the cytoplasm, delivery of LDL to lysosomes and finally degradation by proteases to form small peptides and amino acids (Brown et al. 1981; Goldstein et al. 1985). LDL in their modified forms (acetyl-LDL, malondialdehyde-modified LDL, and oxidised LDL), can be taken up by type A scavenger receptor in macrophages and lead to lipid accumulation and foam cell formation (Goldstein et al. 1979; Brown and Goldstein 1983).

1.1.4 High density lipoproteins

HDL are the smallest (Stokes' diameter 7.4 to 12 nm) and densest ($1.063 < d < 1.25$ g/mL) lipoproteins. One of the best known functions of HDL is their ability to remove cholesterol from the peripheral tissues back to the liver, a process also known as reverse cholesterol transport (RCT) (Glomset 1968). This pathway is considered an important mechanism by which HDL protect against atherosclerosis. HDL will be considered in much greater detail below.

1.2 Plasma lipoproteins and atherosclerosis

1.2.1 Population studies

Elevated plasma cholesterol and LDL-cholesterol (LDL-C) levels are associated with increased future risk of coronary heart disease in population studies. The most notable large scale, prospective population studies include the Multiple Risk Factor Intervention Trial (MRFIT) (Martin et al. 1986; Stamler et al. 1986; Neaton et al. 1992), the Framingham Heart Study (Anderson et al. 1987) and the Prospective Cardiovascular Munster (PROCAM) Study (Assmann et al. 1998).

In contrast to the relationship between LDL cholesterol and CHD, an elevated plasma HDL-cholesterol (HDL-C) level has been shown to be a strong negative predictor of coronary heart disease in a prospective population studies. In some of the studies, the level of HDL-C was found to be the single most important predictor of future CHD events. The major prospective studies include the Framingham Heart Study (Castelli et al. 1986), the PROCAM study (Assmann et al. 1998), the Helsinki Heart Study (Manninen et al. 1992) and the MRFIT study (Stamler et al. 1986; Neaton et al. 1992). It has been concluded in these studies that, for every 0.025 mmol/L increase in HDL-C, the CHD risk is reduced by 2%-5% (Gordon et al. 1989). However, if the LDL-C level is low (< 2.5 mmol/L), a low HDL-C level may be of little importance (Bravo et al. 1989; Barter and Rye 1996).

1.2.2 Interventional studies

LDL-C lowering has been demonstrated as an effective therapeutic target in reducing the incidence of atherosclerotic cardiovascular disease in primary and secondary prevention settings. Large scale primary prevention trials include the Lipid Research Clinics-Coronary

Primary Prevention Trial (LRC-CPPT) (Lipid Research Clinics Program 1984a; Lipid Research Clinics Program 1984b), West of Scotland Coronary Prevention Study (WOSCOPS) (Shepherd et al. 1995), and Air Force/ Texas Coronary Atherosclerosis Prevention Study (AFCAPS/TexCAPS) (Downs et al. 1998). Large scale secondary prevention trials include the Scandinavian Simvastatin Survival Study (4S) (Scandinavian Simvastatin Survival Study Group 1994), the Cholesterol and Recurrent Events (CARE) study (Sacks et al. 1996), the Australian and New Zealand Long-term Intervention with Pravastatin in Ischaemic Disease (LIPID) study (LIPID study Group 1998). The Heart Protection Study (HPS) included both primary and secondary prevention arms (Heart Protection Study Group 2002). In general, a 1.0 mmol/L reduction in plasma total cholesterol translates into an approximately 20% reduction in the risk of future coronary events (Gould et al. 1998; LaRosa et al. 1999). The benefits are apparent in people with or without CHD and patients with a wide range of baseline total cholesterol and LDL-C levels. There is no evidence from these studies that specific groups (eg. the elderly, females and patients with hypertension, diabetes or non-coronary vascular disease) responded differently from the group as a whole.

The possibility that raising HDL may reduce the risk of CHD has been supported by the results of some studies in which fibrates were used as the active drug intervention, although these studies do not prove that the benefits relate solely to the increase in level of HDL. The more important studies of fibrates include the World Health Organisation (WHO) clofibrate study (Report from the Committee of Principal Investigators 1978) (although HDL was not measured in this study), the Helsinki Heart Study (Frick et al. 1987), the Veterans Administration-HDL Intervention Trial (VA-HIT) (Rubins et al. 1999) and the Bezafibrate Infarction Prevention (BIP) Study (The BIP Study Group 2000). Overall, treatment with fibrates reduces plasma triglyceride and raises HDL-C levels. In the Helsinki Heart Study,

fibrate therapy in the primary prevention setting significantly increased HDL-C by 10% and reduced the risk for nonfatal myocardial infarction and CHD death by 34%. In the VA-HIT study (Rubins et al. 1999), fibrate therapy in the secondary prevention setting in subjects with low levels of both LDL-C and HDL-C increased HDL-C by 6% and reduced nonfatal myocardial infarction or death from coronary causes from 21.7% to 17.3%. There is evidence that the benefit of fibrates is greater in people whose baseline triglyceride level is elevated (>1.5-2 mmol/L) (Frick et al. 1987). It should be noted that interpretation of the results of these fibrate studies is difficult because fibrates have potentially anti-atherogenic effects beyond their ability to raise HDL levels (Barbier et al. 2002). Furthermore, the magnitude of HDL elevation in these studies has only been modest and the mechanisms of HDL elevation by these agents have not been fully elucidated. It is possible that the benefits of fibrates in reducing the risk of CHD relate to effects on the metabolism of HDL that is not reflected by a change in plasma concentration. There is a clear need for much greater understanding of HDL metabolism, especially in an *in vivo* setting. The following sections address HDL structure function and metabolism in some detail.

1.3 High density lipoproteins

1.3.1 HDL lipids

The main lipids in HDL are phospholipids, cholesteryl esters (CE), unesterified cholesterol (UC) and triglyceride. These lipids are arranged in a hydrophobic core of CE and triglyceride surrounded by a surface monolayer of phospholipids, a small amount of UC and apolipoproteins.

1.3.1.1 Phospholipids

The major source of phospholipids in HDL is derived from the liver. The small intestine also contributes to the phospholipids pool in HDL in the form of transfers from chylomicrons. Given that the acyl chains of chylomicron phosphatidylcholines are of dietary origin, thus the dietary fat composition has a major influence on the phospholipids composition in HDL. Phospholipids are also integral components of cell membranes and there is considerable exchange of phospholipids between plasma lipoproteins and cell membranes. Phospholipids in HDL play an important role in maintaining non-polar lipids, such as triglycerides and cholesterol esters, in a soluble state (Jackson and Gotto 1974b; Jackson and Gotto 1974a). The most abundant phospholipids in human HDL are 1-palmitoyl-2-linoleoyl-*sn*-glycero-3-phosphocholine (PLPC), 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC) and 1-palmitoyl-2-arachidonyl-*sn*-glycero-3-phosphocholine (PAPC).

1.3.1.2 Unesterified cholesterol

Cholesterol is a sterol, possessing a four-ringed steroid nucleus and a hydroxyl group. The hydroxyl group in UC convey a slight polarity in the molecule and allows its interaction with the aqueous phase. Thus, UC in HDL is found mainly on the surface of the lipoproteins Unesterified cholesterol is a major component of cell membranes in most tissues and almost all tissues can synthesise cholesterol. Under normal circumstances, all newly synthesised unesterified cholesterol in HDL is derived from the liver and dietary source in the small intestine. Unesterified cholesterol in HDL can be esterified by the enzymes LCAT or ACAT or readily exchanged with other cell membranes.

1.3.1.3 Cholesteryl esters

More than two-thirds of the cholesterol in HDL is esterified. This esterification reaction renders CE non-polar and leads to the migration of CE into the core of HDL. The

predominant cholesterol esters in HDL are cholesterol linoleate and cholesterol oleate. A major proportion of cholesteryl esters in HDL is formed via the action of LCAT in plasma but a minor proportion is generated via the action of ACAT in the small intestine and the liver.

1.3.1.4 Triglyceride

Triglycerides are fatty acid esters of glycerol, usually containing a mixture of two or three different fatty acids. Triglyceride is a minor component of HDL and a large proportion is derived from exchange of neutral lipids between HDL and triglyceride-rich lipoproteins via the action of CETP in plasma.

1.3.2 HDL apolipoproteins

1.3.2.1 ApoA-I

ApoA-I accounts for about 70% of the protein in HDL. Human apoA-I is a 243-residue polypeptide that has its origin in the liver and intestine (Miller et al. 1983). ApoA-I sequences from a number of other species have also been determined, with sizes ranging between 258 and 267 amino acids (Frank and Marcel 2000). The secondary structure of the lipid-binding domain is dominated by 10 helical repeats that have been classified as amphipathic α-helices. Eight of these repeats are 22 residues in length, while two repeats are 11 residues in length, with most of these amphipathic α-helical repeats punctuated by a proline residue at the beginning of the repeat (Segrest et al. 1974; Segrest et al. 1994). The hydrophobic face of the apoA-I α-helices is oriented parallel to the lipoprotein lipid surface and penetrate no deeper than the ester linkages of the phospholipids, while the hydrophilic face is exposed to the aqueous phase (Segrest et al. 1994). ApoA-I exists physiologically in a lipid-free (or lipid-poor) form or as a component of discoidal or spherical HDL as described below.

1.3.2.2 ApoA-II

ApoA-II accounts for about 25% of the protein in HDL. It is synthesised in the liver and released into the circulation as a component of preformed lipid complexes and possibly also as lipid-free protein. Human apoA-II exists as a dimer of two 77-amino acid chains linked by a disulphide bridge (Brewer et al. 1972; Brewer et al. 1986). The fact that lipid-free apoA-II has not been identified in plasma may relate to its high affinity for lipids and its ability to displace apoA-I from HDL (Rosseneu et al. 1981; Edelstein et al. 1982).

1.3.2.3 Other apolipoproteins

HDL also contain small amounts of apoA-IV, the C-apolipoproteins, apoD, apoE and apoJ.

1.3.3 HDL structure

1.3.3.1 Discoidal HDL (nascent particles)

There is evidence that HDL begin their existence as nascent, discoidal particles. Discoidal HDL comprise a phospholipid bilayer surrounded by two or more molecules of apoA-I. HDL discs have a prebeta electrophoretic and as outlined below are rapidly converted into the alpha-migrating spherical HDL that predominate in normal plasma. Three models have been proposed to account for the structure of apoA-I on HDL particles: the "belt" model (Segrest et al. 1999; Segrest et al. 2000), the "helical hairpin" model (Rogers et al. 1998a; Rogers et al. 1998b) and the "picket fence" model (Phillips et al. 1997) (Figure 1.2). In the "belt" model, two apoA-I molecules are wrapped together in an anti-parallel belt around a discoidal bilayer of phospholipids and cholesterol (Segrest et al. 1999; Segrest et al. 2000). In the "helical hairpin" model, the two apoA-I molecules position opposite to each other but each of them occupies only half of the outer edge of the disc (Rogers et al. 1998b). In the "picket fence"

model, the amphiphatic α-helices are perpendicular to the plane of the disc (Phillips et al. 1997).

1.3.3.2 Spherical HDL (mature particles)

Discoidal HDL are transformed into spherical HDL as LCAT converts more UC to CE and partitions the cholesterol from the surface into the core of the particles. Spherical HDL consist of a surface monolayer of apolipoproteins, phospholipids and a small amount of UC surrounding a hydrophobic core of CE and small amount of triglyceride.

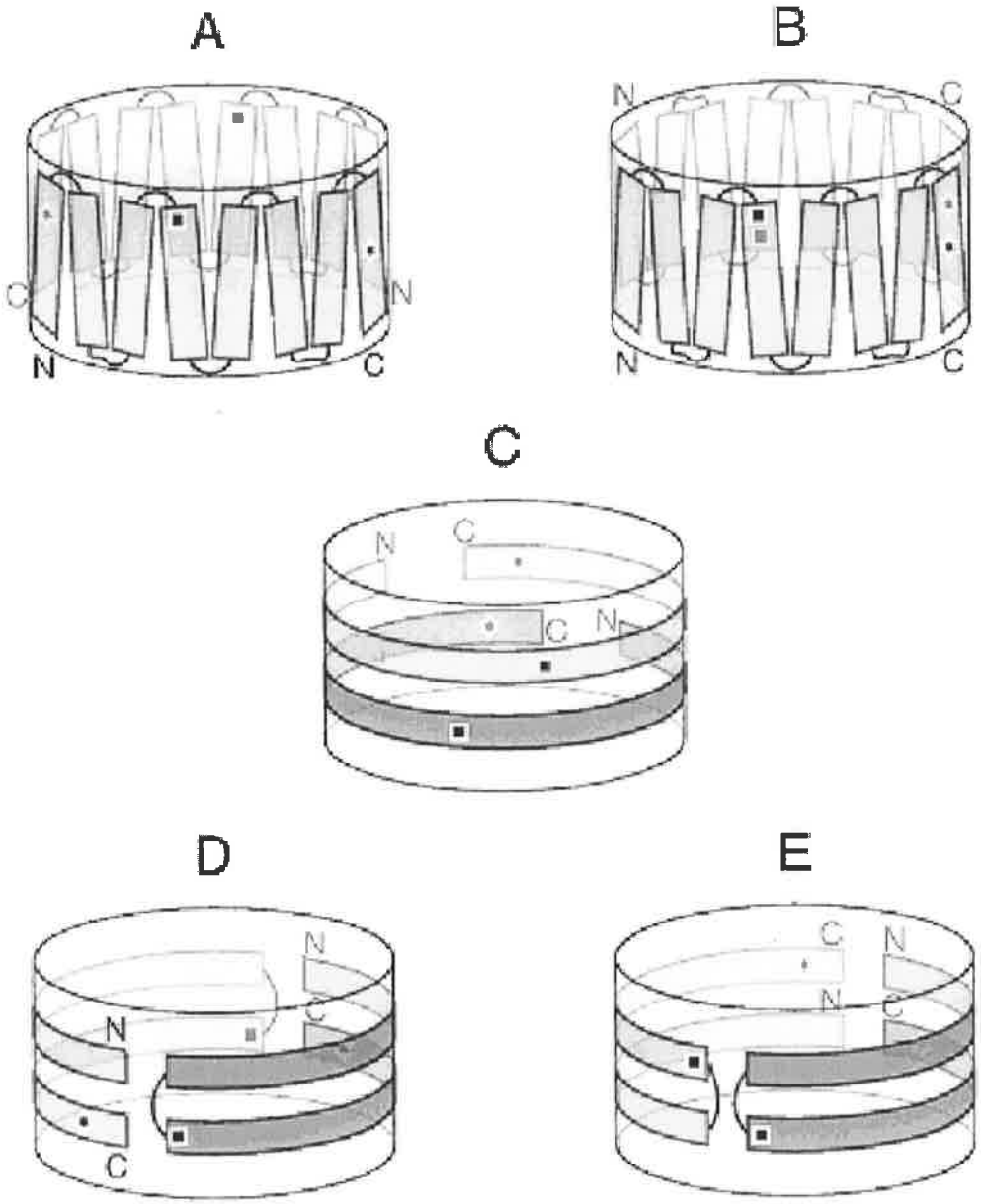

Figure 1.2: Diagrams of rHDL models showing the positions of the labels in the sequences of the two apoA-I molecules. A: Head-to-tail picket fence model; B: Head-to-head picket fence model; C: Belt model; D: Head-to-tail helical hairpin model; E. Head-to-head helical hairpin model (Source: Tricerri MA, Behling AK, Sanchez SA, Bronski, J and Jonas A. Arrangement of Apolipoprotein A-I in Reconstituted High-Density Lipoprotein Disks: An Alternative Model Based on Fluorescence Resonance Energy Transfer Experiments. Biochemistry 2001, 40, 5065-5074).

1.3.4 HDL subpopulations

1.3.4.1 Separated by density

HDL subpopulations can be classified according to their density as separated by density gradient ultracentrifugation into HDL_1 (d = 1.050 to 1.063 g/mL), HDL_2 (d = 1.063 to 1.125 g/mL) and HDL_3 (d = 1.125 to 1.21 g/mL). A minor subpopulation is the very high density lipoproteins (d = 1.21 to 1.25g/mL).

1.3.4.2 Separated by electrophoresis

HDL subpopulations can also be identified by their electrophoretic mobility on agarose gel electrophoresis (Sparks and Phillips 1992).Alpha-migrating HDL are spherical particles and account for the majority of HDL in plasma. They include the HDL_1, HDL_2 and HDL_3 subpopulations. Preβ-migrating HDL include discoidal HDL (and also lipid-free or lipid-poor apoA-I); they account for a small fraction (5-15%) of HDL in plasma. Pre-β HDL are more abundant in peripheral lymph (Asztalos et al. 1993).

1.3.4.3 Separated by immunoaffinity chromatography

HDL subpopulations can also be identified by their apoA-I and apoA-II composition as separated by immunoaffinity chromatography. Two main subpopulations can be identified: one consists of apoA-I without apoA-II (A-I HDL) and the other includes particles containing both apoA-I and apoA-II (A-I/A-II HDL) (Cheung and Albers 1982). There is also a minor subpopulation that consists of apoA-II alone (A-II HDL). ApoA-I is approximately equally distributed between A-I HDL and A-I/A-II HDL. However, more than 90% of apoA-II is found in A-I/A-II HDL (Bekaert et al. 1992). Most of the A-I/A-II HDL are found in the

HDL_3 density range, while A-I HDL are present in both HDL_2 and HDL_3 (Cheung and Albers 1982). The origins of these HDL subpopulations will be discussed later.

1.3.5 HDL functions

Every day, about 9mg of cholesterol per kilogram body weight is synthesised by the extrahepatic tissues and must be transferred to the liver for excretion or recycling (Dietschy et al. 1993). The most recognised function of HDL relates to their role in returning cholesterol from peripheral tissues back to the liver. Other functions include anti-oxidant, anti-inflammatory, anti-thrombotic and endothelial stabilization properties.

1.3.5.1 Reverse cholesterol transport

A role of HDL in RCT was first proposed by Glomset (Glomset 1968). There are several discrete steps in this pathway (Figure 1.1).

1.3.5.1.1 Efflux of unesterified cholesterol from extrahepatic tissues to HDL

The efflux of UC from extrahepatic cells is the first step in RCT. It involves the desorption of cell cholesterol from plasma membrane and the transfer of cell cholesterol to extracellular compartments. The primary plasma acceptor of cell cholesterol is lipid-free or lipid-poor apoA-I although other apolipoproteins and discoidal HDL are also known to function as initial acceptors (Castro and Fielding 1988). Three mechanisms have been proposed to describe the efflux of cholesterol from cell membrane, these include: (1) passive aqueous diffusion of cholesterol down a chemical gradient (Rothblat et al. 1999), (2) efflux of cholesterol via a scavenger receptor-BI (SR-BI) mediated pathway (Ji et al. 1997; de la Llera-Moya et al. 1999), and (3) active cholesterol efflux by the ATP-binding cassette transporter A1 (ABCA1) (Oram and Vaughan 2000; Wang et al. 2000; Haidar et al. 2001).

Aqueous diffusion of cholesterol occurs down a concentration gradient from cell membrane to HDL. This process is considered to be rather inefficient and contribute to a small fraction of net cholesterol efflux to HDL (Rothblat et al. 1999). It involves the desorption of cholesterol molecules from the plasma membrane, diffusion of cholesterol through the aqueous phase, and subsequent incorporation into phospholipid-containing acceptor particles. It does not require the binding of HDL to specific membrane sites.

On the other hand, binding of HDL to cell surface can occur in cells expressing SR-BI. The binding of HDL to cell surface alone, however, is not sufficient to mediate cholesterol efflux. Rather, SR-BI mediates alterations to membrane UC domains and leads to an enhanced bi-directional transfer of UC between cells and HDL (de la Llera-Moya et al. 1999). The level of expression of SR-BI in various cell types directly correlates with the rate of efflux of cell cholesterol mediated by HDL (Ji et al. 1997).

The most efficient cholesterol efflux pathway is the ABCA1-mediated efflux of unesterified cholesterol and phospholipids from cell membranes to lipid-free or lipid-poor apoA-I in the extracellular space (Oram and Yokoyama 1996; von Eckardstein et al. 2001). This pathway was first discovered when studying subjects with Tangier Disease (Luciani et al. 1994; Lawn et al. 1999; Rust et al. 1999). The absence of ABCA1 expression in these subjects results in a virtual absence of plasma HDL and the accumulation of cholesterol in peripheral organs. The exact mechanism of cholesterol efflux by ABCA1 is still being elucidated. ABCA1 appears to form a channel within the plasma membrane through which phospholipids and cholesterol are transferred from the inner leaflet to the outer leaflet of the membrane (Bungert et al. 2001).

Different species of apolipoproteins can accept cholesterol from cells. While lipid-free and lipid-poor apoA-I are the preferred acceptors of cell cholesterol in the process of cell cholesterol efflux (Oram and Yokoyama 1996), other apolipoproteins such as apoA-II (Forte et al. 1995) and apoE (Huang et al. 1994) and albumin (Zhao and Marcel 1996) can also serve as acceptors of cell cholesterol.

Discoidal HDL have also been shown to promote the efflux of cholesterol from cells (Fielding and Fielding 1995) and to reduce the cholesterol content of foam cells (Miyazaki et al. 1992). The ability of discoidal HDL to promote cell cholesterol efflux appears to correlate positively with the size of the lipoprotein particle (Zhao et al. 1996).

1.3.5.1.2 Fate of unesterified cholesterol after incorporation into HDL

Unesterified cholesterol incorporated into HDL has four potential fates. It may be transferred to other lipoproteins, taken up by tissues, including the liver, exchange with erythrocytes or converted to CE by LCAT.

Unesterified cholesterol is exchangeable between plasma lipoproteins and between lipoproteins and a variety of tissues, especially the liver. The exchange of UC between lipoproteins is mediated by passive diffusion in plasma.

There are two possible pathways for the uptake of HDL UC by the liver. Due to the freely exchangeable nature of UC, HDL UC can passively exchange with the cholesterol pool in the cell membranes of hepatocytes. Alternatively, HDL UC can be actively taken up by the liver following binding of the particle to hepatic SR-BI receptors (Ji et al. 1999). It has been reported that SR-BI accounts for a substantial proportion of the clearance of HDL UC from

plasma (Ji et al. 1999). Studies have also demonstrated the preferential use of UC in HDL rather than VLDL/LDL for biliary secretion and excretion (Halloran et al. 1978; Esnault-Dupuy et al. 1987; Bravo et al. 1989; Bravo and Cantafora 1990). In a study using bile-fistula rats, a large proportion of the injected UC in HDL_2 and HDL_3 was taken up by the liver and excreted as bile salt (from HDL_2) or unchanged as UC (from HDL_3) in the bile (Bravo et al. 1989). Similar results were also observed in humans (Halloran et al. 1978). Those results were also confirmed by a liver infusion study using a non-exchangeable steroid, sitostanol, obtained from patients with phytosterolaemia (Robins and Fasulo 1997). Unesterified cholesterol is rapidly transported and excreted as a component of biliary cholesterol (Schwartz et al. 1978b). Its appearance in the bile occurs as early as 5-10 mins after its injection into the circulation (Robins and Fasulo 1997).

Erythrocytes represent a major exchangeable pool of UC in the circulation. When introduced into plasma as a component of lipoproteins, a proportion of the radiolabelled UC is rapidly incorporated into erythrocyte membranes (Quarfordt and Hilderman 1970; Schwartz et al. 1978b; Lund-Katz et al. 1982). The rate and magnitude of the transfer of radiolabelled UC from lipoproteins to erythrocyte membranes appear to vary according to the surface composition and partitioning of UC among various lipoprotein classes (Lund-Katz et al. 1982; Schwartz et al. 1982).

A proportion of UC in discoidal HDL is converted to CE by LCAT. As lipid-poor apoA-I acquires phospholipids and UC it is converted into a discoidal HDL particle. This particle has limited capacity to transport UC. The conversion of UC to CE by LCAT and the subsequent partitioning of CE into the lipoprotein core depletes the surface of cholesterol and generate a cholesterol concentration gradient which facilitates the uptake of more UC from cell

membranes to HDL (Jonas 1991). The rates of esterification by LCAT are estimated at 30-50 μmol/L/hr in rabbits (Rose 1972) and 50-120 μmol/L/hr in man (Glomset 1968).

The LCAT reaction accounts for virtually all of the cholesteryl esters transported in HDL (Jonas 1991). Once formed, the cholesteryl esters in HDL can be selectively taken up by hepatocytes and steroid hormone-producing cells without internalising HDL proteins in a process involving SR-B1 (Glass et al. 1983; Acton et al. 1996; Krieger and Kozarsky 1999) or can be transferred to other lipoproteins by CETP.

1.3.5.1.3 Selective uptake of HDL cholesteryl esters

The mechanism for the selective uptake of cholesterol esters by SR-BI is unclear. The binding of apoA-I to SR-BI is not sufficient to ensure efficient lipid transfer (Liu et al. 2002). The fact that SR-BI receptor is more efficient than CD36 (another member of the SR-B gene family and share structural similarities to SR-BI) in selective cholesterol esters uptake suggests that cofactor(s) may be required in the process (Connelly et al. 1999). Such cofactors may include HL (Lambert et al. 1999; Lambert et al. 2000), apoE (Arai et al. 1999) and CETP (Collet et al. 1999). SR-BI accounts for more than 90% of selective HDL CE uptake in rodents, which do not express CETP (Glass et al. 1983; Stein et al. 1983), and about 20% of HDL CE uptake in rabbits, a species that does express CETP (Goldberg et al. 1991). In CETP-rich animals, the transfer of CE from HDL to apoB-containing lipoproteins may reduce the availability of CE in HDL for selective uptake by SR-BI. A subset of HDL particles that contain apoE can be taken up as a whole particle by apoE receptors and other HDL receptors (Fidge 1999).

1.3.5.1.4 Transfer of HDL cholesteryl esters from HDL to VLDL/LDL.

In animals expressing CETP in plasma, a proportion of cholesteryl esters in HDL are transferred to VLDL and LDL in exchange for triglyceride. The actions of CETP lead to a redistribution of cholesteryl esters and triglyceride in the donor and acceptor lipoproteins. CETP catalyses both net transfer and heteroexchange of neutral lipids between donor and acceptor particles, and that these processes can be differentiated. Under usual condition, CETP-mediated lipid transfer is rapid relative to the catabolism of HDL and LDL (Barter et al. 1982). If the plasma activity of CETP is increased, the rate of lipid transfer between HDL and LDL increases without altering the distribution of lipids in the two lipoprotein fractions. On the other hand, if the plasma activity of CETP is reduced, it may reach a point that its activity may become rate-limiting. Under that condition, the reduced plasma CETP activity may become significant in limiting the re-distribution of cholesteryl esters between HDL and LDL. The rate of transfer also depends on the concentrations of cholesteryl ester and triglyceride pools between donor and acceptor lipoproteins (Lagrost 1994). When the concentration of VLDL is increased, the quantity of CETP is certainly an important determining factor in the rate of transfer of cholesteryl esters out of HDL (Mann et al. 1991).

1.3.5.1.5 Fate of cholesteryl esters transferred to VLDL and LDL

Once HDL CE are transferred to VLDL and LDL, CE can be removed from the circulation as a component of the LDL that is taken up by LDL receptors in the liver and other tissues (Tall et al. 2000). In CETP-rich species such as rabbits, this indirect pathway of RCT via VLDL/LDL accounts for the removal of up to 70% of the CE that originate in HDL (Goldberg et al. 1991).

1.3.5.1.6 Secretion in bile

Compartmental analysis suggest that HDL UC may enter a cholesterol compartment in the liver that is distinct from that of HDL CE (Scobey et al. 1989). Two possible mechanisms have been proposed for the transport of HDL UC from the blood to the bile. One involves a membrane transporter known as phospholipid flippase, a phospholipid vesicle that transports UC from the sinusoidal membrane to the canalicular membrane of the hepatocytes (Smit et al. 1993; Elferink et al. 1997). Another process may involve a bile salt export pump that stimulates the secretion and solubilisation of HDL UC into bile (Verkade et al. 1995). In contrast, CE in HDL is selectively taken up by SR-BI into the hepatocytes. Upon binding of HDL to SR-BI, SR-BI and HDL undergo endocytosis into the early endosome system in which independent sorting of apolipoproteins and CE (also known as transcytosis) occurs (Silver et al. 2001). Cholesteryl esters are then translocated to the canalicular membrane and released into the bile. However, it is now clear that SR-BI is only one of the mechanisms for the excretion of HDL CE into bile, ABC transporters such as ABCG5, ABCG8 and MDR2 are also important for the secretion of HDL CE into bile (Berge et al. 2000; Lee et al. 2001).

1.3.5.1.7 Rate limiting steps in RCT

The inverse relationship between plasma HDL cholesterol concentration and the incidence of premature CHD does not necessarily indicate a causal relationship between the two. In fact, in the absence of other cardiac risk factors, subjects with HDL deficiency as a result of apoA-I or apoA-II deficiency (Assmann et al. 1993), Tangier Disease (Serfaty-Lacrosniere et al. 1994), LCAT deficiency or fish-eye disease (Santamarina-Fojo et al. 2000) are not at especially high risk of premature CHD.

It is uncertain whether any of the steps in RCT are rate limiting under normal physiological conditions. An absence or deficiency of apoA-I (Jolley et al. 2000), HDL (Groen et al. 2001), ABCA1 (Groen et al. 2001) or LCAT (Gylling and Miettinen 1992) does not alter the net cholesterol flux from extrahepatic tissues to the liver into the bile. Even hepatic SR-BI may not represent a rate limiting step in RCT. Although targeted mutation of hepatic SR-BI markedly reduces the selective uptake of CE (Varban et al. 1998) and UC (Ji et al. 1999) from HDL, the excretion of cholesterol in bile is not significantly reduced (Ji et al. 1999). These observations suggest that RCT is a fundamental pathway for the survival of the organism. Consequently, it is possible that multiple levels of redundancy may exist to compensate for the loss of one of the RCT pathways. For example, (1) several plasma apolipoproteins other than apoA-I and even albumin have the ability to accept cell cholesterol in apoA-I deficiency, (2) SR-BI-mediated uptake and passive diffusion appear to compensate for the lack of ABCA1 expression in Tangier Disease, and (3) there is a clear direct uptake of HDL UC by the liver in the absence or deficiency of LCAT expression.

As stated above, HDL also have other potential anti-atherogenic properties beyond their involvement in RCT.

1.3.5.2 Anti-inflammatory properties of HDL

One of the earliest events of atherosclerosis is the expression of adhesion molecules and the adhesion of leukocytes to endothelial cells (Poston et al. 1992; van der Wal et al. 1992). HDL can inhibit the expression of adhesion molecules, VCAM-1, ICAM-1 and E-selectin in activated human umbilical vein endothelial cells (HUVECs) (Cockerill et al. 1995; Calabresi et al. 1997). TNF-α is a potent activator of endothelial cell adhesion molecule expression via the sphingosine kinase pathway (Xia et al. 1998) and have been shown to inhibit TNF-

stimulated sphingosine kinase activity (Xia et al. 1999). The ability of HDL to inhibit sphingosine kinase is related to the phospholipid composition rather than the apolipoprotein content of the HDL (Ashby et al. 1998; Baker et al. 2000).

1.3.5.3 Anti-oxidant properties of HDL

The oxidation of LDL and their subsequent uptake by monocyte-derived macrophages has been regarded as an important initiating event in the development of atherosclerosis (Diaz et al. 1997). HDL have been shown to inhibit LDL oxidation (Hessler et al. 1979; Parthasarathy et al. 1990). The anti-oxidant effects of HDL have been attributed to the anti-oxidative properties of apoA-I and the presence of paraoxonase (Watson et al. 1995; Aviram et al. 1998).

1.3.5.4 Anti-thrombotic properties of HDL

HDL inhibit the synthesis of platelet-activating factor by endothelial cells (Sugatani et al. 1996), regulate coagulation in plasma (Epand et al. 1994), and stimulate prostacyclin synthesis in endothelial cells (Fleisher et al. 1982). HDL have also been reported to inhibit tissue factor (Rosenson and Lowe 1998).

1.3.5.5 Endothelial stabilisation by HDL

Endothelial dysfunction is one of the first hallmarks in the pathogenesis of atherosclerosis (Vanhoutte 1997). Endothelium-dependent vasorelaxation in response to acetylcholine, serotonin, histamine, and bradykinin is considerably decreased in animal models and in patients with hypercholesterolaemia (Ludmer et al. 1986; Hodgson and Marshall 1989; Werns et al. 1989; Shimokawa 1999). In hypercholesterolaemic patients, reconstituted HDL infusion rapidly normalises endothelial function as measured by acetylcholine induced forearm-

mediated dilatation, a receptor-independent marker of nitric oxide-dependent vasodilatation (Spieker et al. 2002). HDL and apoA-I maintain endothelial cell viability and integrity (Tamagaki et al. 1996) and prevent endothelial apoptosis induced by oxidised LDL (Suc et al. 1997), TNF-α (Sugano et al. 2000) and triglyceride-rich lipoproteins (Speidel et al. 1990). HDL also prevent endothelial cell damage and necrosis resulting from the activation of the complement system (Rosenfeld et al. 1983; Packman et al. 1985).

1.3.6 HDL metabolism

1.3.6.1 Origin of HDL

The precursors of A-I HDL are thought to be nascent discoidal particles containing two molecules of apoA-I complexed to phospholipids and unesterified cholesterol (Hamilton et al. 1976). These discoidal particles are secreted by the liver and the intestines or generated as redundant surface remnants from hydrolysis of chylomicron by lipoprotein lipase (Redgrave and Small 1979; Tall et al. 1982). These particles are excellent substrates for LCAT, which rapidly esterifies the UC to form CE. The newly formed CE partitions into the lipoprotein core in a process that converts the discoidal particles into spherical A-I HDL (Nichols et al. 1985). The high degree of reactivity of these discoidal particles with LCAT may explain why most of the circulating HDL are spherical particles.

Discoidal apoA-II/phospholipid complexes are also secreted into plasma from the liver. However, unlike discoidal A-I HDL, discoidal A-II HDL do not react with LCAT to form spherical HDL. The origin of the minor subpopulation of spherical A-II HDL is therefore unknown. Most of the apoA-II circulating in plasma exists as a component of HDL that contain both apoA-I and apoA-II (A-I/A-II HDL).

One mechanism for the formation of A-I/A-II HDL involves the fusion of A-I HDL with discoidal A-II HDL in a reaction promoted by LCAT that culminates in the formation of spherical A-I/A-II HDL (Clay et al. 2000).

1.3.6.2 Remodelling of HDL in plasma

1.3.6.2.1 Overview

HDL subpopulations are converted from one form to another by plasma factors in processes known as HDL remodelling. Factors such as LCAT and PLTP can increase HDL size. Other factors such as CETP, hepatic lipase (HL) and Type II secretory phospholipase A_2 ($sPLA_2$) can decrease HDL size. Lipid-poor A-I can be generated during remodelling by LCAT, CETP, PLTP or HL.

1.3.6.2.2 Remodelling by LCAT

LCAT is a 68 kDa hydrophobic glycoprotein (Albers et al. 1976) that is synthesized in the liver and circulates in the plasma bound to HDL (Cheung et al. 1986; Francone et al. 1989). LCAT is responsible for generating almost all of the CE in plasma (Castro and Fielding 1988; Francone et al. 1989). This enzyme catalyses the transfer of an unsaturated fatty acid from the sn-2 position of phosphatidylcholine to UC, generating CE and lysophosphatidylcholine. The lysophosophatidylcholine which is formed during the LCAT reaction either binds to albumin in the plasma, or acts as an acyl group acceptor and is converted back to phosphatidylcholine by LCAT (Subbaiah et al. 1985; Czarnecka and Yokoyama 1993). The preferred lipoprotein substrates for LCAT are HDL, probably reflecting their content of apoA-I, a highly efficient activator of LCAT. Discoidal HDL have higher reactivity with LCAT than spherical HDL,

although HDL_3 is also moderately reactive as a substrate (Kosek et al. 1999). HDL_2 has relatively little reactivity with LCAT (Kosek et al. 1999).

LCAT converts UC in discoidal HDL into CE. The CE, being hydrophobic, partition into the interior of the disc to create a hydrophobic core in a process that converts discoidal HDL into the spherical particles that predominate in normal human plasma.

Small spherical HDL continue to acquire UC from peripheral tissues; esterification of this UC by LCAT further increases the CE content of the core and expands the size of the particle. Coincident with the LCAT-mediated increase in HDL size, the lipoprotein surface acquires additional apoA-I, either in the lipid-poor form (Liang et al. 1995) or in a process which involves particle fusion (Liang et al. 1996). The importance of LCAT in the generation of spherical HDL particles is evident from mutations of the LCAT gene, which result in familial LCAT deficiency and Fish-eye Disease (Wiebusch et al. 1995; Contacos et al. 1996; Kuivenhoven et al. 1997). Both of these conditions result in an absence of normal, spherical HDL in the circulation (von Eckardstein et al. 1995; Kuivenhoven et al. 1997). Both groups of patients have reduced plasma concentrations of apoA-I and apoA-II due to increased catabolism of the nascent HDL (Rader et al. 1994).

In vitro studies suggested a role of LCAT in the generation of A-I/A-II HDL. LCAT probably participates in the fusion of A-I discoidal HDL and A-II discoidal HDL (Clay et al. 2000) rather than its direct action on A-II discoidal HDL because of the poor reactivity between A-II discoidal HDL and LCAT (Clay et al. 1999).

1.3.6.2.3 Remodelling by CETP

CETP is a hydrophobic glycoprotein that is secreted mainly from the liver and circulates in plasma largely in association with HDL (Tall 1993). It mediates the transfer of CE from HDL to triglyceride-rich lipoproteins and LDL in exchange for triglyceride. To a lesser extent, it also transfers phospholipids between plasma lipoproteins.

When HDL and triglyceride-rich lipoproteins are incubated *in vitro* in the presence of CETP, the amount of CE transferred out of HDL tends to be greater than the amount of triglyceride transferred into HDL (Barter et al. 1982; Liu and Bagdade 1995). The net effect of this is a depletion of the HDL total core lipid content and the reduction in HDL size (Barter et al. 1990) and a dissociation of lipid-free apoA-I from the HDL surface (Liang et al. 1994; Rye et al. 1995). CETP also promotes the fusion of HDL particles, leading to a change in the size and the number of apoA-I molecules in HDL particles (Rye et al. 1997).

1.3.6.2.4 Remodelling by hepatic lipase

Hepatic lipase is a 476-amino-acid glycoprotein that is synthesised by hepatocytes and is anchored to external surfaces of hepatocytes and hepatic endothelial cells by heparan sulphate proteoglycans (Kuusi et al. 1979; Doolittle et al. 1987; Sanan et al. 1997). It belongs to the lipase family that includes other lipases such as pancreatic lipase, endothelial lipase and lipoprotein lipase. In mice, guniea pigs, and golden hamsters, hepatic lipase circulates as free enzyme in plasma. In rabbits and humans, its activity can be measured in the blood plasma after intravenous heparin injection to release the enzyme from the hepatic extracellular matrix.

The role of hepatic lipase in atherogenesis remains unclear but is probably related to its role in hydrolysing lipids in chylomicron and VLDL remnants. When hepatic lipase is deficient or inhibited, there is an accumulation of these atherogenic remnants (Goldberg et al. 1982; Sultan et al. 1990; Hegele et al. 1993) that may predispose to atherosclerosis. The effects of hepatic lipase on HDL are substantial although the relationship of such changes to atherosclerosis is unclear. (Jansen et al. 2002).

Hepatic lipase can remodel HDL by hydrolysing both their triglyceride and phospholipids (Waite et al. 1991). The hepatic lipase-mediated hydrolysis of triglyceride and phospholipids in HDL results in a depletion of triglyceride in the lipoprotein core and of the phospholipid in the surface layer of HDL. The resulting reduction in HDL particle size may be accompanied by the dissociation of lipid poor apoA-I (Clay et al. 1990; Clay et al. 1991; Clay et al. 1992; Barrans et al. 1994; Marques-Vidal et al. 1997).

1.3.6.2.5 Interaction of CETP and hepatic lipase in remodelling of HDL

The effects of hepatic lipase on HDL are enhanced when CETP is also active. As outlined above, activity of CETP increases the amount of triglyceride in the HDL core. This increases the amount of triglyceride in the HDL core that is available for hydrolysis by hepatic lipase. Thus, HDL that have been enriched in triglyceride by the activity of CETP will display an enhanced reduction in particle size when exposed to hepatic lipase. This process may account for the small particle size of HDL in human subjects in whom elevated concentrations of triglyceride-rich lipoproteins are accompanied by an increase in CETP-mediated transfers of triglyceride into HDL. This synergistic action of CETP and HL is particularly effective in reducing HDL size and promoting the dissociation of lipid-poor apoA-I (Clay et al. 1991; Clay et al. 1992; Barrans et al. 1994).

1.3.6.2.6 Remodelling of HDL by other factors

Several other plasma factors can also alter the composition and particle size of HDL.

Remodelling of HDL by phospholipid transfer protein (PLTP) results in the formation of two distinct subpopulations of larger and smaller HDL particles (Jauhiainen et al. 1993). The mechanism of PLTP remodelling of HDL may involve HDL fusion (Lusa et al. 1996). PLTP activity is enhanced in the presence of triglyceride-enriched HDL (Rye et al. 1998).

Phospholipase A2 belongs to the phospholipase family of enzymes. Both the Type II secretory phospholipase A2 ($sPLA_2$) and the lipoprotein-associated PLA2 are implicated in atherogenesis (Leitinger et al. 1999). The non-pancreatic sPLA2 hydrolyses the sn-2-acyl group of glycerophospholipids of lipoproteins and cell membranes to produce lyso-PC and free fatty acids. It is expressed on the surface of endothelial cells of a variety of tissues such as hepatocytes, macrophages and arterial wall smooth muscle cells, in response to a variety of inflammatory mediators (Crowl et al. 1991). It can hydrolyse the phospholipid on a number of plasma lipoproteins, including HDL (Pruzanski et al. 1998). It has been shown to reduce the size of HDL (Rye and Duong 2000; Tietge et al. 2002).

Endothelial lipase (EL) has predominantly phospholipase activity and relatively less triglyceride lipase activity. It has been shown to hydrolyse HDL phospholipids, although its physiological role remains uncertain. Its effects on HDL composition and size are unclear. The overexpression of EL in transgenic mice has been reported to reduce the concentration of both HDL-C and apoA-I (Jaye et al. 1999).

Lipoprotein lipase (LPL) has greater triglyceride lipase than phospholipase activity and acts mainly on chylomicrons and VLDL. Its main effects on HDL are probably indirect, resulting from the generation of surface remnants during hydrolysis of triglyceride-rich lipoproteins (Tam and Breckenridge 1983; Clay and Barter 1996).

1.3.6.3 Catabolism of HDL

The apoA-I, UC and CE in HDL follow distinct catabolic pathways. Overall, the rates of catabolism of UC and CE are faster than that of apoA-I. The mechanisms for the excretion of UC and CE in HDL into bile have been described earlier. The catabolism of apoA-I will be addressed in this section.

The half-life of apoA-I in human HDL is about 3-5 days. The kidney is a major catabolic site for apoA-I (Glass et al. 1985). Cubilin coexpresses with megalin on the luminal aspect of the proximal renal tubule and are involved in the endocytosis of apoA-I (Moestrup and Kozyraki 2000). Small HDL particles (< 8 nm) and lipid-free apoA-I are filtered by the glomeruli and removed by cubilin and megalin receptors (Hammad et al. 1999; Kozyraki et al. 1999; Moestrup and Kozyraki 2000). This may explain the longer residence time of larger HDL in the circulation (Brinton et al. 1989; Horowitz et al. 1993) and the short residence time of the small lipid-poor HDL that circulate in plasma in subjects with Tangier Disease (Schaefer et al. 1978). The liver is also an potential site of catabolism of apoA-I (Glass et al. 1983).

Recently, a high-affinity hepatic HDL receptor for apoA-I has been identified (Martinez et al. 2003). This receptor is identical to the β-chain of ATP synthase, a principal protein complex of the mitochondrial inner membrane. Interactions between apoA-I and the receptor triggers

holoparticle uptake of HDL, which is dependent on the generation of ADP. The overall contribution of this new receptor in HDL catabolism is yet to be determined.

1.3.6.4 Cycling of apoA-I between lipid associated and lipid-poor pools

The dissociation of lipid-free or lipid-poor apoA-I from spherical HDL can occur during HDL remodelling by plasma factors. Plasma factors such as LCAT, HL, CETP and PLTP can generate lipid-free and/or lipid-poor apoA-I from spherical HDL. Two mechanisms are involved in generating lipid-free apoA-I from HDL. Firstly, plasma factors can hydrolyse the contents of the core lipid and/ or surface phospholipids in processes that reduce the volume and surface area of HDL and lead to the dissociation of lipid-free (or lipid-poor) apoA-I from HDL. In this process, the core lipid composition is also found to be quite important. Triglyceride-enriched HDL are better substrate for HL (Hopkins and Barter 1986; Clay et al. 1991) and CETP (Liang et al. 1994) than other HDL. This probably accounts for the actions of HL (Hopkins and Barter 1986; Clay et al. 1991; Clay et al. 1992) and CETP (Clay et al. 1992; Liang et al. 1994). Secondly, plasma factors can promote the fusion of HDL particles to form unstable fusion particles and result in the release of lipid-poor or lipid-free apoA-I. This probably accounts for the actions by LCAT (Liang et al. 1996) and PLTP (Lusa et al. 1996; von Eckardstein et al. 1996).

This lipid-free or lipid-poor apoA-I has a number of potential fates. It can be excreted by the kidneys and irreversibly lost from the circulation (Moestrup and Kozyraki 2000). It may enter the interstitial space or lymphatics and participate in the assembly of discoidal A-I HDL by accepting cholesterol and phospholipids from peripheral tissues (Forte et al. 1995). Lipid-poor apoA-I may accept phospholipids from other plasma lipoproteins in a process linked to the hydrolysis of tirglyceride-rich lipoproteins by LPL (Tam and Breckenridge 1983). It can

incorporate into pre-existing small spherical HDL in the circulation and increase the number of apoA-I in the HDL particles from two to three molecules of apoA-I during interaction with LCAT (Liang et al. 1995).

1.4 Use of reconstituted HDL to study HDL metabolism

1.4.1 ApoA-I

ApoA-I can potentially exist in three different forms – lipid-free apoA-I, discoidal A-I HDL and spherical A-I HDL. Most of the circulating HDL are large spherical HDL but the presence or absence of certain plasma factors can vary their size distribution in plasma as well. It is therefore important to understand how these three forms of apoA-I interrelate with each other and how they are regulated by various plasma factors.

Native HDL consist of heterogenous subpopulations that vary in size, shape, electrophoretic mobility, apolipoprotein composition and lipid composition. This heterogeneity of HDL subpopulations makes it difficult to isolate homogeneous preparations of even the major HDL subpopulations for metabolic studies. Minor HDL subpopulations such as discoidal HDL are metabolic labile and exist in very small quantities in plasma. It is virtually impossible to isolate such particles in any amounts from plasma.

In contrast, using techniques of HDL reconstitution, homogeneous preparations of reconstituted HDL (rHDL) can be assembled from their constituents. These rHDL have defined size, shape, electrophoretic mobility, apolipoprotein composition and lipid composition. Discoidal A-I rHDL can also be prepared in sufficient quantity without the need of ultracentrifugation.

Discoidal rHDL have been used in both *in vitro* and *in vivo* studies in animals and humans. The injection of discoidal rHDL into the circulation leads to an initial increase in the level of preβ-migrating A-I HDL followed by a subsequent increase in the level of α-migrating A-I HDL (Nanjee et al. 1999). Discoidal rHDL promote cholesterol efflux from fibroblasts (Stein et al. 1976; Picardo et al. 1986) and inhibit the expression of adhesion molecules *in vitro* (Baker et al. 1999) and *in vivo* (Cockerill et al. 1995). The kinetics of radioiodinated apoA-I in discoidal rHDL were essentially the same as the kinetics of apoA-I in endogenous HDL in man (Malmendier et al. 1983). All these results highlight the resemblances of rHDL with the native HDL in terms of remodelling, functions and metabolism.

The ability to prepare rHDL of defined characteristics in sufficient quantity, the ability to prepare minor HDL subpopulations and the possibility of incorporating radiolabelled component(s) into rHDL particles make this a powerful technique for the study of HDL remodelling and metabolism *in vivo*. This technique can potentially prepare radiolabelled HDL with more uniform and desirable characteristics than any of the previous techniques.

With this technique, it is possible to directly compare the interrelationships between the three forms of apoA-I (lipid-free apoA-I, discoidal A-I rHDL, spherical A-I rHDL) and their metabolic fates both *in vitro* and *in vivo*.

1.4.2 Unesterified cholesterol

Discoidal rHDL have comparable size, electrophoretic mobility and lipid composition to nascent HDL of intestinal and hepatogenous origin (Green et al. 1978; Johnson et al. 1986). Like nascent HDL, discoidal rHDL also promote efflux of UC from fibroblasts (Stein et al. 1976; Picardo et al. 1986) and become enriched of UC after injection into the circulation

(Stein et al. 1976; Picardo et al. 1986). Discoidal rHDL therefore represent an ideal model for the study of the earliest steps of RCT. HDL reconstitution allows the incorporation of radiolabelled UC. These discoidal rHDL contain radiolabelled UC as the sole radiolabel and are suitable for the study of *in vivo* metabolism of UC in discoidal HDL.

1.4.3 Cholesteryl esters

Spherical rHDL have comparable size, electrophoretic mobility and lipid composition to native mature HDL in the circulation. Spherical rHDL can interact with plasma factors and undergo similar HDL remodelling and lipid exchange as native HDL (Rye and Barter 1994; Rye et al. 1995; Rye et al. 1997). Spherical rHDL can be prepared by incubating discoidal rHDL with LDL and LCAT (Rye et al. 1993). With some modifications to the technique, it is also possible to enrich the spherical rHDL with radiolabelled CE. Reconstituted HDL can provide specific radiolabelling of the component of interest, in this case, cholesteryl esters. Such spherical rHDL containing radiolabelled CE are ideal for the study of *in vivo* metabolism of HDL CE.

1.5 Use of rabbit as experimental model

Rabbits provide a well-characterised animal model for the study of lipoprotein metabolism and lipid-modifying therapies. The plasma lipoprotein profile of the rabbit is well defined (Chapman 1980; Greeve et al. 1993). However, there are some key differences in the concentrations of HDL apolipoprotein and plasma factor. The plasma concentration of apoA-II is low in rabbits, thus the majority of rabbit HDL contain apoA-I alone (Chapman 1980). In contrast, a large proportion of human HDL contains both apoA-I and apoA-II. The fact that levels of expression of hepatic lipase, a key component of the lipoprotein metabolic pathways,

are very low in rabbits can be exploited to help to understand the pathophysiological importance of this enzyme, which is markedly different between rabbits and humans.

Rabbit is a high CETP animal (Ha and Barter 1982). Previous study has shown that as much as 70% of HDL CE is cleared from plasma after redistributed to VLDL and LDL by CETP (Goldberg et al. 1991). Therefore, CETP provides a substantial indirect HDL CE clearance pathway. An ability to inhibit CETP in rabbits thus provides a powerful tool for understanding the role of this protein in HDL metabolism and reverse cholesterol transport. The availability of various methods of CETP inhibition, such as anti-sense oligonucleotides (Sugano and Makino 1996; Sugano et al. 1998), tetanus toxoid conjugated CETP vaccine (Rittershaus et al. 2000) and oral CETP inhibitors (Okamoto et al. 2000), can be exploited in rabbit studies designed to determine the relative contribution of the CETP pathway to the removal of HDL cholesterol from plasma. It may also help to define the maximal cholesterol flux mediated by the direct and indirect clearance pathways of HDL CE.

Rabbits have low endogenous activity of HL related to a low level of HL mRNA expression (Warren et al. 1991). Compared with humans, rabbits have about one-tenth of plasma HL activity (Connelly et al. 1990; Warren et al. 1991). This natural hepatic lipase deficiency in rabbits is associated with the presence of larger and more triglyceride-enriched HDL in plasma (Clay et al. 1989). Human subjects with familial hepatic lipase deficiency also have high plasma levels of triglyceride-rich HDL (Blades et al. 1993; Connelly et al. 1999). In contrast, transgenic rabbits over-expressing HL have markedly reduced HDL levels, consistent with the role of HL in the metabolism of triglyceride-enriched HDL (Fan et al. 1994). The natural deficiency of HL in rabbits closely mimics the genetic deficiency state in the humans. The availability of transgenic rabbits expressing human HL makes rabbit an

excellent model for the study of the effects of HL on the remodelling and metabolism of HDL.

1.6 Scope of this thesis

The studies in this thesis aim at determining the *in vivo* metabolism of apoA-I, unesterified cholesterol and cholesteryl esters in HDL and how those components of HDL are altered in the presence and absence of hepatic lipase or CETP. Specific aims are as follows:

- to determine the *in vivo* remodelling of the three forms of apoA-I (lipid-free apoA-I or as a component of discoidal or spherical rHDL particles) in the rabbits.

- to determine whether there is any difference in the rate of clearance of the three forms of apoA-I in the rabbits.

- to determine how the remodelling of the three forms of apoA-I lead to the predominance of large α-migrating spherical HDL in the circulation.

- to determine whether the size of spherical HDL is reduced in the presence of hepatic lipase expression in the rabbits that naturally express low activity of hepatic lipase.

- to determine the metabolism of unesterified cholesterol and cholesteryl ester in HDL and their relationship with other lipoproteins in the rabbits.

- to determine the effects of CETP inhibition on the metabolism of unesterified cholesterol and cholesteryl ester in HDL and their relationship with other lipoproteins in the rabbits.

2 Materials and Methods

2.1 Isolation of apoA-I

HDL were isolated from pooled rabbit plasma (Quality Australia Farmed Rabbits, Laura, Victoria) by ultracentrifugation in the $1.07 < d < 1.21$ g/mL density range, with a single 16h spin at d = 1.07 g/mL and two 26h spins at d = 1.21 g/mL (Rye et al. 1992; Rye et al. 1993). The ultracentrifugations were carried out using a 55.2 Ti rotor at a speed of 55,000 rpm in a Beckman L8-M ultracentrifuge maintained at 4 °C. Density adjustments were made by the addition of solid KBr. The ultracentrifugally isolated HDL were dialysed against 3 × 5 L of 5 mM ammonium bicarbonate solution, then delipidated as described by Osborne (Osborne 1986). The resulting apoHDL was dissolved in 20 mM Tris, pH 8.2, lyophilised, and stored at –20 °C.

Rabbit apoA-I was isolated from the apoHDL by anion exchange chromatography on an XK 26/40 column of Q Sepharose Fast Flow (Amersham Pharmacia Biotech, Uppsala, Sweden) attached to a Fast Performance Liquid Chromatography (FPLC) system (Amersham Pharmacia Biotech) (Weisweiler 1987; Rye 1990). The apoA-I appeared as a single band after electrophoresis on a 20% sodium dodecyl sulphate (SDS) polyacrylamide gel (Phast System, Amersham Pharmacia Biotech), stained with Coomassie Blue. The purified apolipoprotein was dialysed against 3 × 5 L of 20 mM ammonium bicarbonate, lyophilised, and stored at –20 °C. Prior to use, it was reconstituted in 10 mM Tris/ 3 M guanidine-HCl/0.01% (w/v) EDTA-Na_2, pH 8.2 for 1 h, then dialysed against 5 × 1 L of Tris-buffered saline (10 mM Tris/ 150 mM NaCl, pH 7.4) containing 1 mM EDTA-Na_2 and 0.01% (w/v) NaN_3 (TBS).

2.2 Isolation of LCAT

LCAT was isolated from samples of pooled human plasma as described, with some modifications (Mahadevan and Soloff 1983; Rajaram and Barter 1985). Two litres of pooled human plasma were subjected to precipitation at 35% saturation of ammonium sulphate. The precipitated proteins were removed by centrifugation at a speed of 12,000 rpm then 125 mL of 1 M citric acid was added dropwise to the supernatant. The precipitated proteins were resuspended in 200 mL of Milli Q water and the pH of the sample was raised to 7.4 by the addition of a saturated solution of Na_2CO_3. The solution was dialysed against 2 × 5 L of Milli Q water then ultracentrifuged at a density of 1.25 g/mL. This procedure was carried out at 55,000 rpm for 26 h using a 55.2 Ti rotor in a Beckman L8-M ultracentrifuge maintained at 4 °C. After the initial spin, the $d < 1.25$ g/mL fraction was recovered by tube slicing and subjected to a further 26 h of ultracentrifugation at a density of 1.25 g/mL. The $d > 1.25$ g/mL fractions from both of the spins were pooled and applied to an XK 60/60 containing Phenyl Sepharose 6 Fast Flow (Amersham Pharmacia Biotech) pre-equilibrated with 1 L of 3 M NaCl. After washing with 0.15 M NaCl, LCAT was eluted with Milli Q water/0.02% (w/v) NaN_3/0.01% (w/v) EDTA-Na_2 at a flow rate of 10 mL/min. The active fractions were pooled and dialysed against 2 × 5 L of 20 mM tris, pH 7.4, containing 1 ml β-mercaptoethanol/L. The same buffer was used to pre-equilibrate an XK 26/40 column packed with DEAE Sepharose Fast Flow (Amersham Pharmacia Biotech). The LCAT was loaded onto this column and eluted with 20 mM Tris/500 mM NaCl, pH 7.4, containing 1 ml β-mercaptoethanol/L. Ten mL fractions were collected at a flow rate of 10 mL/min. Fatty acid-free bovine serum albumin (BSA, final concentration 1 mg/mL) was added to the fractions that contained LCAT. The fractions were stored in 10 mL aliquots at –70 °C until use.

2.3 Radioiodination of apoA-I

ApoA-I was reconstituted with 10 mM Tris/3 M guanidine-HCl/0.01% (w/v) EDTA-Na_2, pH 8.2 and dialysed against 5 × 1L of TBS. The purified rabbit apoA-I was iodinated with $Na^{125}I$ (NEN Life Sciences) and IODO-BEADS iodination reagent (Pierce). Two IODO-BEADS were washed with 2 × 1 mL of TBS and dried on filter paper. About 1.2 mCi of $Na^{125}I$ was incubated with the IODO-BEADS for about 2 mins, followed by the addition of 10 mg of purified apoA-I (protein concentration of 9 mg/mL) for another 15 mins. After the iodination procedure, free iodine was separated from ^{125}I-apoA-I on a PD-10 column (Pharmacia Biotech AB). The active ^{125}I-apoA-I fractions were pooled and dialysed against 5 × 1L of TBS. The specific activity of labelled apoA-I ranged from 60 to 120 cpm/ng protein.

2.4 Preparation of reconstituted HDL (rHDL) for tracer experiments

The preparation of ^{125}I-apoA-I-discoidal and spherical rHDL is summarised in Figure 2.1.

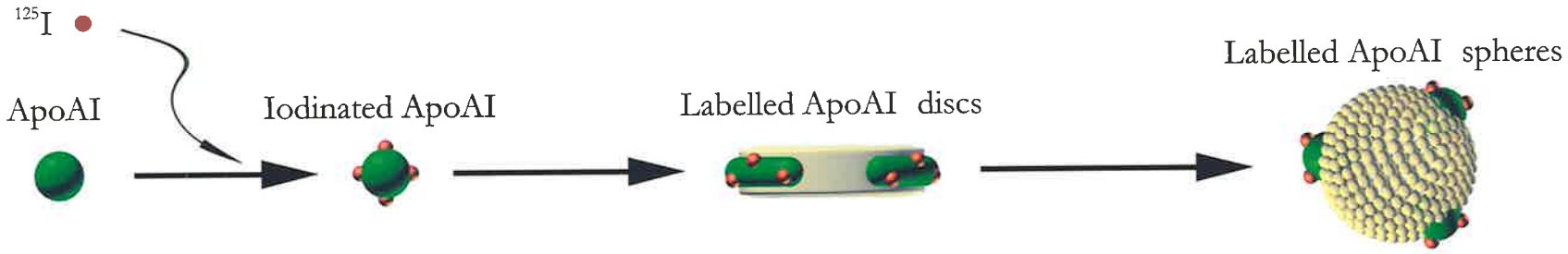

Figure 2.1. Preparation of ^{125}I-apoA-I discoidal and spherical rHDL. Purified rabbit apoA-I is radioiodinated with Na^{125}I (NEN life sciences) and IODOBEAD iodination reagent (Pierce). The ^{125}I-apoA-I discoidal and spherical rHDL are prepared as described in the Section 2.4.

2.4.1 Preparation of ^{125}I apoA-I discoidal rHDL

Discoidal rHDL were prepared by the cholate dialysis method using 1-palmitoyl-2-linoleoylphosphatidylcholine (PLPC), unesterified cholesterol (UC), and ^{125}I apoA-I (Matz and Jonas 1982), at a molar ratio of 100:10:1. The PLPC and UC were both dissolved in chloroform: methanol solution 2:1 (v/v) at a concentration of 100 and 10 mg/mL, respectively. The PLPC and UC were placed in clean dry test tubes and dried as a thin film onto the wall of the tube using nitrogen. The lipids in test tubes were then dried at about 40 °C under nitrogen for 2 h. Sodium cholate (30 mg/mL in TBS) was added to the dried lipids to give a molar ratio of PLPC:UC:cholate of 100:10:100. The final volume was adjusted to 500 μL with TBS. The test tubes were kept in ice and vortexed every 15 min until the mixtures became clear. Two milligrams of ^{125}I apoA-I was then added into each tube. After another 2 h on ice, the contents of the tubes were pooled and dialysed against 5 × 1 L of saturated TBS solution containing 10mM Tris, 150 mM NaCl, 50 μM diethylenetriamine pentaacetic acid (DETAPAC), 0.006% (w/v) NaN_3, 10 μM BHT, and chelex at pH 7.4 to remove the sodium cholate.

2.4.2 Preparation of ^{125}I apoA-I spherical rHDL

Spherical rHDL containing CE as the sole core lipid were prepared as described (Rye et al. 1993; Rye and Barter 1994). Discoidal rHDL (final ^{125}I apoA-I concentration 5.15 mg/mL) was incubated with fatty acid-free BSA (final concentration 40 mg/mL), β-mercaptoethanol (final concentration 4 mM), LDLs (final protein concentration 1.4 mg/mL) and LCAT for 24 h at 37 °C. The spherical rHDL were isolated by ultracentrifugation in the $1.07 < d < 1.21$ g/mL density range with two 24 h spins at the lower density and a single 16 h spin at the upper density. The spins at d 1.07 g/mL were carried out at 55,000 rpm using a Beckman Ti 55.2 rotor in a Beckman L8-M ultracentrifuge. The d = 1.21 g/mL spin was carried out at a

speed of 100,000 rpm using a Beckman TLA 100.4 rotor in a Beckman TL-100 Tabletop ultracentrifuge. These procedures were all conducted at 4 °C. The spherical rHDL were dialysed against 3 × 1 L of TBS before use.

2.4.3 Preparation of discoidal rHDL containing [^{3}H] unesterified cholesterol

Discoidal rHDL containing [^{3}H] unesterified cholesterol were also prepared by the cholate dialysis method as described in section 2.4.1 with some modifications. The main differences are (1) the addition of 600 μL of [1α,2α(n)-^{3}H] cholesterol ([^{3}H]UC) in the beginning, and (2) the addition of nonlabelled rabbit apoA-I in the final stage of the preparation. The mass of the [^{3}H]UC was treated as negligible in the calculation of the molar content of total UC. The final molar ratio of PLPC: UC: cholate: apoA-I remained at 100:10:100:1. The contents of the tubes were pooled and dialysed against 5 × 1 L of TBS to remove the sodium cholate. The specific activity of labelled [^{3}H]UC ranged between 8 X 10^8 and 9 X 10^8 cpm/μmoL of cholesterol.

2.4.4 Preparation of spherical rHDL containing [^{3}H] cholesteryl esters

The preparation of spherical rHDL containing [^{3}H]cholesteryl esters ([^{3}H]CE) as the sole core lipid was similar to that described in section 2.4.2 with some modifications. The main difference was the omission of LDL in the incubation to allow enrichment of radioactivity in spherical rHDL without the transfer of [^{3}H]UC from discoidal rHDL to LDL. Discoidal rHDL containing [^{3}H]UC (final apoA-I concentration 2 mg/mL), fatty acid-free BSA (final concentration 40 mg/mL), β-mercaptoethanol (final concentration 4 mM) and LCAT were incubated for 6 h at 37 °C. The spherical rHDL were isolated by ultracentrifugation in the d > 1.21 g/mL at 55,000 rpm using a Beckman 55.2 rotor in a Beckman L8-M ultracentrifuge at 4

°C. The spherical rHDL were dialysed against 3 × 1 L of TBS before use. The specific activity of labelled [^{3}H]CE ranged between 6×10^8 and 7×10^8 cpm/μmoL of cholesterol.

2.5 Compositional analyses

Compositional analyses were carried out using a Roche/Hitachi 902 analyzer (Roche Diagnostics, Zurich, Switzerland). ApoA-I concentrations were determined either by the method of Lowry (Lowry et al. 1951), using BSA as a standard, or by an immunoturbidometric assay (Riepponen et al. 1987; Hopkins and Barter 1989). Enzymatic kits (Boehringer Mannheim GmbH, Germany) were used to determine PL, UC and TC concentrations. CE concentrations were calculated as the difference between TC and UC concentrations.

2.6 Electrophoresis

2.6.1 Non-denaturing polyacrylamide gradient gel electrophoresis

The hydrated diameter of rHDL was determined by electrophoresis on 3-40% nondenaturing polyacrylamide gradient gels, using a GE-2/4 LS Gel Electrophoresis Apparatus (Amersham Pharmacia Biotech). The gels were prepared according to the method of Rainwater (Rainwater et al. 1992). Samples were pre-mixed with 40% (w/v) sucrose/0.01% (w/v) bromophenol blue solution then applied to the gel. Electrophoresis was carried out in 0.09 M Tris/0.08 M boric acid/0.003 M EDTA-Na_2 buffer, pH 8.4, at 150-180 volts for a total of 3,000 volt-hours. The gel was fixed with 10% (w/v) 5-sulphosalicylic acid for 30-60 min, stained with 0.04% (w/v) Coomassie Brilliant Blue G in 3.5% (v/v) perchloric acid for 1-2 h, and destained with 5% (v/v) acetic acid until the background was clear. The gels were scanned by a Sharp JX-610 scanner (Sharp, Japan). The particle size was determined by the

ImageMaster Software (Amersham Pharmacia Biotech) by reference to a series of high molecular weight standards of known hydrated diameter (Amersham Pharmacia Biotech).

2.6.2 Agarose gel electrophoresis

The electrophoretic mobility of the lipoproteins was determined using the Titan Gel lipoprotein electrophoresis system (Helena Laboratories, USA) according to the manufacturer's instructions. The gels were scanned by a Sharp JX-610 scanner (Sharp, Japan). The migration distance from the origin was determined by the ImageMaster Software (Amersham Pharmacia Biotech). Electrophoretic mobility was calculated as described by Sparks and Phillips (Sparks and Phillips 1992).

$$\text{Mobility} = \frac{\text{Migration distance } (\mu\text{m}) \div \text{Time (sec)}}{\text{Voltage (v)} \div \text{length of the gel (cm)}}$$

2.6.3 SDS-polyacrylamide gel electrophoresis

SDS polyacrylamide gel electrophoresis was carried out on 20% homogeneous polyacrylamide gels in the Phast System (Amersham Pharmacia Biotech). Ten microlitres of sample (protein concentration ~ 1 mg/mL) was boiled with 10 μL of SDS sample buffer (0.01 M Tris/0.001 M EDTA-Na_2/2.5% (w/v) SDS/0.025% (w/v) bromophenol blue) for 5 min, and then applied to the gel. The electrophoresis was carried out for 95 volt-hours. The gels were stained with Coomassie Brilliant Blue R, destained with methanol: acetic acid: water 3:1:6 (v/v/v), and preserved with glycerol:acetic acid:water 1:1:8 (v/v/v).

2.7 Autoradiography

The electrophoretic migration of ^{125}I-apoA-I was determined by exposing the gels to a Phosphorimager plate for about 16 hours. The Phosphorimager plate was read by a Molecular

Dynamics PhosphorImager (Molecular Dynamics, USA). The size and electrophoretic mobility of the lipoproteins were determined using the ImageMaster Software (Amersham Pharmacia Biotech).

2.8 Quantification of radioactivity

2.8.1 Quantification of ^{125}I in apoA-I

Blood samples were drawn from the right marginal ear vein of the rabbit and stored at 4 °C in tubes containing 0.1 mg/mL EDTA Na_2 and 2 mmol/L DTNB. Plasma was separated from red cells by centrifugation at 3000 rpm for 15 mins. Radioactivity in apoA-I was determined by liquid scintillation counting in a Ready Safe cocktail (Beckman Coulter, Fullerton, CA), using a Beckman LS 6000TA liquid scintillation counter (Model LS 6000TA; Beckman Coulter, Fullerton, CA).

2.8.2 Quantification of [^{3}H] in unesterified cholesterol and cholesteryl esters in lipoproteins

Separation of UC and CE was performed in experiments using discoidal rHDL containing [^{3}H]UC. In contrast, all the plasma counts were presumed to be that of CE when the injected tracer was spherical rHDL containing [^{3}H]CE.

2.8.3 Injection of discoidal rHDL containing [^{3}H]UC

Plasma was separated from whole blood sample by centrifugation at 3000 rpm for 15 mins at 4 °C. ApoB-containing lipoproteins and HDL in plasma samples were separated by precipitating apoB-containing lipoproteins with heparin and manganese chloride (Burstein et al. 1970). The lipids in the total plasma and HDL supernatant samples were extracted with the Folch extraction. Briefly, 100 μL of sample was sequentially mixed with 3 ml solution of

chloroform methanol, 1:2 (v/v), 1 ml of chloroform and 1 ml of TBS. The mixture was centrifuged at 1000 rpm for 10 mins and 2 ml of infranatant (methanolic layer containing lipids) was collected with a Hamilton needle. The lipid extract was dried down under nitrogen at 40 °C and later resuspended in multiple washes of diethyl ether. The lipid extract was subjected to thin layer chromatography (TLC), with hexane-diethylether-methanol-acetic acid, 90:20:3:2 used as solvent to separate UC and CE, and later assayed for ^{3}H. The recovery of ^{3}H after TLC was 65% to 80%; values were corrected back to a recovery of 100%. Radioactivity in UC and CE was determined by liquid scintillation counting in a Ready Safe cocktail (Beckman Coulter, Fullerton, CA), using a Beckman LS 6000TA liquid scintillation counter (Model LS 6000TA; Beckman Coulter, Fullerton, CA).

2.8.4 Injection of spherical rHDL containing [^{3}H]CE

Plasma was separated from whole blood sample by centrifugation at 3000 rpm for 15 mins. ApoB-containing lipoproteins and HDL in plasma samples were separated by precipitating apoB-containing lipoproteins with heparin and manganese chloride (Burstein et al. 1970). The radioactivity in the total plasma and HDL supernatant samples was measured in a Beckman LS 6000TA scintillation counter. The difference between total plasma and HDL count equals to the count in apoB-containing lipoproteins.

2.9 Assay for plasma factor activity

2.9.1 LCAT

Activity of LCAT was determined using discoidal rHDL containing β-oleoyl-γ-palmitoyl-L-α-phosphatidylcholine (POPC): unesterified cholesterol (UC): apoA-I labelled with [1α,2α(n)-^{3}H] cholesterol ([^{3}H]UC) (Amersham Pharmacia Biotech) as a substrate (Piran and Morin 1979). The final concentration of UC in the substrate was 0.13 nmol/μL. Twenty-five

μL of substrate, together with 50 μL of 10 mg/mL fatty acid-free BSA, 5 μL of β-mercaptoethanol (final concentration 48.5 mM), and 47 μL of TBS were pre-incubated at 37 °C under nitrogen for 30 min. LCAT (20 μL), TBS or the d > 1.25 g/mL fraction of pooled, human plasma (positive control) were added to the mixtures and the incubations were continued at 37 °C under nitrogen for a further 1 h. The final volume of the incubation mixture was 147 μL. The reaction was stopped by addition of 0.5 mL of 1% digitonin in 95% ethanol. The mixtures were vortexed for 15 sec to denature the proteins and extract lipids. Excess UC (25 μL of 5 mg/mL UC in ethanol) was then added and the mixtures were vortexed again then centrifuged at 1,500 × g for 10 mins. A 400 μL aliquot, which contained radiolabelled CE, was taken from the supernatant, added to 10 mL of aqueous scintillation cocktail (Ready safe, Beckman, USA) and counted in a Beckman LS 6000 TA liquid scintillation system (Beckman Instruments, Inc., Fullerton, CA, USA) for 5 min. The assay was linear when less than 30% of the [^{3}H] UC was esterified. LCAT activity was expressed as μmol of CE formed/mL LCAT/h

2.9.2 Hepatic lipase

Activity of hepatic lipase was measured in postheparin rabbit plasma using a triolein-based substrate (Cheng et al. 1985). After an overnight fast, 10 units/kg of heparin was injected intravenously into the rabbit. Blood sample was drawn 10 minutes after the injection and stored in a tube containing 0.1 mg/mL EDTA Na_2. Plasma was separated from whole blood sample by centrifugation at 3000 rpm for 15 minutes. Ten microlitres of postheparin plasma was incubated with 75 μL of [^{3}H] triolein substrate (containing 1130 nmol of free fatty acid) in the presence of either low (0.15 M NaCl) or high (5 M NaCl) salt solution for 60 minutes at 37 °C. Oleic acid released by the incubation was extracted with a solution of methanol-chloroform-heptane, 1.4:1.25:1 (v:v:v) and carbonate borate buffer (containing 0.1M K_2CO_3

and 0.1M H_3BO_3, with pH adjusted to 10.5 with KOH). The mixture was centrifuged at 3000 rpm for 15 mins. Two millilitres aliquot of supernatant was aspirated and placed in 10 mL of aqueous scintillation cocktail and counted in a Beckman LS 6000 TA liquid scintillation system for 10 min. The recovery of ^3H after extraction was between 45 to 70%; values were corrected back to recovery of 100%. The activity is expressed in μmol of free fatty acid released by 1 mL of enzyme source in 1 hour of incubation at 37 °C.

2.9.3 CETP

Activity of CETP was assessed as the transfer of [^{3}H]CE and [^{3}H]CE-HDL_3 to LDL (Burstein et al. 1970; Tollefson et al. 1988). This assay is based on exogenous donor and acceptor particles for CE. [^{3}H]CE-HDL_3 (final total cholesterol (TC) concentration 80 nmol/mL), LDL (final TC concentration 240 nmol/mL), and 50 μL of rabbit plasma, or the d > 1.25 g/mL fraction of pooled, human plasma (positive control), were incubated in the presence of 10 μL of 14.2 mg/mL 5,5'-dithio-bis(nitrobenzoic acid), DTNB (to inhibit LCAT activity). TBS was added to a total volume to 175 μL. The incubations were carried out at 37 °C for 3 h. At the end of incubation, LDL were precipitated with 25 μL of heparin and $MnCl_2$ solution (2500 IU/mL heparin/1 M $MnCl_2$), and centrifuged for 5 min at 1,500 × g. Two hundred microlitres aliquot of the supernatant was placed into 10 mL of aqueous scintillation cocktail and counted in a Beckman LS 6000 TA liquid scintillation system for 5 min. The assay was linear when less than 30% of [^{3}H]CE transferred from HDL_3 to LDL. Activity is expressed in units/mL, with 1 unit being the transfer activity of 1 mL of a preparation of pooled, human lipoprotein-deficient plasma.

2.10 Protocol for rabbit experiments

Studies were carried out in mature male wild-type New Zealand White (NZW) rabbits and human hepatic lipase transgenic rabbits. The rabbits were fed a normal rabbit chow diet. Experiments were performed at the Hanson Institute in Adelaide, Australia and the Gladstone Institute of Cardiovascular Disease in San Francisco in the United States. The experimental protocols were reviewed and approved by animal ethics committees at the Institute of Medical and Veterinary Science, Adelaide, Australia, and the University of California, San Francisco, USA. A 21-gauge cannula was inserted into the central ear artery prior to the injection of radioactive tracer into the marginal ear in the opposite ear. The first 0.5 mL of blood was rejected and 2 mL of blood was collected from the arterial cannula at each time point. One millilitre of sterile saline solution (0.9% $NaCl_2$) was injected into the arterial cannula to maintain patency. Blood samples were stored at 4 °C in tubes containing 0.1 mg/mL EDTA Na_2 and 2 mmol/L DTNB. At the end of each experiment, the rabbits were euthanased by injecting 5 mL of Lethabarb solution into the marginal ear vein. The radioactive carcasses were packaged and disposed according to established institutional procedures.

2.11 Chemicals and reagents

Agarose	Sigma Chemicals A-6013
4-Aminoantipyrine	Sigma Chemicals A-4382
Ammonium bicarbonate	BDH chemicals 103025 E
Ammonium sulphate	BDH chemicals 10033.6 E
Antifoam A	Sigma Chemicals A-5758
Anti-sheep/goat Ig, HRP conjugated	Silenus Lab, UAH
Barbitone	BDH Chemicals 10415 3 P
Baritone, sodium	BDH Chemicals 103654 E
Boric acid	BDH Chemicals 10058.3 R
Bovine serum albumin (BSA)	Sigma Chemicals A-8022
Bovine serum albumin, fatty acid-free	Sigma Chemicals A-3803
Bromophenol blue	BDH Chemicals 44305
Butylated hydroxy toluene (BHT)	Sigma chemicals B-1378
Chelex resin	Bio-Rad 142-2832
Chloroform	BDH Chemicals 10077.6 B
Cholesterol, unesterified	Sigma Chemicals C-8667
[1α,2α (n)-^{3}H] cholesterol	Amersham Pharmacia Biotech TRK 330 B 80
Cholesteryl oleate	Sigma Chemicals C-9253
Cholic acid, sodium salt	Sigma Chemicals C-1254
Citric acid	BDH Chemicals 27781
Coomassie Brilliant Blue G-250	Bio-Rad 161-0406
Coomassie Brilliant Blue R-350 (Phast Gel)	Amersham Pharmacia Biotech 17-0518-01
Diethylenetriamine pentaacetic acid (DETAPAC)	Sigma Chemicals D-1133
Diethyl ether	BDH Chemicals 10094.6 B
Digitonin	Sigma Chemicals D-5628
5,5'-Dithio-bis(nitrobenzoic acid) (DTNB)	Sigma Chemicals D-8130
ECL reagent	Amersham Pharmacia Biotech RPN 2106
Ethanol	BDH Chemicals 10107.7 Y
Ethylenediaminetetraacetic acid, disodium salt (EDTA-Na_2)	BDH Chemicals 10093.5 V
Folin & Ciocalteau's phenol reagent	Sigma Chemicals F-9252

Free cholesterol reagent	Boehringer Mannheim 310328
Glacial acetic acid	BDH Chemicals 100015 N
Glycine	BDH Chemicals 10119, CU
Guanidine (aminomethanamidine) hydrochloride	Sigma Chemicals G-3272
Heparin, sodium salt	Sigma Chemicals H-9399
n-Hexane	BDH Chemicals 152496 G
High molecular weight electrophoresis calibration kit	Amersham Pharmacia Biotech 17-0445-01
20% Homogeneous polyacrylamide gel	Amersham Pharmacia Biotech 17-0624-01
Hydrochloric acid	BDH Chemicals 10307 8 R
Iodine-125	NEN Life Science NEZ033A
IODO-BEAD iodination reagent	Pierce 28665
β-Linoleoyl-γ-palmitoyl-L-α-phosphatidylcholine (PLPC)	Sigma Chemicals P-9648
Low molecular weight standard electrophoresis calibration kit	Amersham Pharmacia Biotech 17-0446-01
Magnesium sulphate	Merck Chemicals 10151
Manganous chloride	Ajax Chemicals D 3247
β-Mercaptoethanol	Merck Chemicals 805740
Methanol	BDH Chemicals 10158. BG
Nitrocellulose membrane	Advantec MFS, Inc., A 020 A304 C
Perchloric acid	BDH Chemicals 101754 W
Peroxidase (POD)	Boehringer Mannheim 413470
Phospholipid reagent	Roche Diagnostics 691844
Polyethylene glycol	Sigma Chemicals P-2139
Potassium bromide	BDH Chemicals 101954 F
SDS buffer strips	Amersham Pharmacia Biotech 17-0516-01
Skim milk powder	Diploma, Bonlac Foods Ltd., Australia
Sodium acetate	BDH Chemicals 10236.4 Q
Sodium azide	Sigma Chemicals S-2002
Sodium carbonate	BDH Chemicals 10240.4 H
Sodium chloride	BDH Chemicals 10241.3000
Sodium dihydrogen orthophosphate	BDH Chemicals 10245
Sodium dodecyl sulphate (SDS)	BDH Chemicals 442444 H

di-Sodium hydrogen orthophosphate	BDH Chemicals 10249
Sodium hydroxide	BDH Chemicals 10252.4 X
Sucrose	BDH Chemicals 010274.0500
5-Sulphosalicylic acid	BDH Chemicals 103464 A
Thimerosal	Sigma Chemicals T-5215
Titan Gel Lipoprotein Kit	Helena Laboratories 3045
Thin layer chromatography (TLC) plates	Merck 1.05721
Total cholesterol reagent	Roche Diagnostics 2016630
Tris (hydroxymethyl) aminomethane	Sigma Chemicals T-1378
Triton X-100	Merck Chemicals 30632
Tween 20	BDH Chemicals 66368
Urea	BDH Chemicals 10290.BG

3 Metabolism of apoA-I as lipid-free protein or as a component of discoidal and spherical reconstituted high density lipoproteins: methodology and feasibility

3.1 Rationale

The discovery that high density lipoproteins (HDL) protect against the development of atherosclerosis (Miller 1987; Gordon *et al.* 1989) has stimulated a major interest in the factors that regulate these lipoproteins. This interest has applied particularly to the regulation of apolipoprotein (apo)A-I, the main protein constituent of HDL.

Most of the HDL in normal plasma are spherical particles that exhibit α-migration when subjected to agarose gel electrophoresis. They consist of a surface monolayer of apolipoproteins, phospholipids and unesterified cholesterol surrounding a core of cholesteryl esters and triglyceride. However, there is a potential for apoA-I also to circulate in a lipid-free (or lipid-poor) form and as a component of pre-β-migrating, discoidal complexes containing apoA-I, phospholipid and unesterified cholesterol (Castro and Fielding 1988).

In vitro studies have shown that apoA-I cycles between the lipid-free and lipid-associated forms in processes mediated by lecithin:cholesterol acyltransferase (LCAT), cholesteryl ester transfer protein (CETP), phospholipid transfer protein (PLTP) and hepatic lipase (HL) (Rye *et al.* 1999). It has also been shown *in vitro* that pre-β-migrating, discoidal HDL are converted into α-migrating, spherical particles by the action of LCAT (Nichols *et al.* 1985; Liang *et al.* 1996). *In vitro* studies have also shown that α-migrating HDL are increased in size by LCAT and decrease in size when they interact with CETP, HL and triglyceride-rich lipoproteins

(Rye *et al.* 1999). The reduction in size is accompanied by a dissociation of lipid-free apoA-I from the HDL (Clay *et al.* 1990; Clay *et al.* 1992; Hime *et al.* 1998).

Despite the abundant evidence that HDL remodelling occurs *in vitro*, its contribution to HDL metabolism *in vivo* remains unclear. Indeed, the fact that apoA-I exists predominantly in α-migrating, spherical HDL in the plasma of most species, including humans, raises questions about the relevance of these *in vitro* findings to HDL metabolism *in vivo*.

The present study investigates how the different forms of apoA-I are related *in vivo* and whether the processes that operate *in vitro* also impact on the metabolism of HDL *in vivo*. Three specific questions have been addressed in this study. (i) Does lipid-free apoA-I incorporate into pre-existing α-migrating HDL *in vivo* and is the process sufficiently rapid to explain why lipid-free apoA-I is normally not detectable in plasma? (ii) Are pre-β-migrating, discoidal HDL converted to α-migrating particles *in vivo* and is the process sufficiently rapid to account for a virtual absence of such particles in normal plasma? (iii) Does the metabolism of apoA-I differ when it is introduced in a lipid-free form or as a component of discoidal or spherical rHDL?

3.2 Methods

3.2.1 Rabbits

The studies were carried out in mature male New Zealand White (NZW) rabbits purchased from the Institute of Medical and Veterinary Science in Adelaide. The rabbits were fed on a normal rabbit chow diet.

3.2.2 Purification and radiolabelling of apoA-I

The isolation, purification and radioiodination of apoA-I from rabbit plasma are described in chapter 2. The specific activity of labelled apoA-I ranged from 60 to 120 cpm/ng protein.

3.2.3 Preparation of discoidal and spherical apoA-I rHDL

The preparation of the radiolabelled discoidal and spherical rHDL is described in chapter 2. The size and homogeneity of these rHDL were analysed by nondenaturing gradient gel electrophoresis on 3 – 40% gradient acrylamide gels.

3.2.4 *In vitro* studies

Five micrograms of ^{125}I-apoA-I, as lipid-free protein, or as a component of discoidal or spherical rHDL was added to 2 ml of rabbit plasma and incubated at 37°C for 0, 5, 10, 30, 90, 180 and 360 min. The size and electrophoretic mobility of the labelled apoA-I were determined by 3 – 40% nondenaturing polyacrylamide gradient gel electrophoresis and agarose gel electrophoresis, respectively.

3.2.5 *In vivo* studies

The experimental protocols were reviewed and approved by animal ethics committees at the Institute of Medical and Veterinary Science, Adelaide. Five hundred micrograms (20 μCi) of

^{125}I-apoA-I, either in the lipid-free form or as a component of discoidal or spherical rHDL was injected into the left marginal ear vein of the rabbit. Blood samples were drawn from the right marginal ear vein at 2, 10, 30 and 60 minutes and at 2, 4, 6, 24, 50, 76 and 100 hours. The samples were stored at 4°C in tubes containing 0.1 mg/ml EDTA Na_2 and 2 mM 5,5'-dithio-bis(nitrobenzoic acid) (DTNB). Plasma samples collected during the first 6 hours were subjected to nondenaturing polyacrylamide gradient gel electrophoresis and agarose gel electrophoresis. All of the samples were used for kinetic analysis.

3.2.6 Kinetic analyses

The fractional catabolic rate (FCR) of ^{125}I-apoA-I when injected in the various forms was determined by fitting a two-compartment model to the tracer data (Lewis *et al.* 1997). The model assumes that labelled HDL were injected into a central compartment and subsequently exchanged with an extravascular compartment. Irreversible loss of HDL was assumed to have occurred from the central compartment.

3.2.7 Other analysis

Non-denaturing polyacrylamide gradient gel electrophoresis, agarose gel electrophoresis, phosphorimaging and chemical assays were carried out as described in chapter 2.

3.2.8 Statistical analysis

Student's t-test and one-way ANOVA were performed with the JMP software (SAS Institute, Cary, NC). Significance level was set at $p < 0.05$.

3.3 Results

3.3.1 Characterisation of lipid-free ^{125}I-apoA-I, discoidal rHDL and spherical rHDL

Discoidal and spherical rHDL containing PLPC, UC and ^{125}I-apoA-I were prepared. The molar ratios of PLPC/CE/UC/apoA-I in the discoidal and spherical rHDL were 56/0/6/1 and 41/10/2/1, respectively. The respective diameters of the discoidal and spherical rHDL were 9.3 and 8.8 nm. The electrophoretic mobility of the discoidal rHDL was –0.49 μm•sec^{-1}/V•cm^{-1} compared to –0.72 μm•sec^{-1}/V•cm^{-1}, for the spherical rHDL. Radiolabelling of apoA-I and its incorporation into discoidal and spherical rHDL had no effect on either the electrophoretic mobility or particle size when compared with the unlabelled counterparts (Figure 3.1).

3.3.2 Metabolism of apoA-I in plasma of rabbits

3.3.2.1 Lipid-free apoA-I

3.3.2.1.1 In vitro

When lipid-free ^{125}I-apoA-I was added to rabbit plasma, its size and electrophoretic mobility changed rapidly. Within 30 minutes of incubation, all of the pre-β-migrating apoA-I was in particles with α mobility (Figure 3.2A). This change in electrophoretic mobility was accompanied by a rapid appearance of ^{125}I-apoA-I in particles the size of HDL (Figure 3.2B).

3.3.2.1.2 In vivo

When lipid-free ^{125}I-apoA-I was injected into rabbits, the changes in electrophoretic mobility (Figure 3.2C) and size (Figure 3.2D) of apoA-I were complete in 2 min (vs. 30 min *in vitro*). All of the ^{125}I was incorporated into large, α-migrating particles.

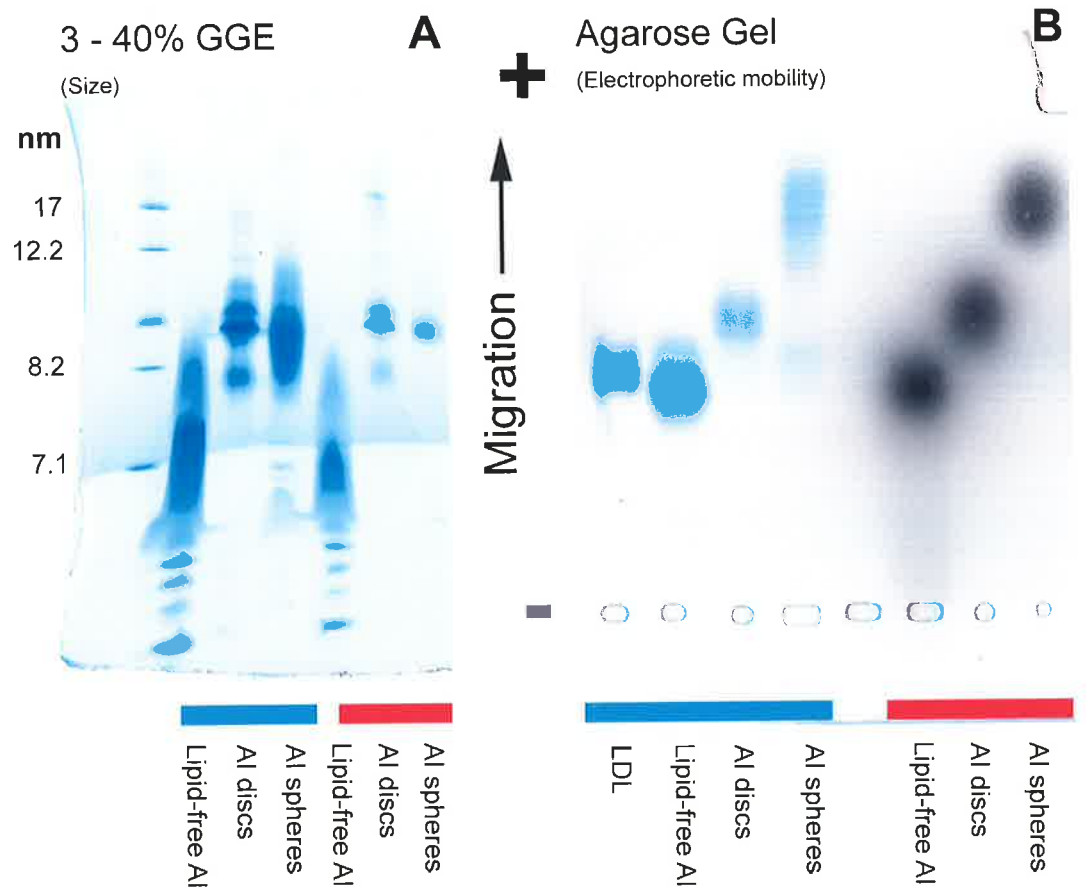

Figure 3.1. The effect of radioiodination on the size distribution (Panel A) and electrophoretic mobility (Panel B) of apoA-I as lipid-free protein or as a component of discoidal or spherical rHDL. Particles labelled by the blue bars are non-radiolabelled particles and red bars are radiolabelled particles. The preparation of the radiolabelled particles are described in chapter 2. Radioiodination of apoA-I did not alter the size or electrophoretic mobility of the three forms of apoA-I. (GGE: gradient gel electrophoresis)

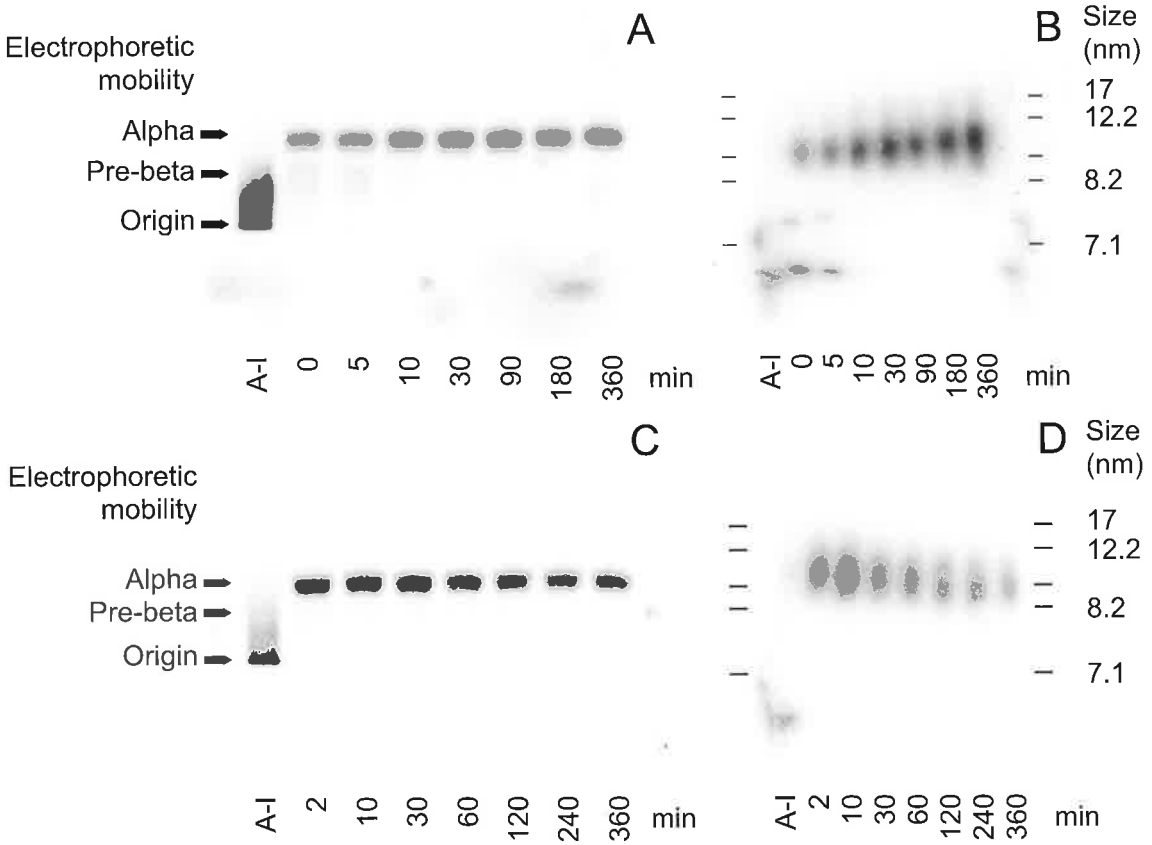

Figure 3.2. Changes in the electrophoretic mobility (Panels A & C) and size distribution (Panels B & D) of lipid-free apoA-I. Lipid-free ^{125}I-apoA-I (A-I) was incubated with rabbit plasma at 37˚C (Panels A & B) or injected into a rabbit (Panels C & D). Serial blood samples were collected at the indicated intervals. The electrophoretic mobility and size distribution of the labelled apoA-I in the serial samples were assessed by agarose gel electrophoresis and 3 – 40% non-denaturing gradient gel electrophoresis respectively. The positions of the radiolabelled apoA-I were detected by phosphorimaging. Samples from individual studies are presented. Each is representative of six such experiments.

3.3.2.2 ApoA-I in discoidal rHDL

3.3.2.2.1 In vitro

When ^{125}I-apoA-I was added to rabbit plasma as a component of discoidal rHDL, its electrophoretic mobility changed from pre-β to α within 10 min (Figure 3.3A). This is consistent with a rapid conversion of the discoidal particles into spheres. There were also changes in the size distribution of the ^{125}I-apoA-I-containing particles. At the earliest time-points the ^{125}I-apoA-I appeared in two populations of particles 7.8 nm and 8.8 nm in diameter (Figure 3.3B). Lipid-free apoA-I was also observed at 0 and 5 min (Figure 3.3B). By one hour, the smaller particles had disappeared, and all of the ^{125}I-apoA-I was incorporated into larger particles.

3.3.2.2.2 In vivo

When ^{125}I-apoA-I was injected into rabbits as a component of discoidal rHDL, the changes in size and electrophoretic mobility were similar to those observed *in vitro*. The electrophoretic mobility of the ^{125}I-apoA-I changed from pre-β to α two minutes after injection (Figure 3.3C). This is consistent with a rapid conversion of the pre-β-migrating discoidal particles into α-migrating spherical HDL. The injected ^{125}I-apoA-I initially appeared in two populations of particles, 7.6 nm and 8.5 nm in diameter (Figure 3.3D). As in the *in vitro* studies, there was a progressive disappearance of the smaller particles, with all of the ^{125}I-apoA-I residing in the larger particles 60 minutes after injection.

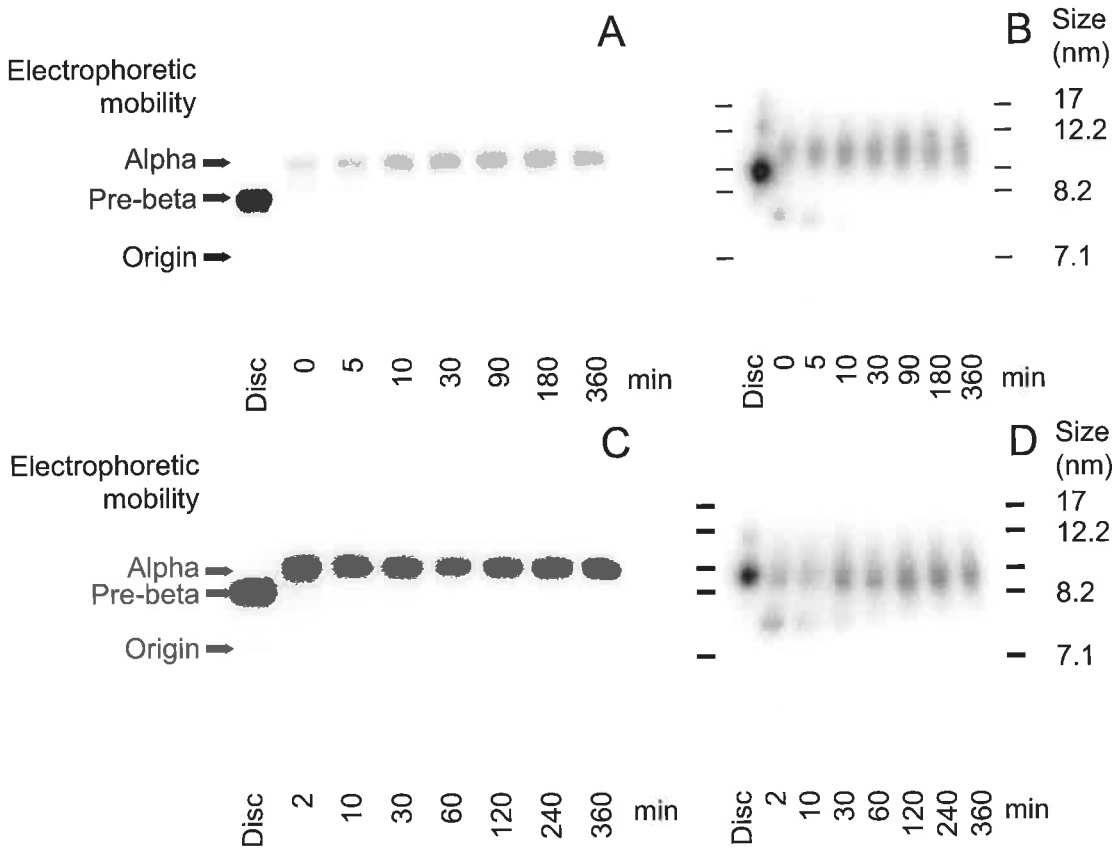

Figure 3.3. Changes in the electrophoretic mobility (Panels A & C) and size distribution (Panels B & D) of discoidal rHDL. ^{125}I-apoA-I as a component of discoidal rHDL (disc) were incubated with rabbit plasma at 37˚C (Panels A & B) or injected into a rabbit (Panels C & D). Serial blood samples were collected and assessed as described in figure 3.2. Samples from individual studies are presented. Each is representative of six such experiments.

3.3.2.3 ApoA-I in pre-labelled spherical rHDL

3.3.2.3.1 In vitro

When ^{125}I-apoA-I was added to rabbit plasma as a component of α-migrating, spherical rHDL its electrophoretic mobility did not change (Figure 3.4A). By 6 hours, larger particles of 9.8 nm in diameter were apparent (Figure 3.4B).

3.3.2.3.2 In vivo

When ^{125}I-apoA-I was injected intravenously into rabbits as a component of spherical rHDL, its electrophoretic mobility (Figure 3.4C) and size distribution (Figure 3.4D) did not change.

3.3.3 Kinetics of the disappearance of ^{125}I apoA-I in rabbits

Rabbits were divided into three groups (six animals per group) and injected intravenously with tracer amounts of ^{125}I-apoA-I either in the lipid-free form or as a component of discoidal rHDL, spherical rHDL. No matter whether apoA-I was in its lipid-free form, discoidal rHDL or spherical rHDL, the ^{125}I-apoA-I displayed virtually identical biphasic decay curves (Figure 3.5). The FCRs of the apoA-I introduced in the lipid-free form or as a component of pre-labelled discoidal or spherical rHDL were respectively 0.78 ± 0.10, 0.89 ± 0.14 and 0.74 ± 0.11 pools/day (mean ± SD). The differences between these values were not statistically significant.

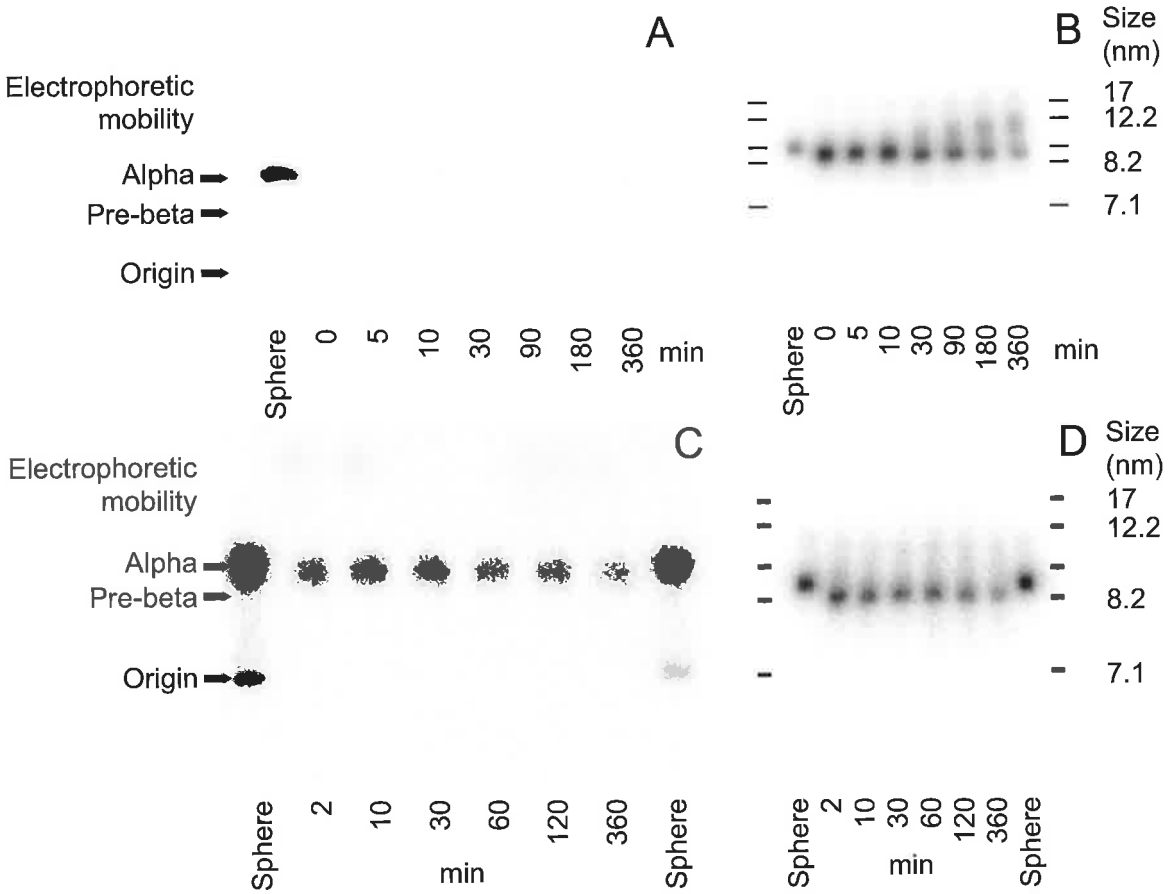

Figure 3.4. Changes in the electrophoretic mobility (Panels A & C) and size distribution (Panels B & D) of spherical rHDL. ^{125}I-apoA-I as a component of spherical rHDL (sphere) were incubated with rabbit plasma at 37˚C (Panels A & B) or injected into a rabbit (Panels C & D). Serial blood samples were collected and assessed as described in figure 3.2. Samples from individual studies are presented. Each is representative of six such experiments.

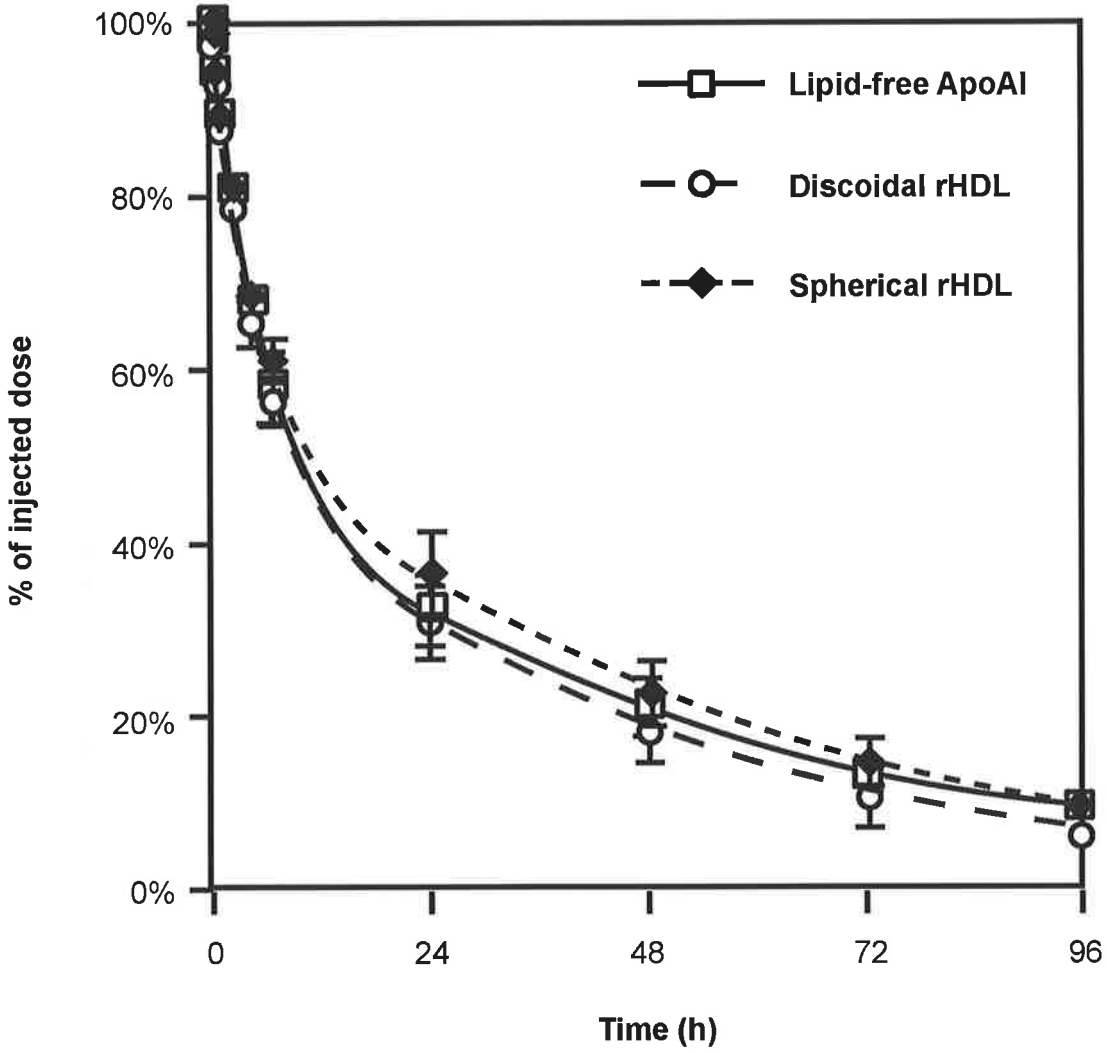

Figure 3.5. In vivo metabolism of ^{125}I-apoA-I in rabbits. Animals were divided into 3 groups (6 animals per group) and injected intravenously with tracer amounts of ^{125}I-apoA-I either in the lipid-free form (n=6) or as a component of discoidal rHDLs (n=6) or spherical rHDLs (n=6). The decay of label is presented as a percentage of injected dose. The FCRs of the apoA-I introduced in the lipid free form or as a component of discoidal or spherical rHDLs (calculated as described in Methods) were, respectively, 0.78±0.10, 0.89±0.14, and 0.74±0.11 pools per day (mean±SD). The differences of FCRs among the three forms of apoA-I were not statistically significant.

3.4 Discussion

This study describes the *in vitro* and *in vivo* metabolism of three forms of apoA-I in plasma: lipid-free apoA-I, pre-β-migrating discoidal HDL and α-migrating spherical HDL. In rabbits, there was (i) a rapid appearance of lipid-free apoA-I in pre-existing α-migrating HDL and (ii) a rapid appearance of the apoA-I injected as a component of pre-β-migrating, discoidal HDL in first smaller and then larger α-migrating particles. These results are consistent with the fact that most of the apoA-I in rabbit plasma circulates in α-migrating particles, with very little existing in either the lipid-free or pre-β-migrating forms.

By using apoA-I in 3 different forms, the present study has given a unique insight into the metabolism of HDL. Previous investigations of the *in vivo* metabolism of apoA-I in humans and other species have used two main techniques. Most commonly, preparations of HDL have been isolated from plasma, labelled exogenously with ^{125}I-apoA-I, then re-injected intravenously into the study subject (Fidge *et al.* 1980; Colvin *et al.* 1999). More recently, experiments have been conducted using an endogenous labelling technique, whereby the kinetics of the incorporation of amino acids (labelled with stable isotopes) into HDL apolipoproteins are analysed (Tilly-Kiesi *et al.* 1997; Velez-Carrasco *et al.* 2000). While the kinetic parameters generated by the two approaches are similar (Ikewaki *et al.* 1993), neither of these techniques can answer the questions being asked in the present studies. The exogenous labelling of native HDL is appropriate for studying α-migrating HDL. However, this approach cannot be used to study pre-β-migrating HDL, which are not normally present in significant quantities in plasma. The endogenous labelling technique, on the other hand, cannot be used to investigate the remodelling of HDL subpopulations because of the rapidity of these processes relative to the overall rate of HDL catabolism.

The *in vivo* relationship between the different forms of apoA-I can only be answered by tracing the metabolism of apoA-I when introduced directly into plasma in each of its forms. To this end, we assembled rHDL of defined shape, size and electrophoretic mobility (Rye and Barter 1994; Rye *et al.* 1995; Rye *et al.* 1997; Hime *et al.* 1998) containing isotopically-labelled apoA-I in each of its forms. The IODO-BEAD iodination technique used in this study exposes the apoA-I to relatively low levels of radiation and thus minimises damage to the protein (Markwell 1982). However, the possibility that the process alters apoA-I in some way cannot be excluded and the results must be interpreted with this reservation. Another reservation in studies of this type relates to possible modification of the injected HDL by prior ultracentrifugation. However, the fact that the FCR was the same when apoA-I was injected as a component of spherical rHDL (that were subject to ultracentifugation) as when it was lipid-free or was a component of discoidal rHDL (that had not been ultracentrifuged) argues strongly against a major effect of the prior centrifugation.

The studies conducted in the rabbits have provided the first demonstration *in vivo* that lipid-free apoA-I is incorporated rapidly and quantitatively into α-migrating HDL. It was also apparent that when injected as a component of pre-β, discoidal rHDL the labelled apoA-I appeared sequentially in small and then in larger α-migrating particles. While this is consistent with a conversion of the discs into first small and then larger spheres, the possibility that apoA-I may have exchanged between discoidal and spherical particles cannot be excluded. The incorporation of lipid-free apoA-I and discoidal apoA-I into α-migrating particles was so rapid *in vivo* that the fate of apoA-I injected into the rabbits was independent of the form in which it was delivered. Within minutes of injection, each form of the apoA-I was found exclusively in α-migrating HDL particles. The rapidity of these processes also

provides an explanation for the virtual absence of the pre-β-migrating apoA-I in normal plasma.

Kinetic analysis showed that the FCRs of ^{125}I-apoA-I as lipid free particle or as a component of prelabelled discoidal and prelabelled spherical rHDL were indistinguishable. Obviously, this is related to the rapidity in the conversion of each form of apoA-I into large α-migrating HDL. The decay profile of apoA-I is reflective of the metabolism of apoA-I in the large α-migrating HDL.

In conclusion, these results show that lipid-free apoA-I and the apoA-I in discoidal HDL rapidly appear in α-migrating HDL, both *in vitro* and *in vivo*. These explain the predominance of large, α-migrating HDL and the virtual absence of lipid-free apoA-I in the circulation. The rapidity in the remodelling process of various forms of apoA-I into large, α-migrating HDL explains why the catabolism of apoA-I is similar when apoA-I is introduced in the three different forms.

4 Remodelling of high density lipoproteins and metabolism of apoA-I in hepatic lipase transgenic rabbits

4.1 Rationale

Studies in the previous chapter have established (1) the rapidity of incorporation of lipid-free apoA-I into pre-existing HDL, (2) the rapidity in the conversion of pre-β discoidal HDL into large α-migrating spherical HDL, and (3) the maintenance of size of large α-migrating spherical HDL in wild-type rabbits. These results offer an explanation of the predominance of large α-migrating spherical HDL and the relative paucity of lipid-free apoA-I or pre-β migrating HDL in the circulation. However, these results also raise the question about the validity of those *in vitro* studies which demonstrated that certain plasma factors, notably CETP and hepatic lipase (HL), can reduce the size of HDL and lead to the dissociation of lipid-free apoA-I (Newnham and Barter 1990; Clay et al. 1992). It remains uncertain whether certain plasma factors are capable in reducing the size of HDL *in vivo*.

Rabbits are naturally deficient in HL. The size of HDL is smaller in transgenic rabbits overexpressing HL than in wild type rabbits (Fan et al. 1994). In contrast to *in vitro* studies, lipid-free apoA-I has not been detected in HL transgenic rabbits. The current study aims to determine (1) whether HL can convert large α-migrating spherical HDL into smaller HDL, (2) whether the conversion of large into small HDL will result in the detection of pre-β-migrating particles or lipid-free apoA-I, and (3) how the metabolism of apoA-I will be altered in the presence of hepatic lipase expression.

4.2 Methods

4.2.1 Rabbits

The studies were carried out in mature male wild-type New Zealand White (NZW) rabbits and human HL transgenic rabbits. The rabbits were fed on a normal rabbit chow diet. The experiments were conducted at the Gladstone Institute of Cardiovascular Disease in San Francisco and involved both wild-type and HL transgenic NZW rabbits of the Charles River strain. The transgenic rabbits were provided by Prof. John Taylor of the Gladstone Institute. The generation of the HL transgenic rabbits and their lipoprotein characteristics have been described in detail (Fan et al. 1994; Fan et al. 1995; Barbagallo et al. 1999). They contain a liver-specific sequence from the human apoE gene, the hepatic control region of the apoE/ C-I locus, and a full-length human HL cDNA. Rabbits containing the HL transgene were identified by Southern blot analysis. Activity of HL in the transgenic rabbits was measured in post-heparin plasma using a triolein-based substrate and is described in Chapter 2 (Cheng et al. 1985). Among the colony of 13 HL transgenic rabbits, the HL activity of the HL transgenic rabbits was 5.2 ± 1.7 (mean ± SE) μmol fatty acid (FA) released • ml^{-1} • h^{-1}. Experiments were performed on two transgenic animals expressing high HL activity (21.6 and 7.8 μmol FA released • ml^{-1} • h^{-1}) and two transgenic animals expressing moderate HL activity of (3.2 and 2.8 μmol FA released • ml^{-1} • h^{-1}). Activity of HL in the wild-type rabbits was 0.6 ± 0.3 μmol FA released • ml^{-1} • h^{-1}. These values should be compared with activity of HL in normal human subjects of 6.6 ± 2.7 μmol FA released • ml^{-1} • h^{-1} (Connelly et al. 1990).

4.2.2 Purification and radio-labelling of apoA-I

The purification and radioiodination of apoA-I were described in Chapter 2.

4.2.3 Preparation of ^{125}I-apoA-I spherical rHDL

The preparation of ^{125}I-apoA-I spherical rHDL was described in Chapter 2.

4.2.4 *In vivo* studies

The experimental protocols were reviewed and approved by animal ethics committees at the University of California, San Francisco. The animal experimental protocol and subsequent analysis of samples were identical to those in Chapter 3.

4.2.5 Kinetic analyses

Kinetic analyses were performed as described in Chapter 3.

4.2.6 Other analysis

Non-denaturing polyacrylamide gradient gel electrophoresis, agarose gel electrophoresis and chemical analysis are described in Chapter 2.

4.3 Results

4.3.1 Lipid profile of wild-type and hepatic lipase transgenic rabbits

The HL transgenic rabbits had lower levels of plasma total cholesterol, HDL-cholesterol and apoA-I when compared to wild-type rabbits (Table 4.1).

TABLE 4.1: Lipid profile of wild-type and hepatic lipase transgenic rabbits*

Subjects	No	Weight, kg	Triglyceride, mmol/L	Total cholesterol, mmol/L	LDL-cholesterol, mmol/L	HDL-cholesterol, mmol/L	ApoA-I, mg/mL
Wild-type	6	3.93 ± 0.14	1.10 ± 0.34	1.32 ± 0.12	0.16 ± 0.11	0.75 ± 0.08	0.64 ± 0.08
Transgenic	4	4.65 ± 0.42	0.54 ± 0.41	0.36 ± 0.15*	0.05 ± 0.12	0.14 ± 0.10†	0.08 ± 0.03†

Values are presented as mean ± SEM. †$p < 0.05$ as assessed by paired t-tests.

* The wild-type and transgenic rabbits described in this table were of the Charles River strain and were investigated at the Gladstone Institute in San Francisco.

4.3.2 Injection of apoA-I in spherical rHDL into wild-type and hepatic lipase transgenic rabbits

When ^{125}I-apoA-I was injected intravenously into wild-type rabbits as a component of spherical rHDL, its electrophoretic mobility (Figure 4.1A) and size distribution (Figure 4.1B) did not change. When ^{125}I-apoA-I was injected intravenously into hepatic lipase transgenic rabbits as a component of spherical rHDL, its electrophoretic mobility did not change (Figure 4.1C). However, the ^{125}I-apoA-I-containing particles progressively decreased in size from 8.8 nm to 7.4 nm (Figure 4.1D).

4.3.3 Kinetics of the disappearance of ^{125}I apoA-I as a component of spherical rHDLs in wild-type rabbits and hepatic lipase transgenic rabbits

Tracer amounts of ^{125}I apoA-I as a component of spherical rHDL were injected into 6 of the wild-type and 4 of the HL transgenic rabbits from the Gladstone Institute (Figure 4.2). The decay of ^{125}I apoA-I following injection of labelled spherical rHDL into the transgenic rabbits was substantially faster than in the non-transgenic rabbits (Figure 4.2). The corresponding fractional catabolic rates of ^{125}I apoA-I in spherical rHDL in the transgenic and non-transgenic rabbits were 0.98 ± 0.03 and 0.42 ± 0.09 (mean $\pm$ SD) pools/ day, respectively ($p < 0.05$).

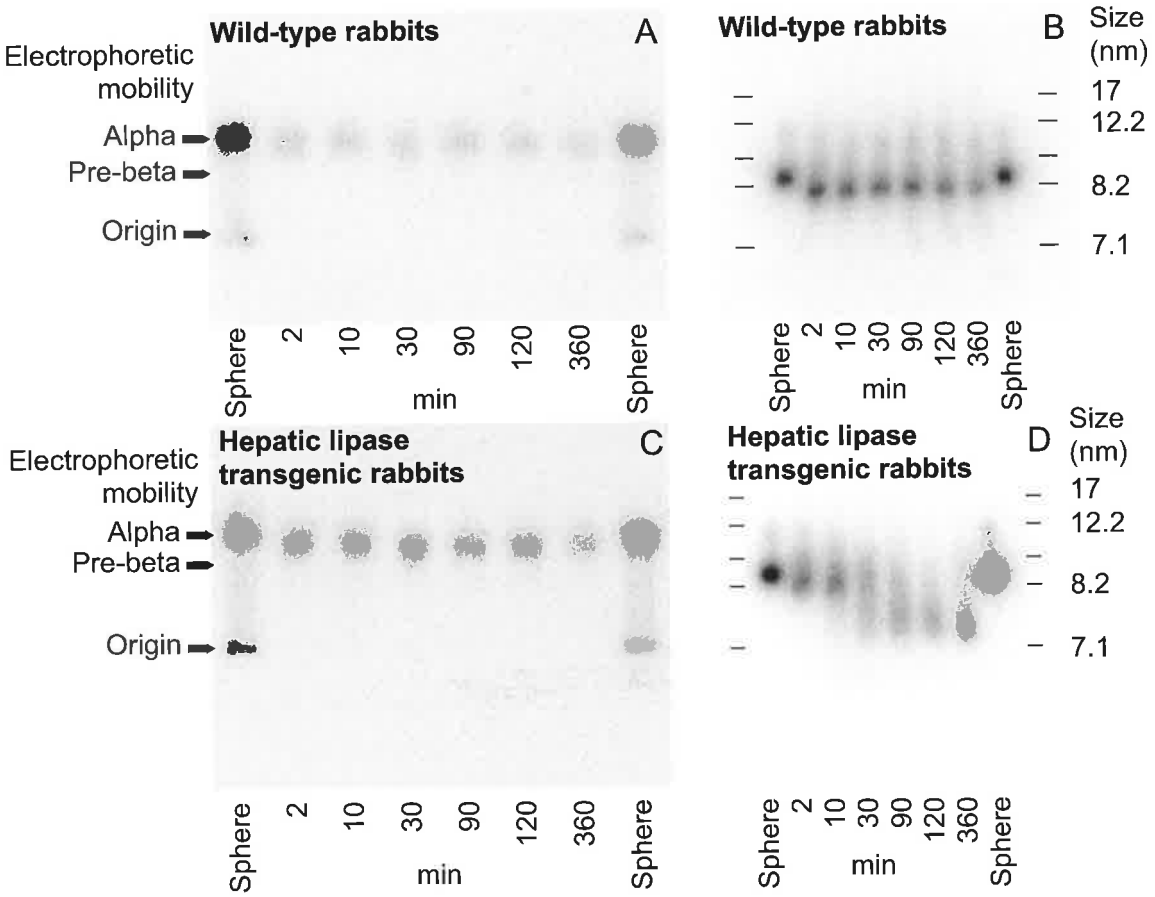

Figure 4.1. Changes in the electrophoretic mobility (A and C) and size distribution (B and D) of spherical rHDL in wild-type rabbits and in HL transgenic rabbits. ^{125}I-apoA-I as a component of spherical rHDL was injected into wild-type (A and B) and HL transgenic (C and D) rabbits. Serial blood samples were taken at the indicated intervals. Analyses of the samples were described in the Methods section. The samples from individual studies are representative of 6 such studies conducted in wild-type rabbits and 4 such studies conducted in HL transgenic rabbits.

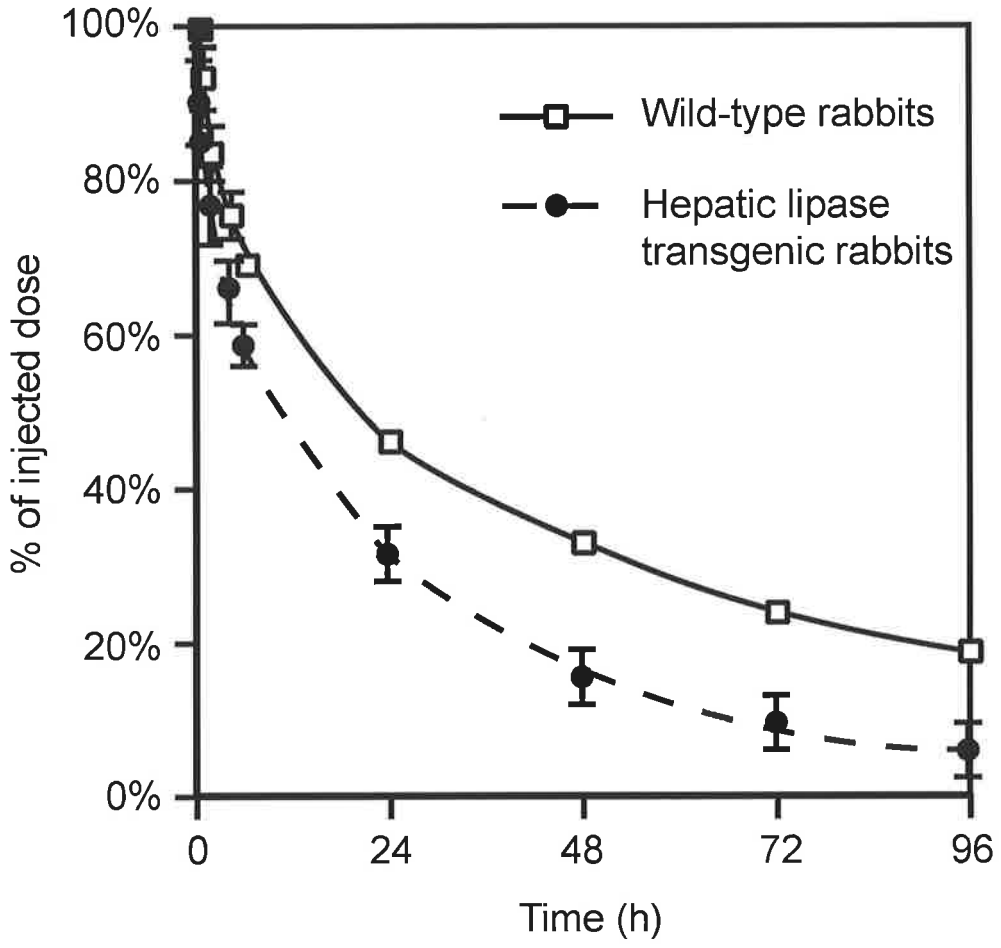

Figure 4.2. In vivo metabolism of ^{125}I-apoA-I in HL transgenic rabbits. Studies were conducted in wild-type and HL transgenic rabbits. Tracer amounts of ^{125}I-apoA-I as a component of spherical rHDL were injected into wild-type rabbits (n=6) and HL transgenic rabbits (n=4). The FCRs of the injected apoA-I in the wild-type and HL transgenic rabbits were, repectively, 0.42±0.09 and 0.98±0.03 (mean±SEM) pools per day ($p<0.05$).

4.4 Discussion

The *in vivo* experiments were carried out in both wild-type and HL transgenic rabbits. In the wild-type rabbits, there was no change in the size or electrophoretic mobility when apoA-I was injected as a component of spherical rHDL. On the other hand, when apoA-I as a component of spherical rHDL was injected into HL transgenic rabbits, there was a progressive reduction in the size of the particles without any concomitant change in the electrophoretic mobility. The smaller particles were small, α-migrating particles. These findings were consistent with previous report of the presence of smaller HDL in these transgenic rabbits. They also demonstrate the action of HL in reducing the size of HDL *in vivo*. In contrast to the *in vitro* studies, neither pre-β HDL nor lipid-free apoA-I was detected after the injection of spherical rHDL into HL transgenic rabbits. When compared with the wild-type rabbits, the catabolism of apoA-I was increased by 2-fold in HL transgenic rabbits.

Rabbit is well suited for the study of the effects of HL on HDL remodelling and metabolism. Rabbits are naturally deficient in activity of HL (Clay et al. 1989), which has been turned into an advantage by using transgenic rabbits expressing human HL.

The observation of an increase in size of α-migrating HDL *in vivo* has been reported previously (Colvin et al. 1999). The fact that the size of injected HDL particles may also be decreased *in vivo* has not previously been reported. The absence of such a decrease in HDL size *in vivo* in wild-type rabbits is predictable from their low activity of HL, since it has been shown *in vitro* that a decrease in HDL particle size depends on the joint activities of CETP and HL (Newnham and Barter 1990; Clay et al. 1992). In this process, CETP transfers cholesteryl esters from HDL to the triglyceride-rich lipoproteins in exchange for a transfer of triglycerides into the HDL to generate triglyceride-enriched HDL. When HL subsequently

hydrolyses the HDL triglyceride, the core lipid content and size of the HDL decreases in a process that is accompanied by dissociation of apoA-I from the particle (Clay et al. 1991; Clay et al. 1992). Thus, given the relative deficiency of HL in wild-type rabbits, it was quite predictable that HDL particle size would not be reduced *in vivo* in these animals. It was, perhaps, also predictable that HDL particle size would be reduced in the transgenic rabbits in which HL was expressed.

In contrast to *in vitro* studies, *in vivo* remodelling of HDL in HL transgenic rabbits did not lead to any detectable dissociation of lipid-free apoA-I or pre-β HDL from HDL. Three possible explanations may account for the finding. Firstly, as shown in the wild-type rabbits in chapter 3, lipid-free apoA-I is rapidly incorporated and become part of the pre-existing α-migrating HDL in the circulation. The circulating HDL has the capacity to bind any free apoA-I which is otherwise dissociated from HDL by HL. Secondly, the amount of lipid-free apoA-I dissociated from HDL is likely to be low and may be beyond the threshold of detection by the available techniques. Finally, lipid-free apoA-I is easily removed from the circulation by glomerular filtration and subsequently cleared by renal mechanisms. Although lipid-free apoA-I or pre-β HDL were not detected in these studies, the possible existence of those particles during HL-mediated HDL remodelling cannot be excluded.

Kinetic analysis showed that the FCR of ^{125}I-apoA-I in HDL was enhanced in the HL transgenic rabbits, although the FCR did not correlate with the level of activity of HL in the individual transgenic rabbits. HL activities of 21.6, 7.8, 3.2 and 2.8 μmol FA released • ml^{-1} • h^{-1} in the four transgenic rabbits were associated with FCRs of 1.05, 0.91, 0.95 and 1.01 pools/day, respectively. This suggests that once the hepatic lipase activity is above a certain threshold level it may no longer be rate-limiting.

The explanation for the increased FCR in the HL transgenic rabbits is not known, although it has been reported that HL participates in the direct uptake of HDL cholesteryl esters by the liver (Lambert et al. 1999). Whether or not this explains the increased FCR of apoA-I is not certain. The increased FCR may also relate to the ability of HL to promote the dissociation of apoA-I from the particle (Clay et al. 1991). Any dissociated lipid-poor apoA-I would not remain in this form for long as evidenced by the rapid disappearance of pre-β migrating apoA-I after addition of lipid-free apolipoprotein to rabbit plasma *in vitro* or after its injection into intact rabbits. Lipid-poor apoA-I has several potential fates. It may be re-lipidated by ABCA1 (Oram and Vaughan 2000), it may be incorporated into pre-existing HDL or it may be filtered by the glomeruli and be lost in the urine (Horowitz et al. 1993). Given the likelihood that urinary loss will be a function of the amount filtered, any increase in the dissociation of lipid-poor apoA-I from HDL would be predicted to increase the urinary excretion and thus contribute to an increased rate of catabolism of apoA-I in the HL transgenic rabbits.

In conclusion, the results show for the first time that HL has the capacity both to decrease the size of α-migrating HDL and to enhance their rate of clearance *in vivo*. The pathophysiological implications of these findings remain to be determined.

5 The contribution of CETP on the metabolism of cholesteryl esters in HDL and VLDL/LDL

5.1 Rationale:

The previous chapter described the metabolism of apoA-I in wild-type and hepatic lipase transgenic rabbits. This chapter will focus on the metabolism of cholesteryl esters (CE), in wild-type rabbits and how their metabolism is altered when plasma CETP activity is altered.

The best-known function of HDL is the transportation of cholesterol from the periphery back to the liver, a pathway also known as reverse cholesterol transport (Glomset 1968). This pathway is regarded anti-atherogenic. Cholesteryl esters (CE), the main form of cholesterol in HDL, can potentially be transported back to the liver via two separate pathways. In the direct pathway, CE in HDL can be taken up via specific hepatic HDL receptors. In the indirect pathway, CE in HDL can be transferred from HDL to VLDL/LDL in animals expressing CETP before taken up by the hepatic LDL receptors. In animals expressing CETP, the proportion of CE cleared by these two pathways has generated a lot of interest because the fate of CE in HDL and LDL may be linked to the development of atherosclerosis. Cholesteryl esters in HDL are delivered to the liver and subsequently excreted in bile, a process that is potentially anti-atherogenic. On the other hand, CE transferred to LDL, via the action of CETP, may re-circulate and accumulate in peripheral tissues, leading to atherosclerosis. Consequently, inhibition of CETP activity may favour the retention of CE in HDL and may be potentially anti-atherogenic. Indeed, a number of experiments have demonstrated the anti-atherogenic effects of CETP inhibition in rabbits, a species expresses high level of CETP (Sugano et al. 1998; Okamoto et al. 2000; Rittershaus et al. 2000). However, the altered CE

kinetics among plasma lipoproteins by CETP inhibition remains speculative and has not been clearly demonstrated. In fact, there is a concern that CETP inhibition may adversely affect reverse cholesterol transport. Up to 70% of plasma CE flux originating from HDL CE is mediated by CETP (Goldberg et al. 1991) and any alteration of the activity of CETP may be potentially pro-atherogenic. In LCAT-transgenic mice with low endogenous CETP, the accumulation of CE in HDL as a result of LCAT overexpression rendered HDL 'dysfunctional' and interfered with the cholesterol efflux from peripheral tissues to HDL (Berard et al. 1997; Albanese et al. 1998). The expression of CETP in these transgenic animals restored the functions of these 'dysfunctional' HDL (Foger et al. 1999). Those transgenic mouse studies need to be interpreted with caution, as the observations may not be relevant in CETP-expressing animals such as rabbits and humans.

It is therefore the aim of this study to measure the effects of CETP inhibition on the CE flux cleared via HDL and VLDL/LDL in an animal model with high endogenous CETP activity. Specifically, rabbits were pre-treated with a potent CETP inhibitor, CP-456,643, before injecting with a tracer amount of rHDL containing [^{3}H]CE. The following parameters were estimated: (1) the cholesterol flux of CE from HDL and VLDL/LDL pools, (2) the proportion of CE cleared via HDL and VLDL/LDL pools and (3) the proportion of CE transported from HDL to LDL.

5.2 Methods

5.2.1 Rabbits

The studies were carried out in mature male New Zealand White (NZW) rabbits purchased from the Institute of Medical and Veterinary Science in Adelaide. The rabbits were fed on a normal rabbit chow diet. The oral CETP inhibitor, CP-456,643, was provided by Pfizer Inc, Connecticut, USA. A stock solution of CP-456,643 (600 mg/mL) was prepared by dissolving the compound in chloroform. The rabbit chow was mixed with CP-456,643 and the solvent was vaporised in a fume hood overnight.

Rabbits were divided into two groups. One group was given normal rabbit chow and the other was given 5 mg/kg of CP-456,643 for 5 days in addition to the normal rabbit chow.

5.2.2 Measurement of CETP activity

Plasma CETP activity was measured before and four days after the administration of the oral CETP inhibitor. Activity of CETP was assessed as the transfer of [^{3}H]CE from [^{3}H]CE-HDL$_3$ to LDL (Burstein et al. 1970; Tollefson et al. 1988) and the methods were described in Chapter 2. Activity of CETP is expressed in nmol of CE transferred per mL of plasma per hour (nmol CE transferred/mL/h).

5.2.3 Purification of apoA-I and preparation of radioactive tracers

The purification of rabbit apoA-I was described in Chapter 2. The methods for preparing spherical rHDL containing [^{3}H]CE were described in Chapter 2.

5.2.4 In Vivo Studies

The experimental protocols were reviewed and approved by the animal ethics committees at the Institute of Medical and Veterinary Science, Adelaide, Australia. 0.8 mCi of spherical rHDL containing [^{3}H]CE was injected into 6 control rabbits and 6 rabbits treated with CP-456,643. Blood samples were collected over 6 hours. The storage, processing and analysis of plasma samples were described in Chapter 2.

5.3 Results

5.3.1 Characterisation of spherical rHDL containing [^{3}H]CE

The molar ratios of PLPC/CE/FC/apoA-I in the spherical rHDL was 40:38:0:1. The diameter of discoidal rHDL was 10.0 nm. The respective electrophoretic mobility of the spherical rHDL was –0.72 $\mu m \cdot s^{-1}/V \cdot cm^{-1}$. The specific activity of labelled [^{3}H]CE ranged between 6×10^8 and 7×10^8 cpm/μmol of cholesterol. LCAT converted more than 95% of [^{3}H]UC to [^{3}H]CE in spherical rHDL.

5.3.2 Lipid profile of rabbits treated with CETP inhibitor

The protocol for the administration of CETP inhibitor to the rabbits has been described in the methods section. As shown in Table 5.1, CETP inhibitor, CP-456,643, effectively inhibited the plasma CETP activity in the treated animals from 33.4 ± 1.7 to 3.5 ± 1.2 nmol CE transferred/ mL plasma/ h, a relative reduction of 89%. CP-456,643 had no effect on the plasma activity of PLTP. The inhibition of plasma CETP activity resulted in a number of changes to the lipid profile of the treated rabbits. The plasma total cholesterol concentration was significantly increased from 0.97 mmol/L to 1.28 mmol/L ($p < 0.05$). The increase in plasma cholesterol was mainly accounted for by an increase in the plasma total CE concentration from 0.67 mmol/L to 0.96 mmol/L while the plasma total UC concentration remained unchanged. CETP inhibition altered the distribution of CE in the lipoprotein classes. There was a significant increase in the concentration of CE in HDL from 0.33 mmol/L to 0.86 mmol/L ($p < 0.05$) and a significant decrease in the concentration of CE in VLDL/LDL from 0.33 mmol/L to 0.11 mmol/L ($p < 0.05$). CETP inhibition also resulted in a significant increase in the plasma concentration of apoA-I from 0.39 mg/mL to 0.66 mg/mL ($p < 0.05$).

Table 5.1: Lipid profile and plasma factor activities of wild-type rabbits before and after CETP inhibition

	Rabbits with no CETP inhibition	Rabbits with CETP inhibition (pre-inhibition)	Rabbits with CETP inhibition (day 4 post-inhibition)
No. of rabbits	6	6	6
Plasma total cholesterol (mmol/L)	0.81 ± 0.10	0.97 ± 0.10	1.28 ± 0.15 †
Plasma unesterified cholesterol (mmol/L)	0.07 ± 0.02	0.30 ± 0.03	0.33 ± 0.04
Plasma cholesteryl esters (mmol/L)	0.74 ± 0.08	0.67 ± 0.06	0.96 ± 0.11 †
HDL total cholesterol (mmol/L)	0.51 ± 0.06	0.50 ± 0.07	1.11 ± 0.11 †
HDL unesterified cholesterol (mmol/L)	0.05 ± 0.02	0.16 ± 0.02	0.25 ± 0.03 †
HDL cholesteryl esters (mmol/L)	0.46 ± 0.05	0.33 ± 0.05	0.86 ± 0.08 †
VLDL/LDL total cholesterol (mmol/L)	0.30 ± 0.04	0.47 ± 0.06	0.18 ± 0.06 †
VLDL/LDL unesterified cholesterol (mmol/L)	0.03 ± 0.01	0.14 ± 0.02	0.07 ± 0.02 †
VLDL/LDL cholesteryl esters (mmol/L)	0.28 ± 0.03	0.33 ± 0.04	0.11 ± 0.04 †
ApoA-I (mg/mL)	0.41 ± 0.08	0.39 ± 0.08	0.66 ± 0.15 †
CETP activity (nmol CE transferred/ mL plasma/h)		33.4 ± 1.7	3.5 ± 1.2 †
PLTP activity (nmol PL transferred/ mL plasma/h)		1413 ± 82	1429 ± 43

Values are presented as mean ± SEM. †$p < 0.05$ as assessed by paired t-tests.
Note: CE: Cholesteryl esters; CETP: Cholesteryl ester transfer protein; PL: Phospholipids; PLTP: Phospholipid transfer protein

5.3.3 Cholesteryl esters kinetics in HDL and LDL/ VLDL lipoprotein particles

A tracer amount of spherical rHDL containing [^{3}H]CE was injected into both treated and untreated rabbits. The time-course of the radioactivity of [^{3}H]CE in HDL and VLDL/LDL is shown in Figure 5.1. In the untreated animals, there was a rapid decline in the radioactivity of [^{3}H]CE in HDL and 50% of the injected radioactivity disappeared within 90 mins. In comparison, the decay of radioactivity of [^{3}H]CE was slower in the treated animals and 50% of the injected radioactivity disappeared within 240 mins. In the untreated animals, the rapid disappearance of [^{3}H]CE in HDL was accompanied by the appearance of [^{3}H]CE in VLDL/LDL and its peak radioactivity reached within 60 mins. In comparison, the appearance of [^{3}H]CE in VLDL/LDL was delayed and the peak radioactivity was lower in the treated animals.

5.3.4 Development of the model

In order to qualify the flux of CE among lipoproteins within plasma as well as the flux of CE out of plasma, a two-compartmental model was developed. The model was based on generally accepted pathways of CE metabolism (Figure 5.2). Assumptions are made in the model are listed as follows: (1) There was no VLDL CE pool in the model as the concentration of CE in VLDL is not directly measured and its contribution to CE transfer is assumed to be insignificant. (2) Based on the known mechanism of CETP in CE transport in plasma, and the observed relationship between the decay of [^{3}H]CE in HDL and the appearance of [^{3}H]CE in LDL, k(2,1) represents the transfer of CE from HDL to LDL. (3) It was also assumed that [^{3}H]CE in LDL could be transferred back to HDL and this pathway was represented by k(1,2) (4) The disappearance of [^{3}H]CE in HDL certainly exceeded the appearance of [^{3}H]CE in

LDL in both the untreated and treated animals, that could only be explained by a net loss of [^{3}H]CE out of plasma. Thus, k(0,1) and k(0,2) represented the net loss of [^{3}H]CE in HDL and LDL, respectively. (5) In the actual analysis, the robustness of the model was scrutinised by forcing either k(0,1) or k(0,2) to zero (i.e. net loss from HDL or LDL out of plasma to zero) and the CE losses from LDL and HDL (i.e. k(0,2) or k(0,1)) were re-calculated.

5.3.5 Parameters of the model

The steady-state fluxes between compartments are shown in Table 5.2. The salient points are as follows: (1) In the control (untreated) animals, no matter whether the net CE flux was lost via HDL or LDL, the total CE fluxes out of plasma are the same in either model. When the net CE flux was lost exclusively via HDL, the CE flux was 3.2 μmol/L/min. When the net CE flux was lost exclusively via LDL, the CE flux was 3.1 μmol/L/min.

The fractional catabolic rates and cholesterol flux of the various pathways are shown in Figure 5.3 and Table 5.2. The concentration of HDL CE significantly increased from 0.46 mmol/L in the untreated animals to 0.86 mmol/L in the treated animals ($p < 0.05$). The FCR of HDL CE was significantly reduced by 56% from 0.0072 pools/min in the untreated animals to 0.0032 pools/min in the treated animals ($p < 0.05$). Thus, the lost of CE from HDL was significantly increased from 1.65 μmol/L/min in the untreated animals to 2.72 μmol/L/min in the treated animals ($p < 0.05$). In contrast, the concentration of LDL CE significantly decreased from 0.28 mmol/L in the untreated animals to 0.11 mmol/L in the treated animals ($p < 0.05$). The FCR of LDL CE was reduced from 0.0062 pools/min in the untreated animals to zero in the treated animals ($p < 0.05$). Thus, the loss of CE from LDL was significantly reduced from 1.46 μmol/L/min in the untreated animals to zero in the treated

animals ($p < 0.05$). In another word, the percentage of CE cleared via HDL increased from 54% in the untreated animals to 100% in the untreated animals.

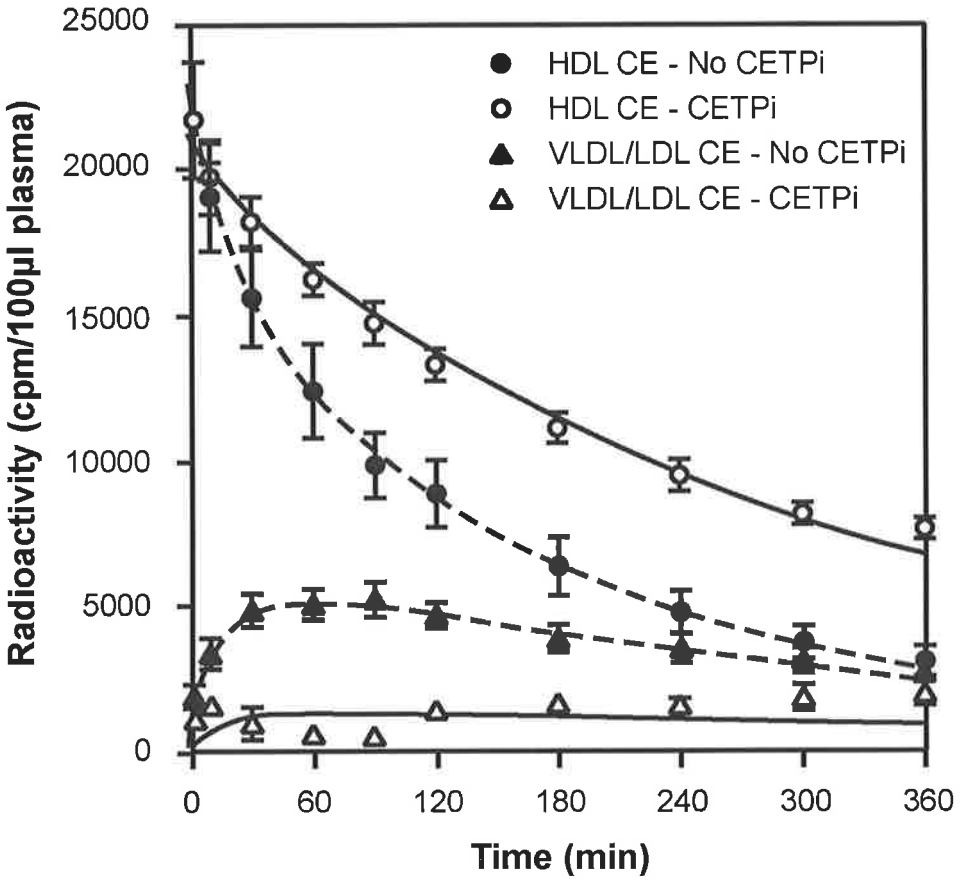

Figure 5.1. In vivo metabolism of [^{3}H]cholesteryl esters ([^{3}H]CE) in rabbits after intravenous injection of spherical rHDL containing [^{3}H]CE. A. Decay curves of [^{3}H]CE in HDL and VLDL/LDL in the presence or absence of CETP inhibition (CETPi).

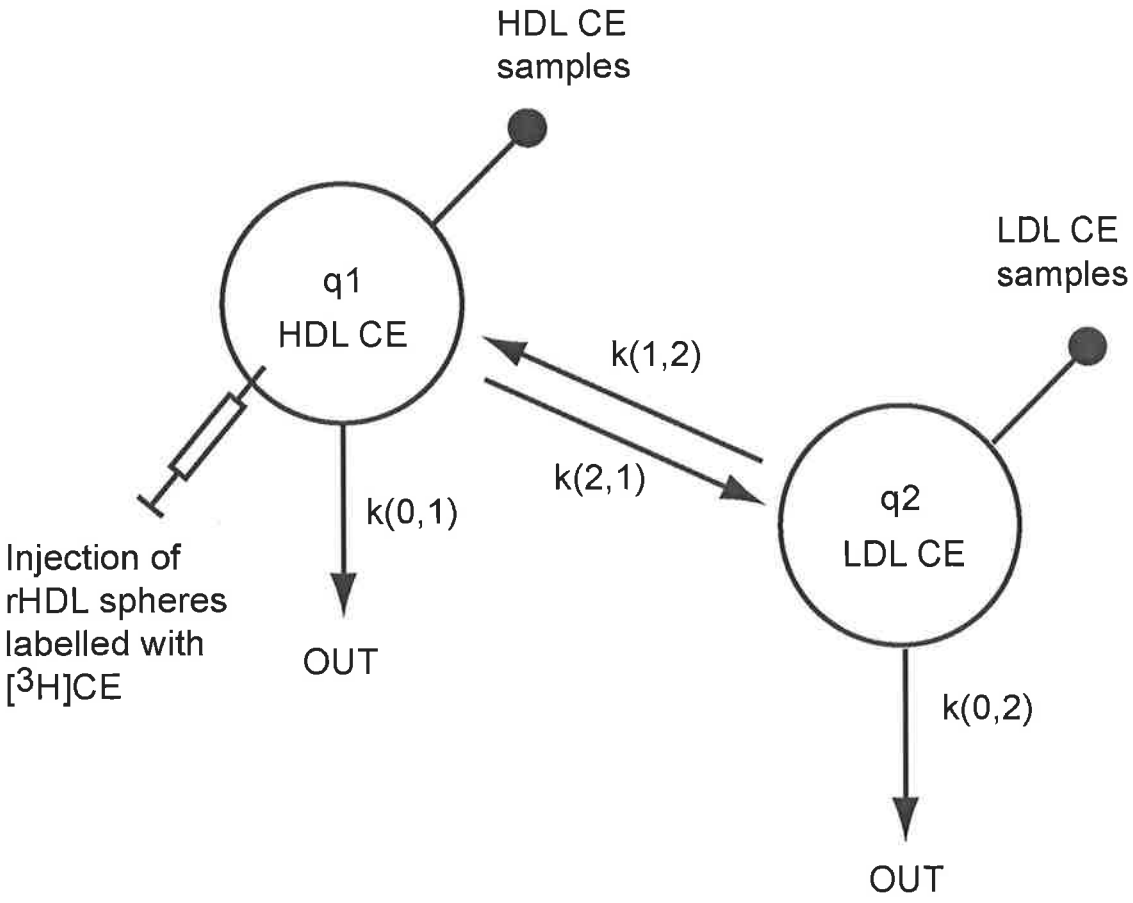

Figure 5.2. Compartmental model for the metabolism of plasma cholesteryl esters (CE) in rabbits. Circles represent standard compartments, each identified by the number in the compartment. Arrows represent transport pathways. Solid circles represent plasma sampling of various compartments.

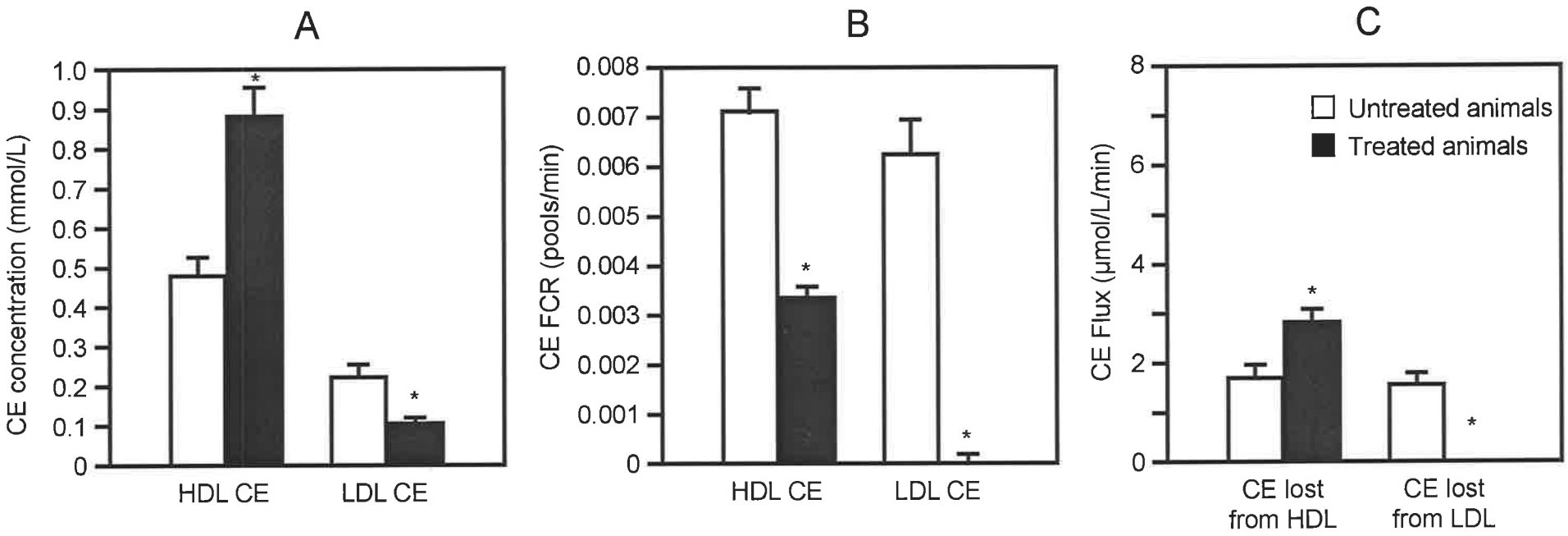

Figure 5.3. The effects of CETP inhibition on the plasma concentration of cholesteryl esters (CE) in HDL and LDL, and fractional catabolic rates (FCRs) and net cholesteryl ester (CE) flux out of HDL and LDL. A. Concentration of HDL CE and LDL CE in untreated and treated animals. B. Fractional catabolic rates (FCRs) of CE in HDL and LDL in untreated and treated animals. C. Net CE flux out of plasma from HDL and LDL in untreated and treated animals.

Table 5.2: Steady-state fluxes and fractional catabolic rates (FCRs) from compartmental analysis

Table 5.2.1: Steady-state fluxes and fractional catabolic rates (FCRs) in untreated animals if CE flux is lost from LDL compartment

Control	C1	C2	C3	C4	C5	C6	Mean	Stdev
				Fluxes (μmol/L/min)				
FLUX(0,1)	0	0	0	0	0	0	0	0
FLUX(0,2)	2.6	4.1	2.7	3.3	3.0	2.6	3.1	0.6
FLUX(1,2)	0.8	15.2	4.6	1.6	15.0	1.0	6.3	6.9
FLUX(2,1)	3.5	19.3	7.3	4.9	17.9	3.6	9.4	7.3
				FCRs (pools/min)				
k(0,1)	0	0	0	0	0	0	0	0
k(0,2)	0.03	0.03	0.04	0.04	0.05	0.15	0.06	0.05
k(1,2)	0.01	0.09	0.08	0.02	0.23	0.06	0.08	0.08
k(2,1)	0.01	0.03	0.02	0.01	0.05	0.01	0.02	0.02

Table 5.2.2: Steady-state fluxes and fractional catabolic rates (FCRs) in untreated animals if CE flux is lost from HDL compartment

Control	C1	C2	C3	C4	C5	C6	Mean	Stdev
				Fluxes (μmol/L/min)				
FLUX(0,1)	2.9	4.2	2.7	3.5	3.0	2.8	3.2	0.6
FLUX(0,2)	0	0	0	0	0	0	0	0
FLUX(1,2)	2.3	17.9	5.8	189.1	17.8	0.2	38.8	74.0
FLUX(2,1)	2.3	17.9	5.8	189.1	17.8	0.2	38.8	74.0
				FCRs (pools/min)				
k(0,1)	0.006	0.008	0.006	0.005	0.007	0.010	0.007	0.001
k(0,2)	0	0	0	0	0	0	0	0
k(1,2)	0.02	0.10	0.09	5	0.30	0.01	0.92	2.00
k(2,1)	0.01	0.03	0.01	0.29	0.05	0	0.06	0.11

Table 5.2 (cont): Steady-state fluxes and fractional catabolic rates (FCRs) from compartmental analysis

Table 5.2.3: Steady-state fluxes and fractional catabolic rates (FCRs) in treated animals, thus net CE flux is lost from HDL compartment

CETPi	Ci1	Ci2	Ci3	Ci4	Ci5	Ci6	Mean	Stdev
				Fluxes (µmol/L/min)				
FLUX(0,1)	3.0	1.6	2.7	2.9	3.4	2.7	2.7	0.6
FLUX(0,2)	0	0	0	0	0	0	0	0
FLUX(1,2)	8.1	0.8	0.8	8.3	10.8	1.4	5.0	4.5
FLUX(2,1)	8.1	0.8	0.8	8.3	10.8	1.4	5.0	4.5
				FCRs (pools/min)				
k(0,1)	0.003	0.004	0.003	0.003	0.003	0.003	0.003	0
k(0,2)	0	0	0	0	0	0	0	0
k(1,2)	0.10	0.40	0.01	0.10	0.12	0.01	0.12	0.14
k(2,1)	0.01	0	0	0.01	0.01	0	0.01	0

Table 5.3: Kinetic parameters of HDL CE and LDL CE in untreated and treated animals

	Untreated animals (n=6)	Treated animals (n=6)
Fractional catabolic rate (pools/ min)		
HDL CE	0.0072 ± 0.0015	0.0032 ± 0.0003 †
LDL CE	0.0062 ± 0.0007	0 ± 0 †
Cholesterol flux (μmol/L/min)		
Total CE	3.11 ± 0.24	2.72 ± 0.24
HDL CE to LDL	5.47 ± 0.86	5.02 ± 1.84
LDL CE to HDL	4.02 ± 0.73	5.03 ± 1.80
CE lost from HDL	1.65 ± 0.20	2.72 ± 0.24 †
CE lost from LDL	1.46 ± 0.27	0 ± 0 †
% CE cleared via HDL	54.0 ± 7.5%	100% ± 0% †
% HDL CE transported to LDL	75.8 ± 3.2%	52.3 ± 10.0% †

Values are presented as mean ± SEM. †$p < 0.05$ as assessed by paired t-tests.
Note: CE: Cholesteryl esters

5.4 Discussion:

The aim of this study was to quantify the contribution of CETP to the fate of CE originating from HDL CE. This kinetic study using rHDL containing [^{3}H]CE has shown that the loss of CE from plasma is not altered in the presence of CETP inhibition. Since there is no loss of CE from LDL in the presence of CETP inhibition, the CE flux out of HDL must be increased in the presence of CETP inhibition. These results are consistent with the observation that HDL CE uptake pathways are not saturable at physiological or elevated levels of HDL, at least in the rabbit model.

The mechanism for the increased uptake of CE from HDL is uncertain. While factors such as gene activation by polyunsaturated fatty acids (Spady et al. 1999) or depletion of hepatic cholesterol stores (Wang et al. 1996) appear to upregulate the expression of one of the HDL receptors, SR-BI, there is currently no evidence to suggest that HDL CE receptors can be upregulated by higher plasma HDL CE concentrations. In fact, two previous study by the same investigators suggested that receptor-dependent HDL CE uptake in the liver and adrenals was saturated at physiological HDL CE concentrations (Woollett and Spady 1997; Spady et al. 1998). By infusing HDL into hamsters or apoA-I knockout mice, they were able to estimate the concentrations of HDL CE necessary to achieve half-maximal transport via receptor-dependent transport (Km) in the liver and adrenal glands were similar to the physiological concentration of HDL CE in the controls in both models. In the current study, The CE uptake from HDL in various tissues was not measured but our results suggests that the overall CE loss from HDL was increased when plasma HDL CE concentration was elevated by CETP inhibition. Previous studies on HDL CE kinetics were mainly carried out in mice which lack endogenous CETP activity. Cholesteryl esters are also mainly transported by

HDL in plasma in mice. The hepatic clearance of HDL CE is likely to be different in mice and CETP-rich animals such as rabbits and humans.

In this study, the CETP-mediated pathway accounts for 46% of CE clearance originating from HDL in the untreated animals. In contrast, a previous study using HDL containing cholesterol-[1-^{14}C]oleate and [^{14}C]cholesteryl-oleyl ether showed that the CETP-mediated pathway might account for up to 70% of CE clearance originating from HDL in rabbits (Goldberg et al. 1991). The reason for this difference is uncertain but may be related to the preparation of the tracer in the current study. In the current study, CE is prepared by exogenous esterification of UC into CE in HDL by LCAT. The resultant cholesteryl ester in spherical rHDL is potentially hydrolysable while the oleate and oleyl ether in Goldberg's study is not. Considering both studies together, the current study and Goldberg's study estimate CETP mediates about 46-70% of CE clearance originating from HDL.

The kinetic analysis used in this study is robust. When evaluating the pathways in the multi-compartmental model, no matter whether the irreversible loss of CE from plasma was constrained to HDL- or LDL-mediated pathways, the CE flux remains similar at 3.19 ± 0.57 μmol/L/min vs 3.06 ± 0.59 μmol/L/min. Even when both pathways are considered simultaneously, the CE flux is still comparable at 3.11 ± 0.24 μmol/L/min. These results suggest that CE equilibrates rapidly between HDL and LDL pools once it is introduced into the plasma in the presence of high CETP activity in rabbits. The kinetics of CE in HDL and LDL pools becomes indistinguishable from each other and behaves like a single pool.

In fact, CETP inhibition does not significantly alter the overall CE loss from plasma. This finding agrees with another study using a different approach. In a species that lacks plasma

CETP activity, it was demonstrated that the flux of cholesterol from the periphery to the liver was unaltered in mice expressing variable amount of simian CETP when compared with controls (Osono et al. 1996). Although the overall CE flux remains the same, the activity of CETP does determine the proportion of CE flux cleared by the HDL- and LDL-mediated pathways.

CETP inhibition leads to a significant increase in plasma cholesterol concentration. This new steady state is the result of the redistribution of CE in the HDL and VLDL/LDL fractions (Table 5.1). As shown in Table 5.3, the percentage of HDL CE transported to LDL is reduced from 75.8% in the untreated animals to 52.3% in the treated animals. This reduced transport of CE from HDL to LDL in the treated animals explains the higher concentration of HDL CE and lower concentration of VLDL/LDL CE in the treated animals. CETP inhibition also leads to an increase in plasma apoA-I concentration. Previous study using a monoclonal antibody against CETP also demonstrated similar changes in the plasma cholesterol profile but failed to demonstrate an increase in apoA-I concentration (Whitlock et al. 1989). The duration of inhibitory activity of the monoclonal antibody was brief and CETP activity recovered to 55% of the treatment level after 48 hours. This may explain how the long-acting oral CETP inhibitor leads to a sustained increase in apoA-I concentration.

The effect of CETP inhibition on cholesterol metabolism was investigated in one previous study (Whitlock et al. 1989). The FCR of [^{3}H]CE in HDL was estimated from the initial slope of each decay curve. It was concluded that the clearance of [^{3}H]CE from HDL was delayed in the presence of CETP inhibition. Unfortunately, that study was difficult to interpret. After the injection of monoclonal antibody, plasma concentration of HDL CE did not reach a steady state until 24 to 48 hours. The inhibitory effect of the monoclonal antibody also deteriorated

during the study period. The initial slope of the decay curve in treated animals may not reflect the true clearance of HDL CE. In contrast, the current study mandated the pre-treatment of treated animals for 5 days. CETP inhibition by the chemical inhibitor was potent and sustainable. After 4 days, plasma CETP activity was inhibited by almost 90%. A new steady lipid profile was achieved prior to the injection of tracer and sustained during the study period. The FCR of HDL CE is 56% higher at 0.0072 pools/min in the untreated animals when compared with 0.0032 pools/min in the treated animals. However, the loss of CE from HDL is much higher at 2.72 μmol/L/min in the treated animals when compared with 1.65 μmol/L/min in the untreated animals due to the higher HDL CE concentration in the treated animals.

The current study has a number of limitations. Firstly, the loss of CE from LDL is based on the transfer of CE from HDL to LDL. This poses some difficulties in estimating the loss of CE from LDL when CETP activity is markedly inhibited in the treated rabbits. The amount of radioactivity of CE in LDL becomes too low for accurately estimating the loss of CE from LDL. This problem could have been overcome by introducing a LDL tracer containing radiolabelled CE. However, this does not hinder the aim of the study, as it is the intention to determine the fate of CE in plasma when introduced as a component of HDL.

Secondly, it is not possible to determine the maximum capacity of the HDL CE uptake pathways in the current study. Contrary to previous studies that demonstrated saturable HDL CE uptake at physiological HDL CE levels (Woollett and Spady 1997; Spady et al. 1998), the loss of CE from HDL does not appear to plateau at physiological HDL CE levels in the control rabbits or at elevated HDL CE levels induced by CETP inhibition. However, the

maximum capacity of HDL CE uptake pathways could be explored by infusing HDL or increasing the cholesterol content in the diet and CETP inhibition.

The uptake of HDL CE by the liver and its subsequent secretion into the bile is thought to be an important pathway in reverse cholesterol transport. Mechanisms that enhances hepatic HDL CE uptake are thought to be anti-atherogenic. This study demonstrated that CETP inhibition increases the concentration of CE in HDL, the flux of CE from HDL and the percentage CE cleared via HDL without altering the overall cholesterol flux out of plasma. These results suggest that CETP inhibition does not impair the total CE flux out of plasma. By achieving a new steady state that favours the retention of cholesterol in HDL and inhibit the concentration of CE in the pro-atherogenic VLDL/LDL. CETP inhibition appears to enhance the clearance of CE via the HDL CE uptake pathways. A number of studies have already demonstrated the usefulness of this strategy in inhibiting atherosclerosis in cholesterol-fed animals (Sugano et al. 1998; Okamoto et al. 2000; Rittershaus et al. 2000). Its usefulness in man is yet to be determined.

6 The feasibility of utilising *in vivo* LCAT activity to convert discoidal rHDL containing isotopic unesterified cholesterol into spherical HDL containing isotopic cholesteryl esters for kinetic studies

6.1 Rationale

In the previous chapter, the *in vivo* metabolism of CE in HDL was studied by injecting spherical rHDL containing radiolabelled CE into rabbits. The preparation of the spherical rHDL is quite complicated and utilises large quantity of purified LCAT. The purpose of this chapter is to explore whether sufficient amount of isotopic UC could be converted into isotopic CE by endogenous LCAT activity when discoidal rHDL containing [^{3}H]UC are injected into rabbits.

It has been established in chapter 3 that when introduced into plasma or injected into rabbits, discoidal rHDL are rapidly remodelled into particles that resemble spherical HDL. This process is mediated by LCAT and the results in chapter 3 suggest that both exogenous and endogenous LCAT can rapidly remodel discoidal HDL to spherical HDL *in vivo*. In the previous chapter, exogenous source of LCAT was used to convert discoidal rHDL containing [^{3}H]UC into spherical rHDL containing [^{3}H]CE. This *in vitro* incubation requires large amount of LCAT purified from human plasma. The preparation of LCAT and the subsequent purification of spherical rHDL containing [^{3}H]CE are labour intensive. If the rate of HDL remodelling is as rapid as shown in the chapter 3, after injecting discoidal rHDL containing [^{3}H]UC into rabbits, endogenous LCAT may have the capacity to convert sufficient amount of HDL containing [^{3}H]CE to study the metabolism of HDL CE in rabbits.

Obviously, apart from the LCAT mediated conversion of HDL UC into HDL CE, UC in HDL has a number of other potential fates. Firstly, it can be transferred to VLDL/LDL and other HDL particles via passive aqueous diffusion. Secondly, it can be transported by HDL to the liver, excreted into the bile and irreversibly lost from the plasma. Thirdly, it can be transported back to the peripheral tissues. CETP is not known to affect any UC metabolism among plasma lipoproteins. However, via the action of LCAT, UC can be converted to CE in HDL and subsequently acted upon by CETP. This chapter will aim to determine whether sufficient amount of HDL containing [^{3}H]CE can be generated by LCAT in the presence of other competing pathways when discoidal rHDL containing [^{3}H]UC are injected into rabbits. If this is successful, it will greatly simplify the preparation of HDL tracer containing isotopic CE for the study of HDL CE kinetics.

Specifically, discoidal rHDL containing [^{3}H]UC were prepared and injected into rabbits. The discoidal rHDL containing [^{3}H]UC were reconstituted from its constituents, i.e. phospholipids, apoA-I and [^{3}H]UC, using cholate dialysis, and resulted in the formation of discoidal rHDL that were radiolabelled only in the UC moiety. These particles resemble the nascent HDL when accepting UC from peripheral tissues. It will be determined whether endogenous LCAT act on these particles to form enough spherical rHDL containing [^{3}H]CE for kinetic study.

6.2 Methods

6.2.1 Rabbits

The studies were carried out in mature male New Zealand White (NZW) rabbits purchased from the Institute of Medical and Veterinary Science in Adelaide. The rabbits were fed on a normal rabbit chow diet. The oral CETP inhibitor, CP-456,643, was provided by Pfizer Inc, Connecticut, USA. A stock solution of CP-456,643 (600 mg/mL) was prepared by dissolving the compound in chloroform. The rabbit chow was mixed with CP-456,643 and the solvent was vaporised in a fume hood overnight.

Rabbits were divided into two groups. One group was given normal rabbit chow and the other was given 5 mg/kg of CP-456,643 for 5 days in addition to the normal rabbit chow.

6.2.2 Measurement of CETP activity

Plasma CETP activity was measured before and four days after the administration of the oral CETP inhibitor. Activity of CETP was assessed as the transfer of [^{3}H]CE from [^{3}H]CE-HDLs$_3$ to LDL (Burstein et al. 1970; Tollefson et al. 1988) and the methods were described in Chapter 2. Activity of CETP is expressed in nmol of CE transferred per mL of plasma per hour (nmol CE transferred/mL/h).

6.2.3 Purification of apoA-I and preparation of radioactive tracers

The purification of rabbit apoA-I was described in Chapter 2. The methods for preparing discoidal rHDL containing [^{3}H]UC were described in Chapter 2.

6.2.4 In Vivo Studies

The experimental protocols were reviewed and approved by the animal ethics committees at the Institute of Medical and Veterinary Science, Adelaide, Australia. 5.4 mCi of discoidal rHDL containing [^{3}H]UC was injected into 6 control rabbits and 6 rabbits treated with CP-456,643. Blood samples were collected over 60 mins. The storage, processing and analysis of plasma samples were described in Chapter 2.

6.3 Results:

6.3.1 Characterisation of discoidal rHDL containing [^{3}H]UC

The molar ratio of PLPC/CE/FC/apoA-I in the discoidal rHDL was 74:0:12:1. The diameter of discoidal rHDL was 10.0 nm and there were two molecules of apoA-I per particle. The respective electrophoretic mobility of the discoidal rHDL was –0.49 $\mu m \cdot s^{-1}/V \cdot cm^{-1}$. The specific activity of labelled [^{3}H]UC ranged between 8×10^8 and 9×10^8 cpm/μmol of cholesterol. Radioactivity was confined to the UC in the discoidal rHDL.

6.3.2 Lipid profile of rabbits treated with CETP inhibitor

The protocol for the administration of CETP inhibitor to the rabbits has been described in the methods section. As shown in Table 6.1, CETP inhibitor, CP-456,643, effectively inhibited the plasma CETP activity in the treated animals from 25.6 ± 2.5 to 2.0 ± 1.3 nmol CE transferred/mL plasma/h, a relative reduction of 92%. CP-456,643 had no effect on the plasma activity of PLTP. The inhibition of plasma CETP activity resulted in a number of changes to the lipid profile of the treated rabbits. The plasma total cholesterol concentration was significantly increased from 0.86 mmol/L to 1.19 mmol/L ($p < 0.05$). HDL UC concentration increased from 0.11 mmol/L to 0.22 mmol/L ($p < 0.05$) while VLDL/LDL UC

concentration remained unchanged (p = ns). CETP inhibition also resulted in a significant increase in the plasma concentration of apoA-I from 0.41 mg/mL to 0.69 mg/mL ($p < 0.05$).

6.3.3 Endogenous conversion of [^{3}H]UC into [^{3}H]CE after injecting discoidal rHDL containing [^{3}H]UC into rabbits

A tracer amount of discoidal rHDL containing [^{3}H]UC was injected into both treated and untreated rabbits. The apparent rates of disappearance of HDL UC were similar in both the treated and untreated animals (Figure 6.1). There was a rapid disappearance of [^{3}H]UC in HDL within 10 mins of injection. By 20 mins, < 10% of the injected [^{3}H]UC remained in the HDL fraction. The radioactivity in HDL CE peaked at about 7% and 11% of the injected dose approximately 5 mins after the injection into the untreated and treated animals. The clearance of HDL CE appeared more rapid in the untreated than in the treated animals. By 60 mins, about 4% and 10% of the injected activity remained in HDL CE in the untreated and treated animals, respectively.

After discoidal rHDL containing [^{3}H]UC was injected into rabbits, it was apparent that a small proportion of the injected activity was detected as [^{3}H]CE in HDL. The rate of transfer of CE from HDL to VLDL/LDL has already been determined in the previous chapter. In the current study, a low level of CE was detected in HDL and peaked at 5 mins after the injection of discoidal rHDL containing [^{3}H]UC. Thus, a large proportion of the injected activity must be distributed to other compartments before any conversion of UC to CE took place. In order to clarify the fates of [^{3}H]UC within the plasma compartments, the clearance of UC in other plasma lipoprotein fractions was measured and the parameters were fitted into a multi-compartmental model.

Table 6.1: Lipid profile and plasma factor activities of wild-type rabbits before and after CETP inhibition

	Rabbits with no CETP inhibition	Rabbits with CETP inhibition (pre-inhibition)	Rabbits with CETP inhibition (day 4 post-inhibition)
No. of rabbits	6	6	6
Plasma total cholesterol (mmol/L)	0.85 ± 0.14	0.86 ± 0.12	1.19 ± 0.21
Plasma unesterified cholesterol (mmol/L)	0.28 ± 0.04	0.19 ± 0.04	0.30 ± 0.05 †
Plasma cholesteryl esters (mmol/L)	0.57 ± 0.11	0.67 ± 0.09	0.89 ± 0.17 †
HDL total cholesterol (mmol/L)	0.34 ± 0.05	0.47 ± 0.08	0.87 ± 0.13 †
HDL unesterified cholesterol (mmol/L)	0.11 ± 0.02	0.11 ± 0.04	0.22 ± 0.04 †
HDL cholesteryl esters (mmol/L)	0.23 ± 0.03	0.37 ± 0.05	0.66 ± 0.10 †
VLDL/LDL total cholesterol (mmol/L)	0.51 ± 0.14	0.39 ± 0.06	0.31 ± 0.10
VLDL/LDL unesterified cholesterol (mmol/L)	0.17 ± 0.04	0.09 ± 0.02	0.08 ± 0.02
VLDL/LDL cholesteryl esters (mmol/L)	0.34 ± 0.04	0.30 ± 0.04	0.24 ± 0.08 †
ApoA-I (mg/mL)	0.41 ± 0.08	0.41 ± 0.07	0.69 ± 0.12 †
CETP activity (nmol CE transferred/ mL plasma/h)		25.6 ± 2.5	2.0 ± 1.3 †
PLTP activity (nmol PL transferred/ mL plasma/h)		1433 ± 61	1303 ± 82

Values are presented as mean ± SEM. †$p < 0.05$ as assessed by paired t-tests.
Note: CE: Cholesteryl esters; CETP: Cholesteryl ester transfer protein; PL: Phospholipids; PLTP: Phospholipid transfer protein

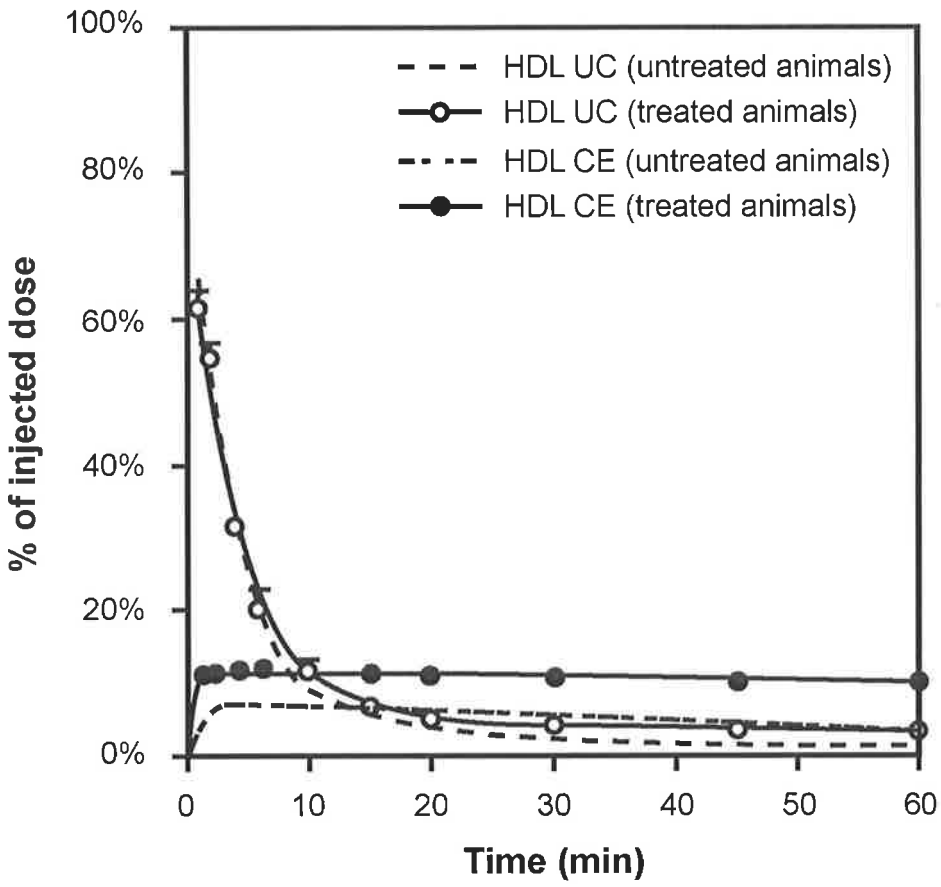

Figure 6.1: In vivo clearance of [^{3}H]unesterified cholesterol ([^{3}H]UC) in rabbits treated and not treated with an oral CETP inhibitor after intravenous injection of discoidal rHDL containing [^{3}H]UC. Dotted line represents the clearance in animals not treated with an oral CETP inhibitor for comparison.

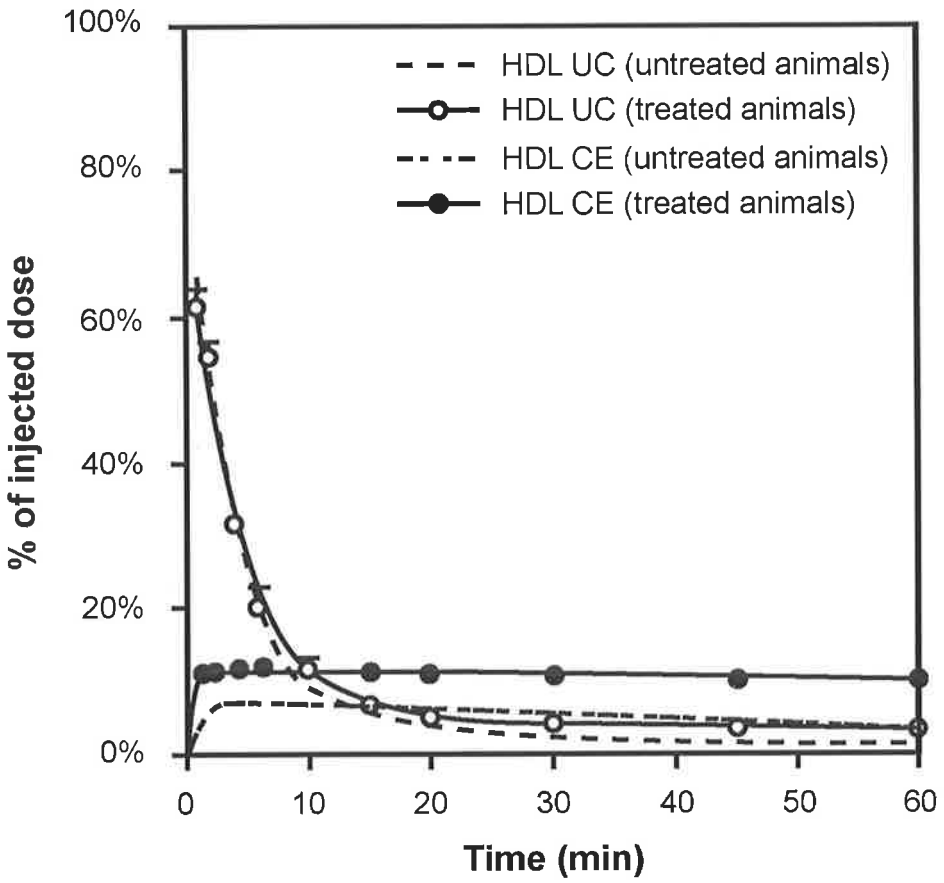

Figure 6.1: In vivo clearance of [^{3}H]unesterified cholesterol ([^{3}H]UC) in rabbits treated and not treated with an oral CETP inhibitor after intravenous injection of discoidal rHDL containing [^{3}H]UC. Dotted line represents the clearance in animals not treated with an oral CETP inhibitor for comparison.

6.3.4 The fate of unesterified cholesterol in HDL within the plasma compartment

After the injection of discoidal rHDL containing [^{3}H]UC into the both treated and untreated rabbits, about 20% of the injected activity appeared as UC in VLDL/LDL at the earliest time point of 1 minute (Figure 6.2). The radioactivity in VLDL/LDL UC decreased rapidly to a level close to zero within 10-20 mins. There was no significant difference in the decay profile between the treated and untreated animals. On the other hand, about 1-2% of the injected radioactivity was detected as CE in VLDL/LDL in both the treated and untreated animals (Figure 6.3). The radioactivity in VLDL/LDL CE reached a plateau at approximately 5-10 mins. The overall clearance of UC in the whole plasma paralleled that of UC in the HDL fraction (Figure 6.4). These observations are consistent with the fact that a large proportion of the injected UC radioactivity in the form of discoidal rHDL was rapidly cleared from the plasma compartment. The tissue sites of uptake cannot be determined from these studies.

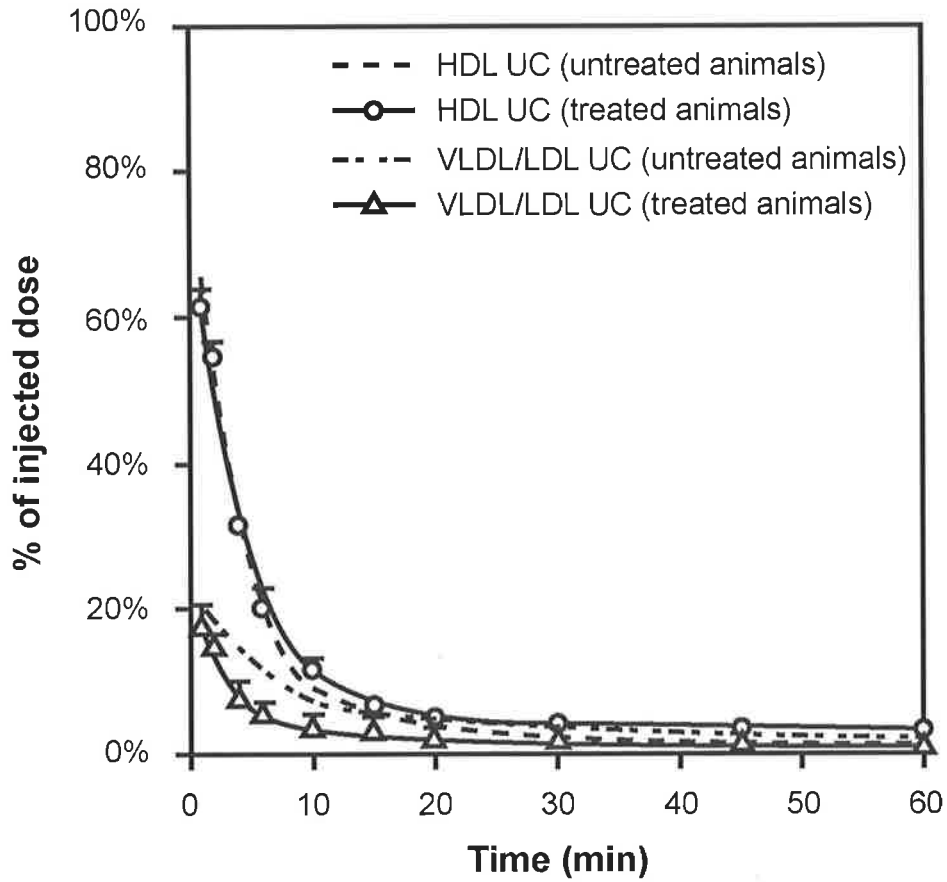

Figure 6.2: In vivo clearance of [^{3}H]unesterified cholesterol ([^{3}H]UC) in rabbits treated and not treated with an oral CETP inhibitor after intravenous injection of discoidal rHDL containing [^{3}H]UC. Treated rabbits (n=6) were given 5mg/kg of oral CETP inhibitor and their average CETP activity was reduced by 91% after 5 days of oral CETP inhibitor administration. All rabbits were injected intravenously with tracer amounts of discoidal rHDL containing [^{3}H]UC. Dotted line represents the clearance in animals not treated with an oral CETP inhibitor for comparison.

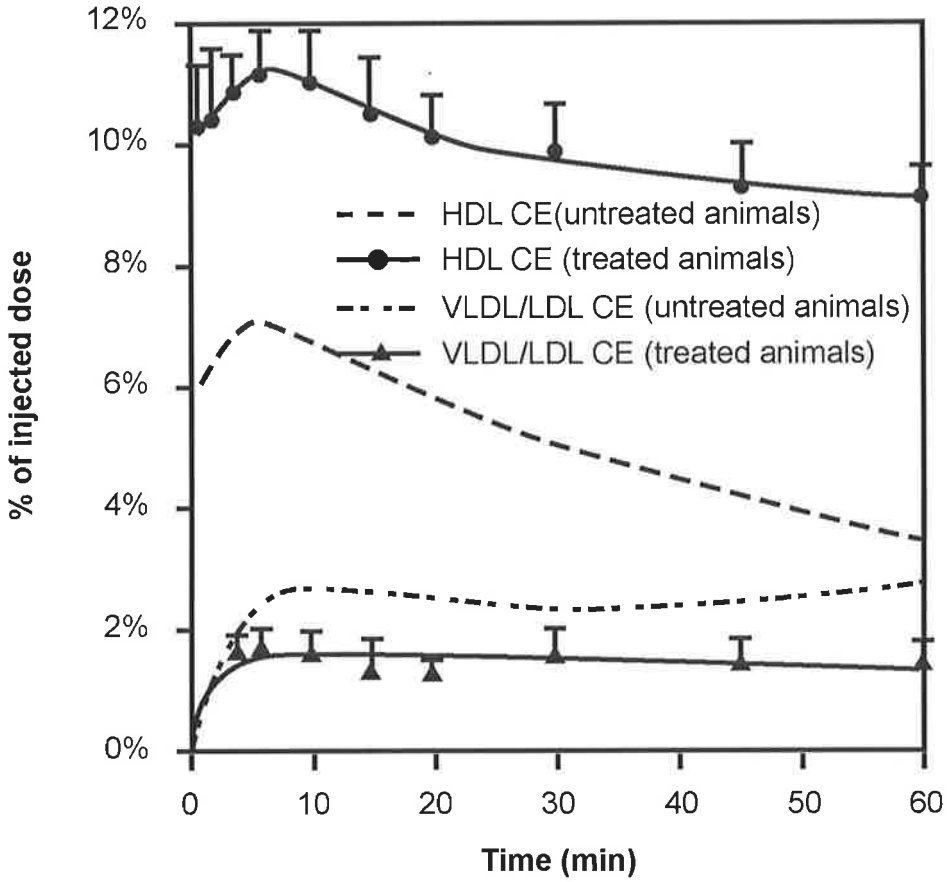

Figure 6.3: In vivo clearance of [^{3}H]cholesteryl esters ([^{3}H]CE) in rabbits treated and not treated with an oral CETP inhibitor after intravenous injection of discoidal rHDL containing [^{3}H]UC. Clearance of [^{3}H]CE from HDL and VLDL/LDL. Dotted line represents the clearance in animals not treated with an oral CETP inhibitor for comparison.

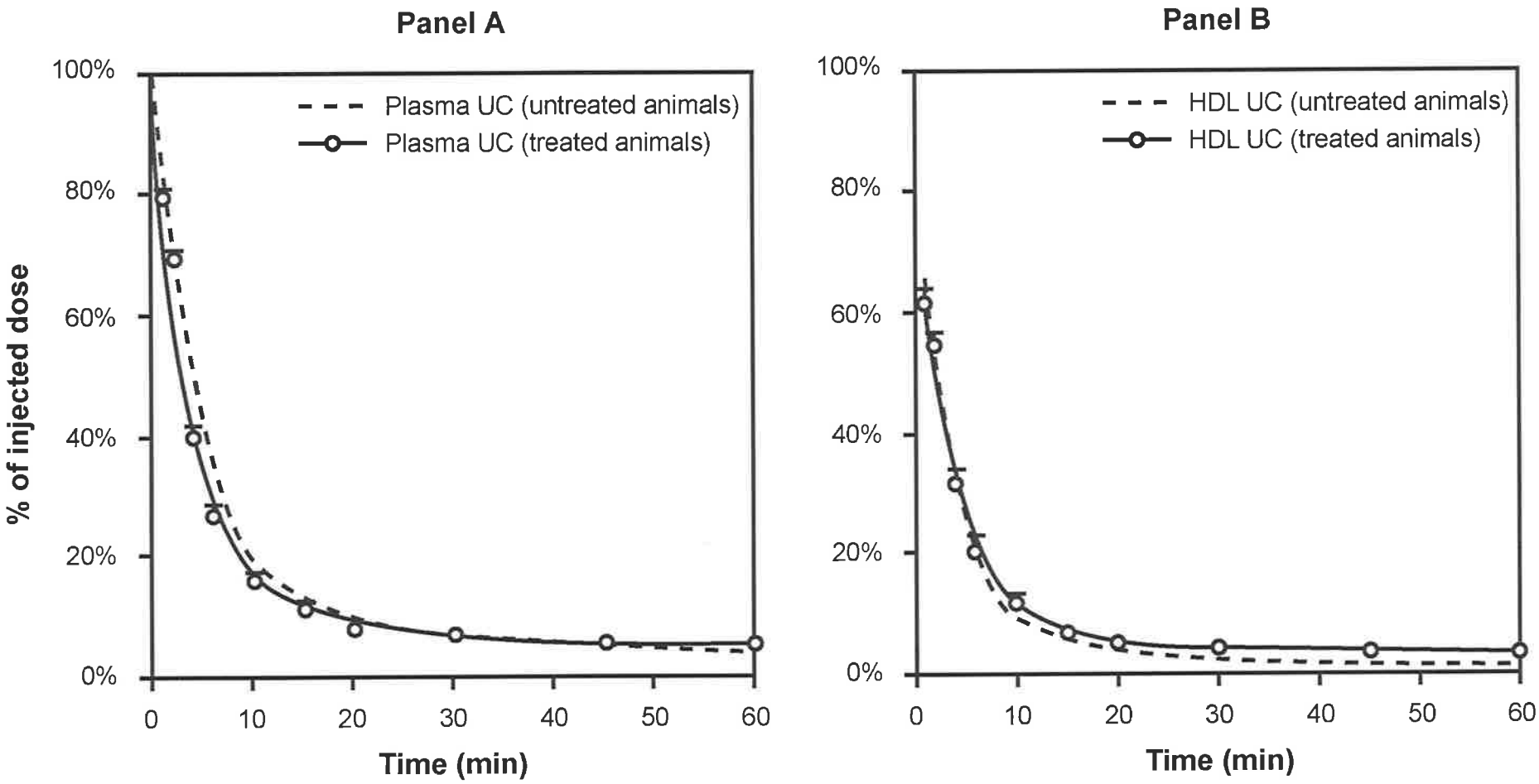

Figure 6.4: In vivo clearance of [^{3}H]unesterified cholesterol ([^{3}H]UC) in rabbits treated and not treated with an oral CETP inhibitor after intravenous injection of discoidal rHDL containing [^{3}H]UC. All animals were fed with normal rabbit chow. Treated rabbits (n=6) were given 5mg/kg of oral CETP inhibitor and their average CETP activity was reduced by 91% after 5 days of oral CETP inhibitor administration. All rabbits were injected intravenously with tracer amounts of discoidal rHDL containing [^{3}H]UC. A. Clearance of [^{3}H]UC from the whole plasma. Dotted line represents the clearance in animals not treated with an oral CETP inhibitor for comparison. B. Clearance of [^{3}H]UC from HDL. Dotted line represents the clearance in animals not treated with oral CETP inhibitor for comparison (see also Figure 5.2). The difference in [^{3}H]UC between total plasma and HDL equals to [^{3}H]UC in VLDL/LDL.

6.3.5 Development of the model

A multi-compartmental model was constructed based on generally accepted pathways of cholesterol metabolism in the plasma compartment (Figure 6.5). Assumptions made in the model are listed as follows: (1) Based on the results in chapter 3 that demonstrated the rapid remodelling of discoidal HDL into particles resembling spherical HDL in plasma, this model assumes that discoidal rHDL containing UC are converted into HDL UC and this conversion is represented by k(2,1). (2) A proportion of UC in discoidal rHDL is also thought to transfer to LDL via passive aqueous diffusion and this transfer is represented by k(3,1). (3) The current study demonstrated the rapid disappearance of UC in HDL from the plasma compartment after injecting the tracer into rabbits. The rate of loss of UC in HDL in plasma paralleled that of UC from the whole plasma compartment. Thus, k(0,2), is introduced into the model to represent the only significant route of UC loss from the plasma compartment. Potential acceptors of UC from HDL via this route include red cell membrane and other peripheral end organs, including the liver. (4) k(3,2) and k(2,3) represent the exchange of UC between the HDL and LDL compartments via passive aqueous diffusion. (5) An exchangeable pool, q5, is created to represent an additional compartment that has the potential to exchange UC with HDL within the plasma. This exchangeable pool may represent red cell membrane and other peripheral tissues. The bi-directional exchange of UC between HDL and compartment 5 is represented by k(5,2) and k(2,5). (6) Passive aqueous exchange of UC between LDL and VLDL is represented by k(4,3) and k(3,4). (8) Finally, based on the results of the current study, the amount of [^{3}H]UC in HDL converted into [^{3}H]CE in HDL and VLDL/LDL by endogenous LCAT activity appeared minor. The plasma compartments for HDL CE and VLDL/LDL CE are therefore not included in the model because the amount of [^{3}H]CE in HDL and VLDL/LDL is low and unreliable.

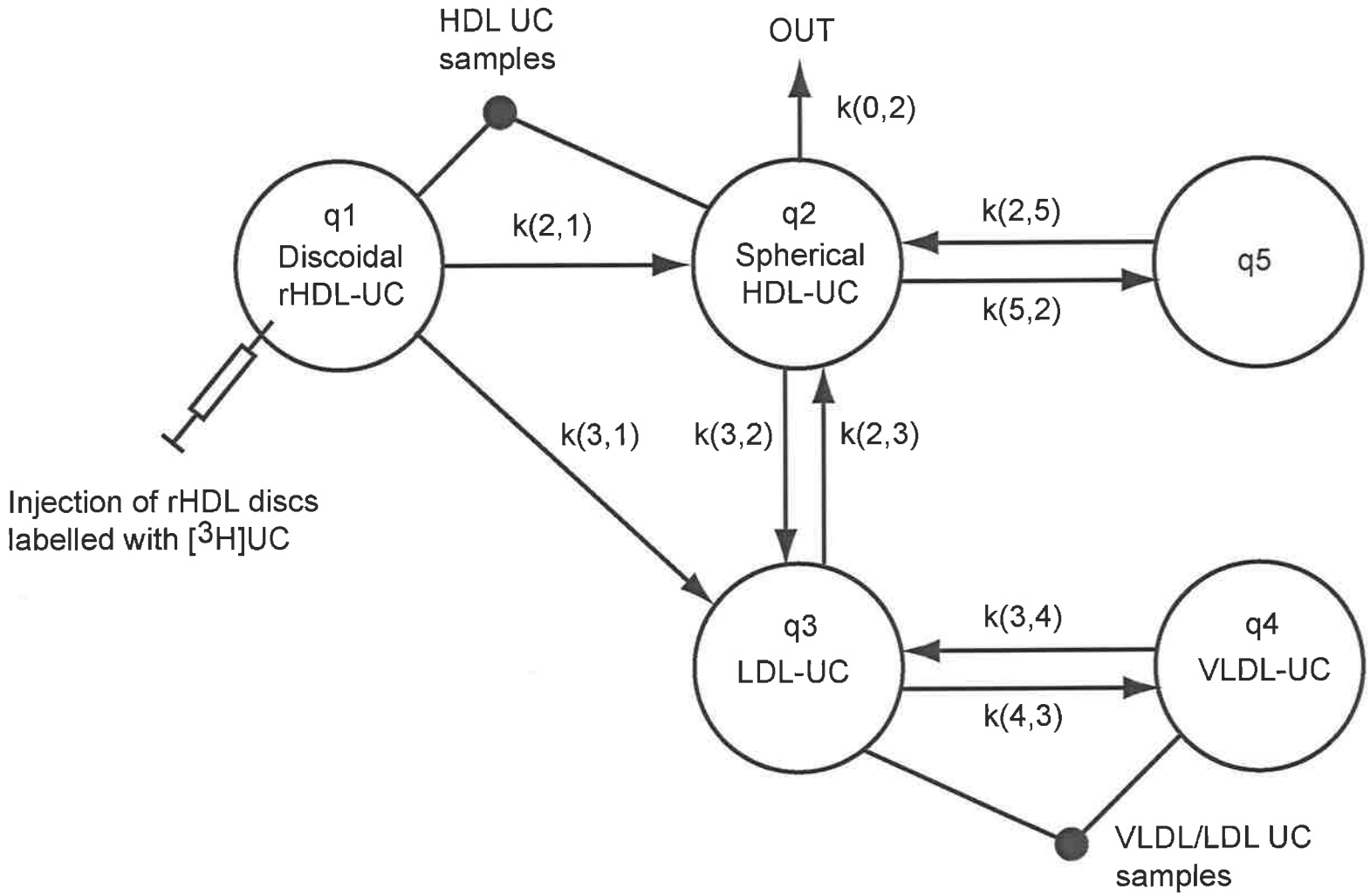

Figure 6.5. Compartmental model for the metabolism of plasma unesterified cholesterol (UC) in rabbits. Circles represent standard compartments, each identified by the number in the compartment. Arrows represent transport pathways. Solid circles represent plasma sampling of various compartments.

6.3.6 Parameters of the model

The steady-state fluxes between compartments are shown in Table 6.2. The salient points are as follows: (1) CETP inhibition increased the UC fluxes between all the compartments. (2) CETP inhibition increased the steady-state bi-directional exchanges between various compartments to the same extent. (3) The net flux of HDL UC out of the plasma compartment, flux(0,2), was increased in the presence of CETP inhibition. Flux(0,2) increased from 15.6 ± 2.4 μmol/L/min in the untreated animals to 27.5 ± 3.1 μmol/L/min in the treated animals ($p < 0.05$). Although the FCRs of HDL UC were similar at 0.19 ± 0.02 pools/min and 0.16 ± 0.02 pools/min in the untreated and treated animals (p = ns), the doubling of the plasma concentration of HDL UC in the treated animals led to a net increase in the total HDL UC flux out of the plasma compartment.

Table 6.2: Steady-state fluxes and fractional catabolic rates (FCRs) from compartmental analysis: Control animals

Control	C1	C2	C3	C4	C5	C6	Mean	Stdev
				Fluxes (μmol/L/min)				
FLUX(0,2)	21.6	18.5	10.1	22.4	9.0	11.9	15.6	6.0
FLUX(2,1)	10.8	9.3	5.0	11.2	4.5	6.0	7.8	3.0
FLUX(2,3)	91.3	40.8	21.2	53.6	21.6	30.1	43.1	26.7
FLUX(2,5)	24.8	18.5	9.0	19.4	10.3	9.6	15.3	6.5
FLUX(3,1)	10.8	9.3	5.0	11.2	4.5	6.0	7.8	3.0
FLUX(3,2)	80.5	31.5	16.1	42.4	17.0	24.1	35.3	24.2
FLUX(3,4)	2.1	0.9	1.1	1.1	0.3	0.5	1.0	0.6
FLUX(4,3)	2.1	0.9	1.1	1.1	0.3	0.5	1.0	0.6
FLUX(5,2)	24.8	18.5	9.0	19.4	10.3	9.6	15.3	6.5
				FCRs (pools/min)				
k(0,2)	0.12	0.18	0.24	0.21	0.16	0.25	0.19	0.05
k(2,1)	0.80	0.56	0.23	0.66	0.33	0.47	0.51	0.21
k(2,3)	1.59	1.69	0.47	1.47	1.23	1.02	1.24	0.45
k(2,5)	0.03	0.05	0.02	0.04	0.05	0.05	0.04	0.01
k(3,1)	0.80	0.56	0.23	0.66	0.33	0.47	0.51	0.21
k(3,2)	0.46	0.30	0.38	0.39	0.29	0.51	0.39	0.09
k(3,4)	0.02	0.01	0.00	0.01	0.00	0.00	0.01	0.01
k(4,3)	0.04	0.04	0.03	0.03	0.02	0.02	0.03	0.01
k(5,2)	0.14	0.18	0.21	0.18	0.18	0.20	0.18	0.03

Table 6.2 (cont): Steady-state fluxes and fractional catabolic rates (FCRs) from compartmental analysis: Animals treated with CETP inhibitor

CETPi	Ci1	Ci2	Ci3	Ci4	Ci5	Ci6	Mean	Stdev
				Fluxes (μmol/L/min)				
FLUX(0,2)	36.3	33.8	16.6	30.7	27.3	20.5	27.5	7.7
FLUX(2,1)	18.1	16.9	8.3	15.4	13.6	10.2	13.8	3.8
FLUX(2,3)	126.6	137.0	21.2	41.8	135.0	31.3	82.2	56.0
FLUX(2,5)	47.1	35.8	12.8	53.9	47.5	33.1	38.4	14.8
FLUX(3,1)	18.1	16.9	8.3	15.4	13.6	10.2	13.8	3.8
FLUX(3,2)	108.5	120.1	12.9	26.4	121.4	21.1	68.4	53.2
FLUX(3,4)	4.2	1.0	0.1	0.2	0.9	1.2	1.3	1.5
FLUX(4,3)	4.2	1.0	0.1	0.2	0.9	1.2	1.3	1.5
FLUX(5,2)	47.1	35.8	12.8	53.9	47.5	33.1	38.4	14.8
				FCRs (pools/min)				
k(0,2)	0.12	0.16	0.27	0.14	0.12	0.14	0.16	0.06
k(2,1)	0.73	0.85	0.54	0.36	0.40	1.47	0.73	0.41
k(2,3)	2.12	1.94	1.26	1.09	2.20	0.97	1.60	0.55
k(2,5)	0.05	0.04	0.07	0.06	0.08	0.02	0.06	0.02
k(3,1)	0.73	0.85	0.54	0.36	0.40	1.47	0.73	0.41
k(3,2)	0.36	0.57	0.21	0.12	0.52	0.14	0.32	0.19
k(3,4)	0.04	0.01	0.00	0.00	0.00	0.01	0.01	0.02
k(4,3)	0.07	0.01	0.01	0.00	0.01	0.04	0.02	0.02
k(5,2)	0.16	0.17	0.21	0.25	0.20	0.23	0.20	0.03

6.4 Discussion

This study was intended to explore the possibility of utilising endogenous LCAT activity to convert the injected discoidal rHDL containing [^{3}H]UC into sufficient amount of HDL containing [^{3}H]CE for the study of *in vivo* HDL CE metabolism. It became apparent that the amount of [^{3}H]CE generated by endogenous LCAT activity was insufficient for HDL CE metabolic study. Within the 10 min of injection, only 5-11% of the injected radioactivity was detected as [^{3}H]CE in HDL. In fact, the amount of [^{3}H]CE in VLDL/LDL was so low that it was not possible to differentiate CETP-mediated transfer of CE from HDL to VLDL/LDL from the possible contamination during lipoprotein separation. The net clearance of [^{3}H]UC in HDL paralleled that of [^{3}H]UC in the whole plasma and this indicated that HDL UC is cleared from the plasma rather than being re-distributed to other lipoproteins or other components in plasma. The results were unexpected given the high *in vitro* and *in vivo* activity of LCAT in plasma. It appears that LCAT is capable of remodelling discoidal rHDL rapidly into spherical particles as demonstrated in chapter 3 but is unable to esterify adequate amount of UC in discoidal HDL into CE in spherical particles *in vivo*. After considering the steady states in multi-compartmental model, it is consistent that the *in vivo* esterification rate of UC in HDL is low in comparison with other UC exchange pathways within plasma and the net loss of UC from HDL. The results suggest that the various uptake, transfer or exchange processes of UC from HDL were more rapid than the rate of *in vivo* esterification of UC to CE in HDL.

However, the results should be interpreted with caution due to a number of limitations in the proposed compartmental model. Firstly, as stated earlier, it was not the intention of this study to examine HDL UC metabolism in plasma. As a result, only discoidal rHDL containing [^{3}H]UC were injected into the rabbits. The net flux of HDL UC out of plasma was high in comparison to the amount of [^{3}H]UC transferred to other lipoproteins or the amount of

[^{3}H]CE generated in plasma. As a result, the specific activity of [^{3}H]UC in VLDL/LDL was low and therefore the relationship of UC in VLDL/LDL with other compartments could not be accurately estimated in this compartmental model. These limitations could only be resolved by simultaneously injecting a VLDL/LDL tracer containing a different isotopic UC (for example, [^{14}C]UC) and a discoidal rHDL tracer containing [^{3}H]UC into rabbits. Secondly, the amount of [^{3}H]UC in red blood cells was not measured. Thus, the model could not differentiate the flux of HDL UC transferred to peripheral tissues from that to the red cells. However, previous studies suggested that the equilibration of UC between HDL and red cells was not complete for 2-3 hours (Halloran et al. 1978; Schwartz et al. 1978b), the net flux of UC from HDL to other peripheral tissues could still be estimated within 60 min in the current study. Thirdly, the amount of [^{3}H]UC in peripheral tissues, liver and bile was not measured. Apart from the bile, it is technically impossible to sample the radioactivity in the liver and other peripheral tissues at regular intervals during the experiment. The fate of [^{3}H]UC in peripheral tissues therefore could not be quantified. Fourthly, the highly exchangeable nature of UC among lipoproteins complicated the isolation process of lipoproteins for analysis. This technical difficulty was apparent when analysing the UC flux data in different animals, which was likely a result of the unavoidable contamination during lipoproteins isolation. Finally, it was not possible to include HDL CE and VLDL/LDL CE compartments into the model due to the very low rate of endogenous esterification. The "noise" in CE in HDL and VLDL/LDL was too high for any kinetic analysis. This limitation could only be overcome by injecting a labelled HDL CE or VLDL/LDL CE in combination with other tracers into the rabbits. Given the limited number of suitable isotopes for labelling UC and CE, the kinetics of various compartments could only be resolved by injecting two tracers at a time into a large number of animals.

Nevertheless, the metabolism of plasma UC has been studied by a number of important human studies (Halloran et al. 1978; Schwartz et al. 1978a; Schwartz et al. 1978b; Schwartz et al. 1993). Schwartz et al. (1978a) injected radiolabelled mevalonic acid and [^{14}C]cholesterol into bile fistula human subjects. Mevalonic acid represented the precursor of the newly synthesised cholesterol while [^{14}C]cholesterol represented UC released into the plasma. Serial samples of bile and plasma were collected and the radioactivities in the bile acids, biliary cholesterol, plasma UC and plasma CE were measured. Based on the observed radioactive decay of UC and CE in the measured components, a multi-compartmental model was developed. According to that model, the major portion of the cholesterol entering the bile acid (58%) and biliary cholesterol (71%) precursor sites was derived from the plasma UC compartment of either HDL or LDL in origin. Unfortunately, the study did not differentiate between the contribution of HDL UC and LDL UC in bile synthesis. The plasma CE compartment contributed 11% of the input into the bile acid site. In contrast, the newly synthesised cholesterol contributed only 31% and 20% of the respective total flux into the bile acid and biliary cholesterol precursor compartments. When comparing with the current study, it appears that a substantial proportion of the plasma clearance of HDL UC could be delivered to the liver for bile acid synthesis. In two other studies, Halloran et al. (1978) and Schwartz et al. (1978b) simultaneously injected HDL containing [^{3}H]UC and LDL containing [^{14}C]UC into bile fistula human subjects. The radioactivities in red blood cells and bile acid were measured. The detection of radioactivities was consistently and significantly lower in the red blood cells than in the bile acid over 2-3 hours. Those two studies were the first reported studies that demonstrated the possibility of estimating hepatic uptake of HDL UC despite of the passive aqueous exchange of UC between HDL and red blood cells. Thus, in the current study that lasted for only 1 hour, a substantial proportion of clearance of UC from the plasma compartment could have been delivered to peripheral tissues and the liver rather than red

blood cells. Halloran et al. (1978) and Schwartz et al. (1978b) have also established the preferential utilisation of HDL UC over LDL UC in bile-acid synthesis, an observation that could not be detected in the current study due to the low level of UC in LDL. Finally, in a comprehensive kinetic study by Schwartz et al. (1992), a number of radiolabelled tracers including radiolabelled albumin-UC, mevalonic acid, HDL UC, HDL CE and LDL FC were prepared. In each bile-fistula subject, two or three of the radiolabelled tracers were simultaneously injected. The number of pools in the multi-compartmental model was expanded when comparing with their previous study in 1978. In particular, plasma lipoproteins were separated into β-lipoproteins (VLDL/LDL) and α-HDL by heparin-$MnCl_2$ precipitation, a method also used in the current study. The plasma UC pool was subdivided into VLDL/LDL UC and HDL UC pools. The plasma CE pool containing VLDL/LDL and HDL was regarded as a single pool. A more detailed multi-compartmental model was developed. In agreement with the current study, no matter whether cholesterol precursor was introduced into the plasma compartment as albumin-UC, mevalonic acid or HDL UC, the specific activity of CE in plasma increased at a slow rate, which suggests a relatively slow *in vivo* esterification rate. When radiolabelled HDL UC was injected into subjects, the specific activities of UC in HDL and VLDL/LDL reached equilibrium within 30 min, which suggests the passive exchange of UC between HDL and VLDL is complete within that duration. In terms of the specific activity in bile, after a delay of 10-20 min after administration of HDL UC, specific activity in bile increased rapidly. The specific activity in bile peaked earlier when isotopic UC was introduced as a constituent of HDL rather than LDL, indicating the preferential utilisation of HDL UC in bile synthesis. In conclusion, these studies demonstrated the possibility of studying HDL UC kinetics before passive exchange with red blood cells and other peripheral tissues is complete. Endogenous esterification of HDL UC into HDL CE is lower than expected in a closed system due to the number of competing pathways *in vivo*.

HDL UC is preferred over HDL CE as a substrate for bile synthesis. Mechanisms that increase plasma concentration of HDL UC, such as CETP inhibition in this study, have the potential of shuttling more UC to the liver for bile synthesis.

Although the initial objective of generating sufficient isotopic HDL CE from discoidal rHDL containing [^{3}H]UC was not realised, the current study does provide interesting insights into the regulation of UC metabolism in plasma. In particular, the results suggest that the flux of HDL UC out of plasma is significantly influenced by the plasma concentration of HDL UC. In this case, elevation of plasma concentration of HDL UC by a CETP inhibitor did not alter the FCR of HDL UC but effectively increased the net flux of HDL UC. Previous kinetic studies suggest that this HDL UC flux is a major contributor towards the precursor pools of bile acids and biliary cholesterol (Schwartz et al. 1978b; Schwartz et al. 1993). It will be of great clinical interest to inject various combinations of isotopic lipoprotein tracers and re-examine the effects of CETP inhibition on cholesterol fluxes and hepatic bile synthesis.

7 General discussion

Numerous clinical trials have established the benefits of LDL-cholesterol lowering, notably by HMG-CoA reductase inhibitors, in reducing the risk of myocardial infarction in both primary and secondary prevention settings. HMG-CoA reductase inhibitors, also known as statins, significantly lower plasma LDL-cholesterol and marginally elevate HDL-cholesterol. The effect of statins on actual plaque regression appears subtle but the clinical benefits have been impressive. This discrepancy between coronary plaque regression and clinical benefits has been attributed to statin's possible plaque stabilisation and anti-inflammatory properties, also known as pleiotropic effects. Despite of the success of statin therapy, coronary heart disease continues to occur. In fact, statin therapy has only resulted in 30 percent reduction in coronary artery disease in the highest risk groups. There are still a substantial number of patients who are not completely protected by LDL-cholesterol lowering. Obviously, other preventive strategies to reduce coronary artery disease should be considered. One of the potential candidates is HDL because of its widely accepted role in the return of "excess" cholesterol from extrahepatic tissues to the liver for excretion.

An important question in reverse cholesterol transport is to determine which form of cholesterol, unesterified cholesterol (UC) or cholesteryl esters (CE), contributes to the major proportion of cholesterol flux from extrahepatic tissues to the liver for excretion rather than re-utilisation. There is no doubt that the main form of cholesterol in plasma is CE. This observation probably led to the prevailing proposition that the bulk of cholesterol destined for hepatic uptake (and elimination) is CE. Cholesterol is effluxed from peripheral cells in the form of UC and is accepted by plasma HDL. In order to conform with the proposed reverse cholesterol transport, an obligatory step of esterification of UC into CE in HDL needs to

occur before its uptake by the liver. A number of studies have re-examined the importance of the two forms of cholesterol in reverse cholesterol transport. In order to determine the relative contribution of HDL UC and HDL CE in bile secretion, HDL labelled with [^{3}H]UC or [^{3}H]CE were separately injected into bile-fistula rats (Bravo et al. 1994). The liver preferentially took up 58.2% of the injected HDL [^{3}H]UC dose when compared with 6.8% of the injected HDL [^{3}H]CE dose during the first 180 min. Furthermore, a larger proportion of HDL UC (8.8%) than HDL CE (3.3%) was recovered in bile during the same duration. These results suggested the HDL UC is preferentially taken up by the liver and subsequently secreted into bile. Similar comparative study was performed in human bile-fistula subjects (Schwartz et al. 1981). Subjects were injected with HDL (or LDL) labelled with [^{3}H]CE, [^{3}H]UC and [^{14}C]UC. Kinetic analysis concluded that lipoproteins UC was the main source of cholesterol in bile. HDL CE only accounted for 20% of biliary steroid production. However, one of the difficulties in studying UC kinetics is related to the rapid exchange of UC among plasma lipoproteins. Lipoproteins associated UC taken up by the liver is also rapidly mixed with hepatically synthesised UC in a single hepatic pool. Thus, the highly exchangeable nature of UC poses a significant problem when determining the origin of UC in bile in kinetic studies. One novel study attempted to overcome this issue by perfusing rat livers with VLDL, LDL and HDL derived from patients with hereditary phytosterolaemia that were rich in plant sterols (Robins and Fasulo 1997). Plant sterols are not normally absorbed in the diet. Plant sterols in donor lipoproteins therefore act as a surrogate of UC and this technique enables the distinction of lipoproteins associated UC from hepatically synthesised UC in bile. When comparing with VLDL or LDL, perfusion of HDL led to a significant three-fold increase in plant sterols in bile. That study and others confirmed three important points: (1) the preferential uptake of UC from HDL over other lipoproteins by the liver, (2) the preferential

uptake of HDL UC over HDL CE by the liver, and (3) the preferential utilisation of HDL UC over HDL CE in bile synthesis.

Nevertheless, HDL CE is sequestered in the liver but to a lesser extent when comparing with the amount of HDL UC taken up by the liver (Bravo et al. 1994). A small proportion of hepatic CE is hydrolysed into UC and entered the hepatic UC pool for bile secretion. The rate of hydrolysis of hepatic CE is closely linked to the rate of appearance of cholesterol of CE origin in bile (Robins et al. 1989) and is probably regulated by the activity of hepatic 7α-hydroxylase (Bjorkhem and Akerlund 1988). The fate of the rest of the un-hydrolysed hepatic CE is unclear but is believed to be re-secreted into plasma via VLDL.

According to the proposed reverse cholesterol transport, the esterification of UC to CE in HDL is a prerequisite to achieve mass transport of cholesterol from extrahepatic tissues back to the liver. Chapter 6 highlighted one potential problem with the proposal. When radiolabelled HDL UC was injected into plasma, endogenous LCAT activity was unable to esterify most of UC to CE in HDL before the exchange or transfer of HDL UC out of plasma took place. Thus, it suggested another inconsistency in the proposed reverse cholesterol transport. Thus, despite of its abundance among plasma lipoproteins, endogenous LCAT does not appear to esterify newly introduced UC into CE in plasma when other competing pathways exist.

In this case, what are the roles of HDL CE and how are they linked to their fates? In humans, the mass transport of CE in HDL appears to be governed by three pathways: (1) selectively uptake by the liver, (2) transfer to VLDL/LDL via the action of CETP and (3) transfer to extrahepatic tissues. Apart from the minor proportion of hepatic CE being hydrolysed and

excreted as bile, it appears that all the other pathways aim at conserving total body cholesterol and promoting cholesterol homeostasis in extrahepatic tissues.

Figure 7.1 illustrates a suggested modification to the proposed reverse cholesterol transport according to the latest cholesterol kinetic studies. It highlights the new roles of HDL UC and HDL CE in cholesterol metabolism. This new proposal supports a role of HDL in shuttling UC from extrahepatic tissues to the liver for excretion. After unloading UC to the liver, HDL recirculate and accept more UC from extrahepatic tissues. A proportion of UC is esterified to CE in HDL. No matter which pathway is followed, a substantial amount of CE ultimately redistributes back to extrahepatic tissues.

A better understanding of HDL cholesterol metabolism enables the identification of potential targets for therapeutic intervention. Chapter 5 and 6 suggest that cholesterol flux from each compartment is not only determined by the fractional catabolic rate of each compartment but also the plasma concentration of HDL-cholesterol (HDL-C). In general, any intervention that results in an elevation of HDL cholesterol is potentially anti-atherogenic. However, the elevated HDL-C must be accomplished by a clear pathway that leads to the elimination of "excess cholesterol" by the liver rather than the result of an accumulation of HDL-C in the circulation. The latter statement needs further clarification. A number of interventions can potentially raise plasma HDL-C concentration. These interventions include (1) an increased production of precursors such as apoA-I and overexpression of ABCA1, (2) an inhibition of plasma factors that normally promotes HDL degradation, (3) an overexpression of plasma factors that leads to HDL-C retention, and (4) the downregulation of hepatic HDL receptors such as SR-BI.

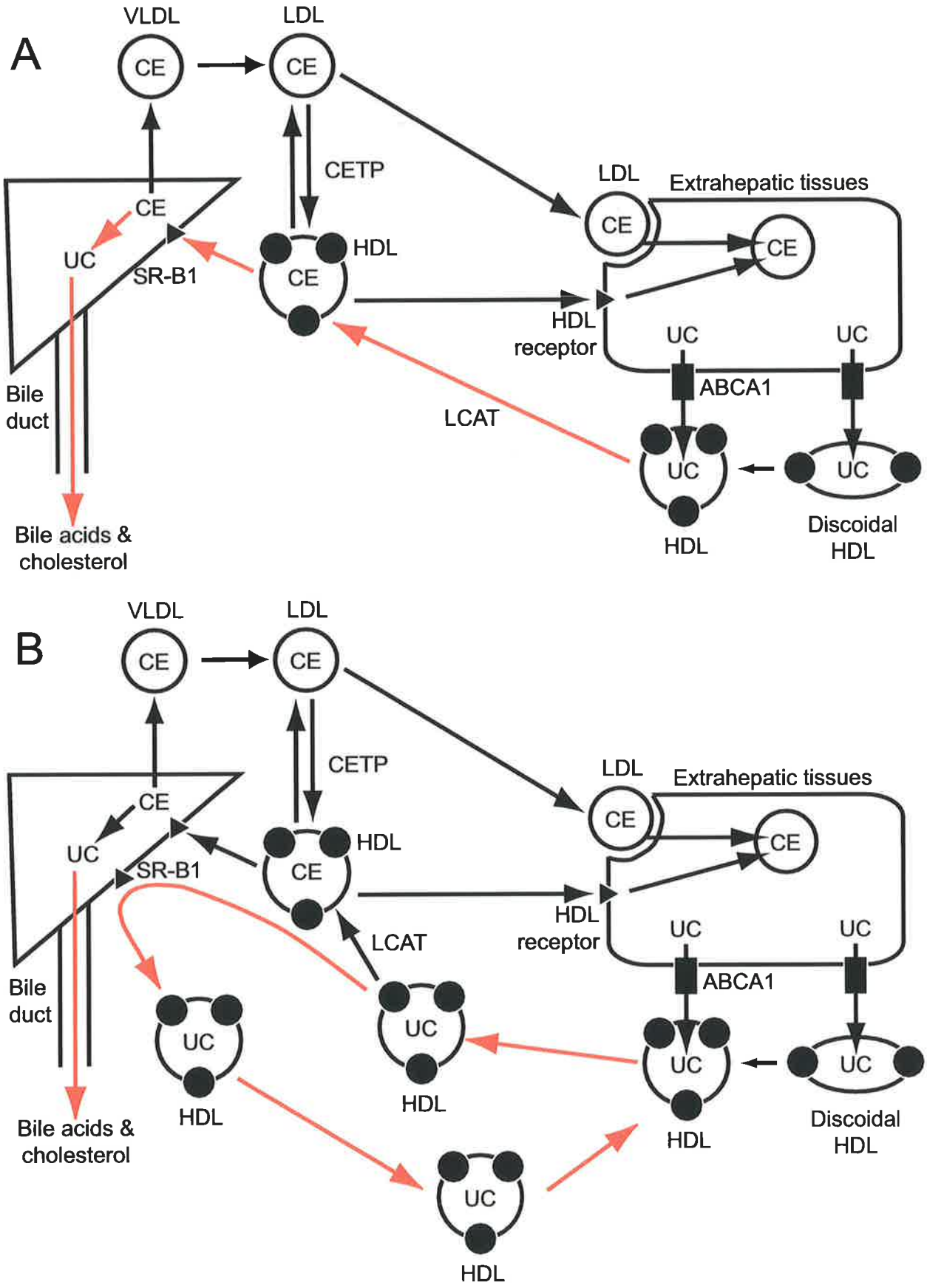

Figure 7.1: Schematic diagram of lipoprotein and cholesterol metabolism. A. Widely accepted pathway. B. Proposed modified pathway. The red lines indicate the predominant reverse cholesterol transport pathway in each respective model.

7.1 Increased production of HDL precursors

Precursors of HDL include lipid-free apoA-I, discoidal HDL, and recombinant apoA-I such as apoA-I (Milano). Overexpression of human apoA-I in transgenic mice has been shown to inhibit early atherogenesis (Rubin et al. 1991). Fibrates such as gemfibrozil can increase HDL production. In the VA-HIT trial, the use of gemfibrozil in subjects with coronary artery disease and normal LDL-C levels has been proven beneficial in reducing coronary risk (Rubins et al. 1999). Plasma HDL can also be transiently raised by HDL infusion. The infusion of native HDL (Badimon et al. 1989; Badimon et al. 1990) or recombinant apoA-I (Milano)/ phospholipid complex have reduced plaque progression in animal models (Ameli et al. 1994; Soma et al. 1995; Shah et al. 1998; Chiesa et al. 2002). Recently, the first human trial using recombinant apoA-I (Milano)/ phospholipid complex has been published and the results are highly encouraging (Nissen et al. 2003). Finally, the recent discovery of the role of ABCA1 in mediating cellular cholesterol efflux has made it a potential target for intervention. The overexpression of ABCA1in cholesterol-fed mice inhibits atherosclerosis but its overexpression of ABCA1 in apoE knockout mice paradoxically increased atherosclerosis (Joyce et al. 2002). The study highlighted the importance of apoE in cholesterol metabolism and atherosclerosis. The clinical application of ABCA1 is yet to be explored.

7.2 Inhibition of plasma factors that increase HDL catabolism

A number of plasma factors have the potential to increase HDL catabolism. These plasma factors include hepatic lipase (HL) and cholesteryl ester transfer protein (CETP). As highlighted in chapter 3 and 4, HL reduces the size of HDL and led to an increases clearance of HDL from plasma. Hepatic lipase transgenic rabbits also have lower plasma HDL-C concentration, presumably from the increased clearance of HDL. The significance of HL in

atherogenesis remains controversial and the clinical application of HL remains a subject of debate (Santamarina-Fojo et al. 1998; Thuren 2000; Jansen et al. 2002). On the other hand, the inhibition of CETP has inhibited the progression of atherosclerosis in some animal models (Sugano et al. 1998; Okamoto et al. 2000; Rittershaus et al. 2000). Both chapter 5 and 6 have highlighted some of the potential mechanisms in inhibiting atherosclerosis. Rabbits treated with the CETP inhibitor have an anti-atherogenic lipid profile consisting of high HDL-C and low LDL-C. This alteration in lipid profile comes about as a result of the redistribution of CE between HDL and LDL. Thus, CE is retained in the anti-atherogenic HDL and diverted from the pro-atherogenic LDL. A CETP inhibitor has been studied in a human phase II trial and similar anti-atherogenic lipid profile was also observed (de Grooth et al. 2002). The clinical efficacy of CETP inhibitor is yet to be determined.

7.3 Overexpression of plasma factors that lead to HDL-C retention

LCAT promotes the esterification of UC to CE in HDL. Overexpression of LCAT in transgenic rabbits led to a significant increase in HDL-C concentration (Hoeg et al. 1996a) and prevented diet-induced atherosclerosis (Hoeg et al. 1996b). However, the benefit of LCAT overexpression in reducing atherosclerosis appears to relate to CETP status of the animal model. LCAT transgenic mice also demonstrated the favourable increase in HDL-C concentration but paradoxically had increased susceptibility to diet-induced atherosclerosis (Berard et al. 1997). The co-expression of CETP and LCAT in transgenic mice attenuated the development of atherosclerosis (Foger et al. 1999). These studies demonstrated how mechanisms resulting in elevated HDL-C concentration could be influenced by the interactions of various plasma factors.

This thesis has made a number of contributions and has demonstrated (1) the sequence of events in the remodelling of HDL by plasma factors, (2) the effects of HL on HDL remodelling and how a reduction in HDL size resulted in the enhanced clearance of HDL, (3) how CETP inhibition led to an increase in CE clearance via HDL without affecting the overall CE flux out of plasma, and (4) the slow *in vivo* esterification of UC to CE in HDL and how such an observation complimented other previously published cholesterol kinetic studies in proposing an alternate reverse cholesterol transport pathway.

8 Appendix

P. Kee, K. Rye, J. L. Taylor, P. Hugh, R. Barrett, and P. J. Barter (2002) Metabolism of ApoA-I as Lipid-Free Protein or as Component of Discoidal and Spherical Reconstituted HDLs: Studies in Wild-Type and Hepatic Lipase Transgenic Rabbits. *Arteriosclerosis, Thrombosis, and Vascular Biology, v. 22 (11), pp. 1912-1917, November 2002*

9 Bibliography

Acton, S., A. Rigotti, K. T. Landschulz, S. Xu, H. H. Hobbs and M. Krieger (1996). "Identification of scavenger receptor SR-BI as a high density lipoprotein receptor." Science **271**(5248): 518-20.

Albanese, M., A. Mancusco, G. Talley, W. Yang, H. Kruth, S. Santamarina-Fojo and H. Brewer Jr (1998). "Effects of dysfunctional HDL on cholesterol efflux in LCAT transgenic mice [abstract]." Circulation **98**: I-450.

Albers, J. J., V. G. Cabana and Y. Dee Barden Stahl (1976). "Purification and characterization of human plasma lecithin:cholesterol acyltransferase." Biochemistry **15**(5): 1084-7.

Ameli, S., A. Hultgardh-Nilsson, B. Cercek, P. K. Shah, J. S. Forrester, H. Ageland and J. Nilsson (1994). "Recombinant apolipoprotein A-I Milano reduces intimal thickening after balloon injury in hypercholesterolemic rabbits." Circulation **90**(4): 1935-41.

Anderson, K. M., W. P. Castelli and D. Levy (1987). "Cholesterol and mortality. 30 years of follow-up from the Framingham study." Jama **257**(16): 2176-80.

Arai, T., F. Rinninger, L. Varban, V. Fairchild-Huntress, C. P. Liang, W. Chen, T. Seo, R. Deckelbaum, D. Huszar and A. R. Tall (1999). "Decreased selective uptake of high density lipoprotein cholesteryl esters in apolipoprotein E knock-out mice." Proc Natl Acad Sci U S A **96**(21): 12050-5.

Ashby, D. T., K. A. Rye, M. A. Clay, M. A. Vadas, J. R. Gamble and P. J. Barter (1998). "Factors influencing the ability of HDL to inhibit expression of vascular cell adhesion molecule-1 in endothelial cells." Arterioscler Thromb Vasc Biol **18**(9): 1450-5.

Assmann, G., P. Cullen and H. Schulte (1998). "The Munster Heart Study (PROCAM). Results of follow-up at 8 years." Eur Heart J **19 Suppl A**: A2-11.

Assmann, G., A. von Eckardstein and H. Funke (1993). "High density lipoproteins, reverse transport of cholesterol, and coronary artery disease. Insights from mutations." Circulation **87**(4 Suppl): III28-34.

Asztalos, B. F., C. H. Sloop, L. Wong and P. S. Roheim (1993). "Comparison of apo A-I-containing subpopulations of dog plasma and prenodal peripheral lymph: evidence for alteration in subpopulations in the interstitial space." Biochim Biophys Acta **1169**(3): 301-4.

Aviram, M., M. Rosenblat, C. L. Bisgaier, R. S. Newton, S. L. Primo-Parmo and B. N. La Du (1998). "Paraoxonase inhibits high-density lipoprotein oxidation and preserves its functions. A possible peroxidative role for paraoxonase." J Clin Invest **101**(8): 1581-90.

Badimon, J. J., L. Badimon and V. Fuster (1990). "Regression of atherosclerotic lesions by high density lipoprotein plasma fraction in the cholesterol-fed rabbit." J Clin Invest **85**(4): 1234-41.

Badimon, J. J., L. Badimon, A. Galvez, R. Dische and V. Fuster (1989). "High density lipoprotein plasma fractions inhibit aortic fatty streaks in cholesterol-fed rabbits." Lab Invest **60**(3): 455-61.

Baker, P. W., K. A. Rye, J. R. Gamble, M. A. Vadas and P. J. Barter (1999). "Ability of reconstituted high density lipoproteins to inhibit cytokine-induced expression of vascular cell adhesion molecule-1 in human umbilical vein endothelial cells." J Lipid Res **40**(2): 345-53.

Baker, P. W., K. A. Rye, J. R. Gamble, M. A. Vadas and P. J. Barter (2000). "Phospholipid composition of reconstituted high density lipoproteins influences their ability to inhibit endothelial cell adhesion molecule expression." J Lipid Res **41**(8): 1261-7.

Barbagallo, C., J. Fan, P. Blanche, M. Rizzo, J. Taylor and R. Krauss (1999). "Overexpression of human hepatic lipase and apoE in transgenic rabbits attenuates response to dietary cholesterol and alters lipoprotein subclass distributions." Arterioscler Thromb Vasc Biol **19**: 625-632.

Barbier, O., I. P. Torra, Y. Duguay, C. Blanquart, J. C. Fruchart, C. Glineur and B. Staels (2002). "Pleiotropic actions of peroxisome proliferator-activated receptors in lipid metabolism and atherosclerosis." Arterioscler Thromb Vasc Biol **22**(5): 717-26.

Barrans, A., X. Collet and R. Barbaras (1994). "Hepatic lipase induces the formation of pre-β_1 high density lipoprotein (HDL) from triacylglycerol-rich HDL_2. A study comparing liver perfusion to in vitro incubation with lipases." J Biol Chem **269**: 11572-11577.

Barter, P. J., L. B. Chang, H. H. Newnham, K. A. Rye and O. V. Rajaram (1990). "The interaction of cholesteryl ester transfer protein and unesterified fatty acids promotes a reduction in the particle size of high-density lipoproteins." Biochim Biophys Acta **1045**(1): 81-9.

Barter, P. J., G. J. Hopkins and G. D. Calvert (1982). "Transfers and exchanges of esterified cholesterol between plasma lipoproteins." Biochem J **208**(1): 1-7.

Barter, P. J. and K. A. Rye (1996). "High density lipoproteins and coronary heart disease." Atherosclerosis **121**(1): 1-12.

Beisiegel, U., W. Weber and G. Bengtsson-Olivecrona (1991). "Lipoprotein lipase enhances the binding of chylomicrons to low density lipoprotein receptor-related protein." Proc Natl Acad Sci U S A **88**(19): 8342-6.

Bekaert, E. D., P. Alaupovic, C. Knight-Gibson, R. A. Norum, M. J. Laux and M. Ayrault-Jarrier (1992). "Isolation and partial characterization of lipoprotein A-II (LP-A-II) particles of human plasma." Biochim Biophys Acta **1126**(1): 105-113.

Bengtsson, G. and T. Olivecrona (1980). "Lipoprotein lipase: some effects of activator proteins." Eur J Biochem **106**(2): 549-55.

Berard, A. M., B. Foger, A. Remaley, R. Shamburek, B. L. Vaisman, G. Talley, B. Paigen, R. F. Hoyt, Jr., S. Marcovina, H. B. Brewer, Jr. and S. Santamarina-Fojo (1997). "High plasma HDL concentrations associated with enhanced atherosclerosis in transgenic mice overexpressing lecithin-cholesteryl acyltransferase." Nat Med **3**(7): 744-9.

Berge, K. E., H. Tian, G. A. Graf, L. Yu, N. V. Grishin, J. Schultz, P. Kwiterovich, B. Shan, R. Barnes and H. H. Hobbs (2000). "Accumulation of dietary cholesterol in sitosterolemia caused by mutations in adjacent ABC transporters." Science **290**(5497): 1771-5.

Bjorkhem, I. and J. E. Akerlund (1988). "Studies on the link between HMG-CoA reductase and cholesterol 7 alpha-hydroxylase in rat liver." J Lipid Res **29**(2): 136-43.

Blades, B., G. Vega and S. Grundy (1993). "Activities of lipoprotein lipase and hepatic triglyceride lipase in postheparin plasma of patients with low concentrations of HDL cholesterol." Arterioscler Thromb **13**: 1227-1235.

Bravo, E., K. M. Botham, M. A. Mindham, P. A. Mayes, T. Marinelli and A. Cantafora (1994). "Evaluation in vivo of the differential uptake and processing of high-density lipoprotein unesterified cholesterol and cholesteryl ester in the rat." Biochim Biophys Acta **1215**(1-2): 93-102.

Bravo, E. and A. Cantafora (1990). "Hepatic uptake and processing of free cholesterol from different lipoproteins with and without sodium taurocholate administration. An in vivo study in the rat." Biochim Biophys Acta **1045**(1): 74-80.

Bravo, E., A. Cantafora and L. Argiolas (1989). "Hepatic uptake and metabolism of free cholesterol from different lipoprotein classes. An in vivo study in the rat." Biochim Biophys Acta **1003**(3): 315-20.

Brewer, H. B., Jr., S. E. Lux, R. Ronan and K. M. John (1972). "Amino acid sequence of human apoLp-Gln-II (apoA-II), an apolipoprotein isolated from the high-density lipoprotein complex." Proc Natl Acad Sci U S A **69**(5): 1304-8.

Brewer, H. B., Jr., R. Ronan, M. Meng and C. Bishop (1986). "Isolation and characterization of apolipoproteins A-I, A-II, and A-IV." Methods Enzymol **128**: 223-46.

Brinton, E., S. Eisenberg and J. Breslow (1989). "Elevated high density lipoprotein cholesterol levels correlate with decreased apolipoprotin A-I and A-II fractional catabolic rate in women." J Clin Invest **84**: 262-269.

Brown, B., P. Kovanen and J. Goldstein (1981). "Regulation of plasma cholesterol by lipoprotein receptors." Science **21**: 628-635.

Brown, M. and J. Goldstein (1983). "Lipoprotein metabolism in the macrophage: implications for cholesterol deposition in atherosclerosis." Annu Rev Biochem **52**: 223-261.

Brown, M. S. and J. L. Goldstein (1986). "A receptor-mediated pathway for cholesterol homeostasis." Science **232**(4746): 34-47.

Bungert, S., L. L. Molday and R. S. Molday (2001). "Membrane topology of the ATP binding cassette transporter ABCR and its relationship to ABC1 and related ABCA transporters: identification of N-linked glycosylation sites." J Biol Chem **276**(26): 23539-46.

Burstein, M., H. Scholnick and R. Morfin (1970). "Rapid method for the isolation of lipoproteins from human serum by precipitation with polyanions." J Lipid Res **11**: 583-587.

Calabresi, L., G. Franceschini, C. Sirtori, A. DePalma, M. Sarasella and P. Ferrante (1997). "Inhibition of VCAM-1 expression in endothelial cells by reconstituted high density lipoproteins." Biochim Biophys Res Commun **238**: 61-65.

Castelli, W. P., R. Garrison, P. Wilson, R. Abbott, S. Kakousdian and W. Kannel (1986). "Incidence of coronary heart disease and lipoprotein-cholesterol levels: the Framingham Study." JAMA **256**: 2835-2838.

Castro, G. R. and C. J. Fielding (1988). "Early incorporation of cell-derived cholesterol into pre-beta-migrating high-density lipoprotein." Biochemistry **27**(1): 25-29.

Chapman, M. J. (1980). "Animal lipoproteins: chemistry, structure, and comparative aspects." J Lipid Res **21**(7): 789-853.

Cheng, C. F., A. Bensadoun, T. Bersot, J. S. Hsu and K. H. Melford (1985). "Purification and characterization of human lipoprotein lipase and hepatic triglyceride lipase. Reactivity with monoclonal antibodies to hepatic triglyceride lipase." J Biol Chem **260**(19): 10720-10727.

Cheung, M. C. and J. J. Albers (1982). "Distribution of high density lipoprotein particles with different apoprotein composition: particles with A-I and A-II and particles with A-I but no A-II." J Lipid Res **23**(5): 747-753.

Cheung, M. C., A. C. Wolf, K. D. Lum, J. Tollefson and J. J. Albers (1986). "Distribution and localization of lecithin:cholesterol acyltransferase and cholesteryl ester transfer activity in A-I containing lipoproteins." J Lipid Res **27**: 1135-1144.

Chiesa, G., E. Monteggia, M. Marchesi, P. Lorenzon, M. Laucello, V. Lorusso, C. Di Mario, E. Karvouni, R. S. Newton, C. L. Bisgaier, G. Franceschini and C. R. Sirtori (2002). "Recombinant apolipoprotein A-I(Milano) infusion into rabbit carotid artery rapidly removes lipid from fatty streaks." Circ Res **90**(9): 974-80.

Clay, M. A. and P. J. Barter (1996). "Formation of new HDL particles from lipid-free apolipoprotein A-I." J Lipid Res **37**(8): 1722-1732.

Clay, M. A., D. A. Cehic, D. H. Pyle, K. A. Rye and P. J. Barter (1999). "Formation of apolipoprotein-specific high-density lipoprotein particles from lipid-free apolipoproteins A-I and A-II." Biochem J **337**(Pt 3): 445-51.

Clay, M. A., G. J. Hopkins, C. P. Ehnholm and P. J. Barter (1989). "The rabbit as an animal model of hepatic lipase deficiency." Biochim Biophys Acta **1002**(2): 173-181.

Clay, M. A., H. H. Newnham and P. J. Barter (1991). "Hepatic lipase promotes a loss of apolipoprotein A-I from triglyceride-enriched human high density lipoproteins during incubation in vitro." Arterioscler Thromb **11**(2): 415-422.

Clay, M. A., H. H. Newnham, T. M. Forte and P. J. Barter (1992). "Cholesteryl ester transfer protein and hepatic lipase activity promote shedding of apo A-I from HDL and subsequent formation of discoidal HDL." Biochim Biophys Acta **1124**(1): 52-58.

Clay, M. A., D. H. Pyle, K. A. Rye and P. J. Barter (2000). "Formation of spherical, reconstituted high density lipoproteins containing both apolipoproteins A-I and A-II is mediated by lecithin:cholesterol acyltransferase." J Biol Chem **275**(12): 9019-9025.

Clay, M. A., K. A. Rye and P. J. Barter (1990). "Evidence in vitro that hepatic lipase reduces the concentration of apolipoprotein A-I in rabbit high-density lipoproteins." Biochim Biophys Acta **1044**(1): 50-56.

Cockerill, G. W., K. A. Rye, J. R. Gamble, M. A. Vadas and P. J. Barter (1995). "High-density lipoproteins inhibit cytokine-induced expression of endothelial cell adhesion molecules." Arterioscler Thromb Vasc Biol **15**(11): 1987-94.

Collet, X., A. R. Tall, H. Serajuddin, K. Guendouzi, L. Royer, H. Oliveira, R. Barbaras, X. C. Jiang and O. L. Francone (1999). "Remodeling of HDL by CETP in vivo and by CETP and hepatic lipase in vitro results in enhanced uptake of HDL CE by cells expressing scavenger receptor B-I." J Lipid Res **40**(7): 1185-93.

Colvin, P., E. Moriguchi, P. Barrett, J. Parks and L. Rudel (1999). "Small HDL particles containing two apoA-I molecules are precusors in vivo to medium and large HDL particles containing three and four apoA-I molecules in nonhuman primates." J Lipid Res **40**: 1782-1792.

Connelly, M. A., S. M. Klein, S. Azhar, N. A. Abumrad and D. L. Williams (1999). "Comparison of class B scavenger receptors, CD36 and scavenger receptor BI (SR-BI), shows that both receptors mediate high density lipoprotein-cholesteryl ester selective uptake but SR-BI exhibits a unique enhancement of cholesteryl ester uptake." J Biol Chem **274**(1): 41-7.

Connelly, P. W., G. F. Maguire, M. Lee and J. A. Little (1990). "Plasma lipoproteins in familial hepatic lipase deficiency." Arteriosclerosis **10**(1): 40-48.

Contacos, C., D. R. Sullivan, K. A. Rye, H. Funke and G. Assmann (1996). "A new molecular defect in the lecithin: cholesterol acyltransferase (LCAT) gene associated with fish eye disease." J Lipid Res **37**(1): 35-44.

Crowl, R. M., T. J. Stoller, R. R. Conroy and C. R. Stoner (1991). "Induction of phospholipase A2 gene expression in human hepatoma cells by mediators of the acute phase response." J Biol Chem **266**(4): 2647-51.

Czarnecka, H. and S. Yokoyama (1993). "Regulation of lecithin-cholesterol acyltransferase reaction by acyl acceptors and demonstration of its "idling" reaction." J Biol Chem **268**(26): 19334-40.

de Grooth, G. J., J. A. Kuivenhoven, A. F. Stalenhoef, J. de Graaf, A. H. Zwinderman, J. L. Posma, A. van Tol and J. J. Kastelein (2002). "Efficacy and safety of a novel cholesteryl ester transfer protein inhibitor, JTT-705, in humans: a randomized phase II dose-response study." Circulation **105**(18): 2159-65.

de la Llera-Moya, M., G. H. Rothblat, M. A. Connelly, G. Kellner-Weibel, S. W. Sakr, M. C. Phillips and D. L. Williams (1999). "Scavenger receptor BI (SR-BI) mediates free cholesterol flux independently of HDL tethering to the cell surface." J Lipid Res **40**(3): 575-80.

Diaz, M. N., B. Frei, J. A. Vita and J. F. Keaney, Jr. (1997). "Antioxidants and atherosclerotic heart disease." N Engl J Med **337**(6): 408-16.

Dietschy, J., S. Turley and D. Spady (1993). "Role of liver in the maintenance of cholesterol and low density lipoprotein homeostasis in different animal species including humans." J Lipid Res **34**: 1637-1639.

Doolittle, M. H., H. Wong, R. C. Davis and M. C. Schotz (1987). "Synthesis of hepatic lipase in liver and extrahepatic tissues." J Lipid Res **28**(11): 1326-34.

Downs, J. R., M. Clearfield, S. Weis, E. Whitney, D. R. Shapiro, P. A. Beere, A. Langendorfer, E. A. Stein, W. Kruyer and A. M. Gotto, Jr. (1998). "Primary prevention of acute coronary events with lovastatin in men and women with average cholesterol levels: results of AFCAPS/TexCAPS. Air Force/Texas Coronary Atherosclerosis Prevention Study." Jama **279**(20): 1615-22.

Edelstein, C., M. Halari and A. M. Scanu (1982). "On the mechanism of the displacement of apolipoprotein A-I by apolipoprotein A-II from the high density lipoprotein surface. Effect of concentration and molecular forms of apolipoprotein A-II." J Biol Chem **257**(12): 7189-95.

Elferink, R. P., G. N. Tytgat and A. K. Groen (1997). "Hepatic canalicular membrane 1: The role of mdr2 P-glycoprotein in hepatobiliary lipid transport." Faseb J **11**(1): 19-28.

Epand, R. M., A. Stafford, B. Leon, P. E. Lock, E. M. Tytler, J. P. Segrest and G. M. Anantharamaiah (1994). "HDL and apolipoprotein A-I protect erythrocytes against the generation of procoagulant activity." Arterioscler Thromb **14**(11): 1775-83.

Esnault-Dupuy, C., F. Chanussot, H. LaFont, C. Chabert and J. Hauton (1987). "The relationship between HDL-, LDL-, liposomes-free cholesterol, biliary cholesterol and bile salts in the rat." Biochimie **69**(1): 45-52.

Fan, J., S. P. McCormick, R. M. Krauss, S. Taylor, R. Quan, J. M. Taylor and S. G. Young (1995). "Overexpression of human apolipoprotein B-100 in transgenic rabbits results in increased levels of LDL and decreased levels of HDL." Arterioscler Thromb Vasc Biol **15**(11): 1889-1899.

Fan, J., J. Wang, A. Bensadoun, S. J. Lauer, Q. Dang, R. W. Mahley and J. M. Taylor (1994). "Overexpression of hepatic lipase in transgenic rabbits leads to a marked reduction of plasma high density lipoproteins and intermediate density lipoproteins." Proc Natl Acad Sci U S A **91**(18): 8724-8728.

Fidge, N., P. Nestel, T. Ishikawa, M. Reardon and T. Billington (1980). "Turnover of apoproteins A-I and A-II of high density lipoprotein and the relationship to other lipoproteins in normal and hyperlipidemic individuals." Metabolism **29**(7): 643-653.

Fidge, N. H. (1999). "High density lipoprotein receptors, binding proteins, and ligands." J Lipid Res **40**(2): 187-201.

Fielding, C. and P. E. Fielding (1995). "Molecular physiology of reverse cholesterol transport." J Lipid Res **36**: 211-228.

Fleisher, L. N., A. R. Tall, L. D. Witte, R. W. Miller and P. J. Cannon (1982). "Stimulation of arterial endothelial cell prostacyclin synthesis by high density lipoproteins." J Biol Chem **257**(12): 6653-5.

Foger, B., M. Chase, M. J. Amar, B. L. Vaisman, R. D. Shamburek, B. Paigen, J. Fruchart-Najib, J. A. Paiz, C. A. Koch, R. F. Hoyt, H. B. Brewer, Jr. and S. Santamarina-Fojo (1999). "Cholesteryl ester transfer protein corrects dysfunctional high density

lipoproteins and reduces aortic atherosclerosis in lecithin cholesterol acyltransferase transgenic mice." J Biol Chem **274**(52): 36912-20.

Forte, T. M., J. K. Bielicki, R. Goth-Goldstein, J. Selmek and M. R. McCall (1995). "Recruitment of cell phospholipids and cholesterol by apolipoproteins A-II and A-I: formation of nascent apolipoprotein-specific HDL that differ in size, phospholipid composition, and reactivity with LCAT." J Lipid Res **36**(1): 148-57.

Francone, O. L., A. Gurakar and C. Fielding (1989). "Distribution and functions of lecithin:cholesterol acyltransferase and cholesteryl ester transfer protein in plasma lipoproteins. Evidence for a functional unit containing these activities together with apolipoproteins A-I and D that catalyzes the esterification and transfer of cell-derived cholesterol." J Biol Chem **264**(12): 7066-72.

Frank, P. G. and Y. L. Marcel (2000). "Apolipoprotein A-I: structure-function relationships." J Lipid Res **41**(6): 853-72.

Frick, M. H., O. Elo, K. Haapa, O. P. Heinonen, P. Heinsalmi, P. Helo, J. K. Huttunen, P. Kaitaniemi, P. Koskinen, V. Manninen and et al. (1987). "Helsinki Heart Study: primary-prevention trial with gemfibrozil in middle-aged men with dyslipidemia. Safety of treatment, changes in risk factors, and incidence of coronary heart disease." N Engl J Med **317**(20): 1237-45.

Gianturco, S. H., M. P. Ramprasad, A. H. Lin, R. Song and W. A. Bradley (1994). "Cellular binding site and membrane binding proteins for triglyceride-rich lipoproteins in human monocyte-macrophages and THP-1 monocytic cells." J Lipid Res **35**(9): 1674-87.

Glass, C., R. C. Pittman, M. Civen and D. Steinberg (1985). "Uptake of high-density lipoprotein-associated apoprotein A-I and cholesterol esters by 16 tissues of the rat in vivo and by adrenal cells and hepatocytes in vitro." J Biol Chem **260**(2): 744-50.

Glass, C., R. C. Pittman, D. B. Weinstein and D. Steinberg (1983). "Dissociation of tissue uptake of cholesterol ester from that of apoprotein A-I of rat plasma high density lipoprotein: selective delivery of cholesterol ester to liver, adrenal, and gonad." Proc Natl Acad Sci U S A **80**(17): 5435-9.

Glomset, J. (1968). "The plasma lecithin: cholesterol acyltransferase reaction." J Lipid Res **9**: 155-167.

Goldberg, D. I., W. F. Beltz and R. C. Pittman (1991). "Evaluation of pathways for the cellular uptake of high density lipoprotein cholesterol esters in rabbits." J Clin Invest **87**(1): 331-46.

Goldberg, I. J. (1996). "Lipoprotein lipase and lipolysis: central roles in lipoprotein metabolism and atherogenesis." J Lipid Res **37**(4): 693-707.

Goldberg, I. J., N. A. Le, J. R. Paterniti, Jr., H. N. Ginsberg, F. T. Lindgren and W. V. Brown (1982). "Lipoprotein metabolism during acute inhibition of hepatic triglyceride lipase in the cynomolgus monkey." J Clin Invest **70**(6): 1184-92.

Goldstein, J., M. Brown, R. Anderson, D. Russell and W. Schneider (1985). "Receptor-mediated endocytosis: concepts emerging from the LDL receptor system." Ann Rev Cell Biol **1**: 1-39.

Goldstein, J., Y. Ho, S. Basu and M. Brown (1979). "Binding site on macrophages that mediates uptake and degradation of acetylated low density lipoprotein, producing massive cholesterol deposition." Proc Natl Acad Sci **76**: 333-337.

Gordon, D., J. Probstfield, R. Garrison, J. Neaton, W. P. Castelli, J. Knoke, D. Jacobs, S. Bangdiwala and H. A. Tyroler (1989). "High-density lipoprotein cholesterol and cardiovascular disease. Four prospective American studies." Circulation **79**: 8-15.

Gould, A. L., J. E. Rossouw, N. C. Santanello, J. F. Heyse and C. D. Furberg (1998). "Cholesterol reduction yields clinical benefit: impact of statin trials." Circulation **97**(10): 946-52.

Green, P. H., A. R. Tall and R. M. Glickman (1978). "Rat intestine secretes discoid high density lipoprotein." J Clin Invest **61**(2): 528-34.

Greeve, J., I. Altkemper, J. H. Dieterich, H. Greten and E. Windler (1993). "Apolipoprotein B mRNA editing in 12 different mammalian species: hepatic expression is reflected in low concentrations of apoB-containing plasma lipoproteins." J Lipid Res **34**(8): 1367-83.

Groen, A. K., V. W. Bloks, R. H. Bandsma, R. Ottenhoff, G. Chimini and F. Kuipers (2001). "Hepatobiliary cholesterol transport is not impaired in Abca1-null mice lacking HDL." J Clin Invest **108**(6): 843-50.

Gylling, H. and T. A. Miettinen (1992). "Non-cholesterol sterols, absorption and synthesis of cholesterol and apolipoprotein A-I kinetics in a Finnish lecithin-cholesterol acyltransferase deficient family." Atherosclerosis **95**(1): 25-33.

Ha, Y. C. and P. J. Barter (1982). "Differences in plasma cholesteryl ester transfer activity in sixteen vertebrate species." Comp Biochem Physiol B **71**(2): 265-269.

Haidar, B., S. Mott, B. Boucher, C. Y. Lee, M. Marcil and J. Genest, Jr. (2001). "Cellular cholesterol efflux is modulated by phospholipid-derived signaling molecules in familial HDL deficiency/Tangier disease fibroblasts." J Lipid Res **42**(2): 249-57.

Halloran, L. G., C. C. Schwartz, Z. R. Vlahcevic, R. M. Nisman and L. Swell (1978). "Evidence for high-density lipoprotein-free cholesterol as the primary precursor for bile-acid synthesis in man." Surgery **84**(1): 1-7.

Hamilton, R. L., M. C. Williams, C. J. Fielding and R. J. Havel (1976). "Discoidal bilayer structure of nascent high density lipoproteins from perfused rat liver." J Clin Invest **58**(3): 667-80.

Hammad, S. M., S. Stefansson, W. O. Twal, C. J. Drake, P. Fleming, A. T. Remaley, H. Brewer Jr and W. Argraves (1999). "Cubilin, the endocytic receptor for intrinsic factor-vitamin B_{12} complex, mediates high density lipoprotein holoparticle endocytosis." Proc Natl Acad Sci **96**: 10158-10163.

Havel, R. J. (1995). "Chylomicron remnants: hepatic receptors and metabolism." Curr Opin Lipidol **6**(5): 312-6.

Heart Protection Study Group (2002). "MRC/BHF Heart Protection Study of cholesterol lowering with simvastatin in 20,536 high-risk individuals: a randomised placebo-controlled trial." Lancet **360**: 7-22.

Hegele, R., J. Little, A. Vezina, G. Maguire, L. Tu and T. Woleyer (1993). "Hepatic lipase deficiency. Clinical biochemical and molecular genetic characteristics." Arterioscler Thromb **13**: 720-728.

Hessler, J. R., A. L. Robertson, Jr. and G. M. Chisolm, 3rd (1979). "LDL-induced cytotoxicity and its inhibition by HDL in human vascular smooth muscle and endothelial cells in culture." Atherosclerosis **32**(3): 213-29.

Hime, N. J., P. J. Barter and K. A. Rye (1998). "The influence of apolipoproteins on the hepatic lipase-mediated hydrolysis of high density lipoprotein phospholipid and triacylglycerol." J Biol Chem **273**(42): 27191-27198.

Hodgson, J. M. and J. J. Marshall (1989). "Direct vasoconstriction and endothelium-dependent vasodilation. Mechanisms of acetylcholine effects on coronary flow and arterial diameter in patients with nonstenotic coronary arteries." Circulation **79**(5): 1043-51.

Hoeg, J., B. Vaisman and S. J. Demosky (1996a). "Lecithin:cholesterol acyltransferase overexpression generates hyperalpha-lipoproteinemia and a nonatherogenic lipoprotein pattern in transgenic rabbits." J Biol Chem **271**: 4396-402.

Hoeg, J. M., S. Santamarina-Fojo, A. M. Berard, J. F. Cornhill, E. E. Herderick, S. H. Feldman, C. C. Haudenschild, B. L. Vaisman, R. F. Hoyt, Jr., S. J. Demosky, Jr., R. D. Kauffman, C. M. Hazel, S. M. Marcovina and H. B. Brewer, Jr. (1996b). "Overexpression of lecithin:cholesterol acyltransferase in transgenic rabbits prevents diet-induced atherosclerosis." Proc Natl Acad Sci U S A **93**(21): 11448-53.

Hopkins, G. J. and P. J. Barter (1986). "Role of triglyceride-rich lipoproteins and hepatic lipase in determining the particle size and composition of high density lipoproteins." J Lipid Res **27**(12): 1265-1277.

Hopkins, G. J. and P. J. Barter (1989). "Apolipoprotein A-I inhibits transformation of high density lipoprotein subpopulations during incubation of human plasma." Atherosclerosis **75**(1): 73-82.

Horowitz, B., I. Goldberg, J. Merab, T. Vanni, R. Ramakrishnan and H. Ginsberg (1993). "Increased plasma and renal clearance of an exchangeable pool of apolipoprotein A-I in subjects with low levels of high density lipoprotein cholesterol." J Clin Invest **91**: 1743-1752.

Huang, Y., A. von Eckardstein, S. Wu, N. Maeda and G. Assmann (1994). "A plasma lipoprotein containing only apolipoprotein E and with gamma mobility on electrophoresis releases cholesterol from cells." Proc Natl Acad Sci U S A **91**(5): 1834-8.

Ikewaki, K., D. J. Rader, J. R. Schaefer, T. Fairwell, L. A. Zech and H. B. Brewer, Jr. (1993). "Evaluation of apoA-I kinetics in humans using simultaneous endogenous stable isotope and exogenous radiotracer methods." J Lipid Res **34**(12): 2207-2215.

Jackson, R. L. and A. M. Gotto, Jr. (1974a). "Phospholipids in biology and medicine (first of two parts)." N Engl J Med **290**(1): 24-9.

Jackson, R. L. and A. M. Gotto, Jr. (1974b). "Phospholipids in biology and medicine (second of two parts)." N Engl J Med **290**(2): 87-93.

Jansen, H., A. J. Verhoeven and E. J. Sijbrands (2002). "Hepatic lipase: a pro- or anti-atherogenic protein?" J Lipid Res **43**(9): 1352-62.

Jauhiainen, M., J. Metso, R. Pahlman, S. Blomqvist, A. van Tol and C. Ehnholm (1993). "Human plasma phospholipid transfer protein causes high density lipoprotein conversion." J Biol Chem **268**(6): 4032-6.

Jaye, M., K. J. Lynch, J. Krawiec, D. Marchadier, C. Maugeais, K. Doan, V. South, D. Amin, M. Perrone and D. J. Rader (1999). "A novel endothelial-derived lipase that modulates HDL metabolism." Nat Genet **21**(4): 424-8.

Ji, Y., B. Jian, N. Wang, Y. Sun, M. L. Moya, M. C. Phillips, G. H. Rothblat, J. B. Swaney and A. R. Tall (1997). "Scavenger receptor BI promotes high density lipoprotein-mediated cellular cholesterol efflux." J Biol Chem **272**(34): 20982-5.

Ji, Y., N. Wang, R. Ramakrishnan, E. Sehayek, D. Huszar, J. L. Breslow and A. R. Tall (1999). "Hepatic scavenger receptor BI promotes rapid clearance of high density lipoprotein free cholesterol and its transport into bile." J Biol Chem **274**(47): 33398-402.

Johnson, F. L., J. Babiak and L. L. Rudel (1986). "High density lipoprotein accumulation in perfusates of isolated livers of African green monkeys. Effects of saturated versus polyunsaturated dietary fat." J Lipid Res **27**(5): 537-48.

Jolley, C. D., J. M. Dietschy and S. D. Turley (2000). "Induction of bile acid synthesis by cholesterol and cholestyramine feeding is unimpaired in mice deficient in apolipoprotein AI." Hepatology **32**(6): 1309-16.

Jonas, A. (1991). "Lecithin: cholesterol acyltransferase in the metabolism of high density lipoproteins." Biochim Biophys Acta **1084**: 205-220.

Joyce, C. W., M. J. Amar, G. Lambert, B. L. Vaisman, B. Paigen, J. Najib-Fruchart, R. F. Hoyt, Jr., E. D. Neufeld, A. T. Remaley, D. S. Fredrickson, H. B. Brewer, Jr. and S. Santamarina-Fojo (2002). "The ATP binding cassette transporter A1 (ABCA1) modulates the development of aortic atherosclerosis in C57BL/6 and apoE-knockout mice." Proc Natl Acad Sci U S A **99**(1): 407-12.

Kosek, A. B., D. Durbin and A. Jonas (1999). "Binding affinity and reactivity of lecithin cholesterol acyltransferase with native lipoproteins." Biochem Biophys Res Commun **258**(3): 548-51.

Kozyraki, R., J. Fyfe, M. Kristiansen, C. Gerdes, C. Jacobsen, S. Cut, E. I. Christensen, M. Aminoff, A. de la Chapelle, R. Krathe, P. J. Verroust and S. K. Moestrup (1999). "The intrinsic factor-vitamin B_{12} receptor, cubilin, is a high-affinity apolipoprotein A-I receptor facilitating endocytosis of high density lipoprotein." Nat Med **5**: 656-661.

Krieger, M. and K. Kozarsky (1999). "Influence of the HDL receptor SR-BI on atherosclerosis." Curr Opin Lipidol **10**(6): 491-7.

Kuivenhoven, J. A., H. Pritchard, J. Hill, J. Frohlich, G. Assmann and J. Kastelein (1997). "The molecular pathology of lecithin:cholesterol acyltransferase (LCAT) deficiency syndromes." J Lipid Res **38**(2): 191-205.

Kuusi, T., P. K. Kinnunen and E. A. Nikkila (1979). "Hepatic endothelial lipase antiserum influences rat plasma low and high density lipoproteins in vivo." FEBS Lett **104**(2).

Lagrost, L. (1994). "Regulation of cholesteryl ester transfer protein (CETP) activity: review of in vitro and in vivo studies." Biochim Biophys Acta **1215**: 209-236.

Lambert, G., M. J. Amar, P. Martin, J. Fruchart-Najib, B. Foger, R. D. Shamburek, H. B. Brewer, Jr. and S. Santamarina-Fojo (2000). "Hepatic lipase deficiency decreases the selective uptake of HDL-cholesteryl esters in vivo." J Lipid Res **41**(5): 667-72.

Lambert, G., M. B. Chase, K. Dugi, A. Bensadoun, H. B. Brewer, Jr. and S. Santamarina-Fojo (1999). "Hepatic lipase promotes the selective uptake of high density lipoprotein-cholesteryl esters via the scavenger receptor B1." J Lipid Res **40**(7): 1294-1303.

LaRosa, J. C., J. He and S. Vupputuri (1999). "Effect of statins on risk of coronary disease: a meta-analysis of randomized controlled trials." Jama **282**(24): 2340-6.

Lawn, R. M., D. P. Wade, M. R. Garvin, X. Wang, K. Schwartz, J. G. Porter, J. J. Seilhamer, A. M. Vaughan and J. F. Oram (1999). "The Tangier disease gene product ABC1 controls the cellular apolipoprotein-mediated lipid removal pathway." J Clin Invest **104**(8): R25-31.

Lee, M. H., K. Lu, S. Hazard, H. Yu, S. Shulenin, H. Hidaka, H. Kojima, R. Allikmets, N. Sakuma, R. Pegoraro, A. K. Srivastava, G. Salen, M. Dean and S. B. Patel (2001). "Identification of a gene, ABCG5, important in the regulation of dietary cholesterol absorption." Nat Genet **27**(1): 79-83.

Leitinger, N., A. D. Watson, S. Y. Hama, B. Ivandic, J. H. Qiao, J. Huber, K. F. Faull, D. S. Grass, M. Navab, A. M. Fogelman, F. C. de Beer, A. J. Lusis and J. A. Berliner (1999). "Role of group II secretory phospholipase A2 in atherosclerosis: 2. Potential involvement of biologically active oxidized phospholipids." Arterioscler Thromb Vasc Biol **19**(5): 1291-8.

Lewis, G. F., B. Lamarche, K. D. Uffelman, C. Heatherington, M. A. Honing, L. W. Szeto and P. H. R. Barrett (1997). "Clearance of post-prandial and lipolytically modified human HDL in rabbits and rats." J Lipid Res **38**: 1771-1781.

Liang, H. Q., K. A. Rye and P. J. Barter (1994). "Dissociation of lipid-free apolipoprotein A-I from high density lipoproteins." J Lipid Res **35**(7): 1187-99.

Liang, H. Q., K. A. Rye and P. J. Barter (1995). "Cycling of apolipoprotein A-I between lipid-associated and lipid-free pools." Biochim Biophys Acta **1257**(1): 31-7.

Liang, H. Q., K. A. Rye and P. J. Barter (1996). "Remodelling of reconstituted high density lipoproteins by lecithin: cholesterol acyltransferase." J Lipid Res **37**(9): 1962-1970.

Lipid Research Clinics Program (1984a). "The Lipid Research Clinics Coronary Primary Prevention Trial results. I. Reduction in incidence of coronary heart disease." JAMA **251**: 351-364.

Lipid Research Clinics Program (1984b). "The Lipid Research Clinics Coronary Primary Prevention Trial results. II. The relationship of reduction in incidence of coronary heart disease to cholesterol lowering." JAMA **251**: 365-374.

LIPID study Group (1998). "Prevention of cardiovascular events and death with pravastatin in patients with coronary heart disease and a broad range of initial cholesterol levels. The Long-Term Intervention with Pravastatin in Ischaemic Disease (LIPID) Study Group." N Engl J Med **339**: 1349-1357.

Liu, T., M. Krieger, H. Y. Kan and V. I. Zannis (2002). "The effects of mutations in helices 4 and 6 of ApoA-I on scavenger receptor class B type I (SR-BI)-mediated cholesterol efflux suggest that formation of a productive complex between reconstituted high density lipoprotein and SR-BI is required for efficient lipid transport." J Biol Chem **277**(24): 21576-84.

Liu, X. and J. Bagdade (1995). "Neutral lipid mass transfer among lipoprotein in plasma from normolipidemic subjects is not an equimolar heteroexchange." J Lipid Res **36**: 2574-2579.

Lowry, O., N. Rosebrough, A. Farr and R. Randall (1951). "Protein measurement with the Folin phenol reagent." J Biol Chem **193**: 265-275.

Luciani, M. F., F. Denizot, S. Savary, M. G. Mattei and G. Chimini (1994). "Cloning of two novel ABC transporters mapping on human chromosome 9." Genomics **21**(1): 150-9.

Ludmer, P. L., A. P. Selwyn, T. L. Shook, R. R. Wayne, G. H. Mudge, R. W. Alexander and P. Ganz (1986). "Paradoxical vasoconstriction induced by acetylcholine in atherosclerotic coronary arteries." N Engl J Med **315**(17): 1046-51.

Lund-Katz, S., B. Hammerschlag and M. C. Phillips (1982). "Kinetics and mechanism of free cholesterol exchange between human serum high- and low-density lipoproteins." Biochemistry **21**(12): 2964-9.

Lund-Katz, S., L. Liu, S. T. Thuahnai and M. C. Phillips (2003). "High density lipoprotein structure." Front Biosci **8**: d1044-54.

Lusa, S., M. Jauhiainen, J. Metso, P. Somerharju and C. Ehnholm (1996). "The mechanism of human plasma phospholipid transfer protein-induced enlargement of high-density lipoprotein particles: evidence for particle fusion." Biochem J **313 (Pt 1)**: 275-282.

Mahadevan, V. and L. A. Soloff (1983). "A method for isolating human plasma lecithin:cholesterol acyltransferase without using anti-apolipoprotein D, and its characterization." Biochim Biophys Acta **752**(1): 89-97.

Mahley, R. W., T. L. Innerarity, S. C. Rall, Jr. and K. H. Weisgraber (1984). "Plasma lipoproteins: apolipoprotein structure and function." J Lipid Res **25**(12): 1277-94.

Malmendier, C. L., C. Delcroix and J. P. Ameryckx (1983). "In vivo metabolism of human apoprotein A-I-phospholipid complexes. Comparison with human high density lipoprotein-apoprotein A-I metabolism." Clin Chim Acta **131**(3): 201-10.

Mann, C. J., F. T. Yen, A. M. Grant and B. E. Bihain (1991). "Mechanism of plasma cholesteryl ester transfer in hypertriglyceridemia." J Clin Invest **88**(6): 2059-66.

Manninen, V., L. Tenkanen and P. Koskinen (1992). "Joint effects of serum triglyceride and LDL cholesterol and HDL cholesterol concentrations on coronary heart disease risk in the Helsinki Heart Study: implications for treatment." Circulation **85**: 37-45.

Markwell, M. A. K. (1982). "A new solid-state reagent to iodinate proteins: conditions for the efficient labeling of antiserum." Anal Biochem **125**: 427-432.

Marques-Vidal, P., M. Jauhiainen, J. Metso and C. Ehnholm (1997). "Transformation of high density lipoprotein 2 particles by hepatic lipase and phospholipid transfer protein." Atherosclerosis **133**: 87-95.

Martin, M. J., S. B. Hulley, W. S. Browner, L. H. Kuller and D. Wentworth (1986). "Serum cholesterol, blood pressure, and mortality: implications from a cohort of 361,662 men." Lancet **2**(8513): 933-6.

Martinez, L. O., S. Jacquet, J. P. Esteve, C. Rolland, E. Cabezon, E. Champagne, T. Pineau, V. Georgeaud, J. E. Walker, F. Terce, X. Collet, B. Perret and R. Barbaras (2003). "Ectopic beta-chain of ATP synthase is an apolipoprotein A-I receptor in hepatic HDL endocytosis." Nature **421**(6918): 75-9.

Matz, C. and A. Jonas (1982). "Micellar complexes of human apolipoprotein A-I with phosphatidylcholines and cholesterol prepared from cholate-lipid dispersions." J Biol Chem **257**: 4535-4540.

Miller, J. C., R. K. Barth, P. H. Shaw, R. W. Elliott and N. D. Hastie (1983). "Identification of a cDNA clone for mouse apoprotein A-1 (apo A-1) and its use in characterization of apo A-1 mRNA expression in liver and small intestine." Proc Natl Acad Sci U S A **80**(6): 1511-5.

Miller, N. E. (1987). "Associations of high-density lipoprotein subclasses and apolipoproteins with ischemic heart disease and coronary atherosclerosis." Am Heart J **113**(2 Pt 2): 589-597.

Miyazaki, A., A. Rahim, T. Ohta, Y. Morino and S. Horiuchi (1992). "High density lipoprotein mediates selective reduction in cholesteryl esters from macrophage foam cells." Biochim Biophys Acta **1126**: 73-80.

Moestrup, S. K. and R. Kozyraki (2000). "Cubilin, a high-density lipoprotein receptor." Curr Opin Lipidol **11**(2): 133-40.

Nanjee, M., J. Doran, P. Lerch and N. Miller (1999). "Acute effects of intravenous infusion of apoAI/ phosphatidylcholine discs on plasma lipoproteins in humans." Arterioscler Thromb Vasc Biol **19**: 979-989.

Neaton, J. D., H. Blackburn, D. Jacobs, L. Kuller, D. J. Lee, R. Sherwin, J. Shih, J. Stamler and D. Wentworth (1992). "Serum cholesterol level and mortality findings for men screened in the Multiple Risk Factor Intervention Trial. Multiple Risk Factor Intervention Trial Research Group." Arch Intern Med **152**(7): 1490-500.

Newnham, H. H. and P. J. Barter (1990). "Synergistic effects of lipid transfers and hepatic lipase in the formation of very small high-density lipoproteins during incubation of human plasma." Biochim Biophys Acta **1044**(1): 57-64.

Nichols, A. V., P. J. Blanche, E. L. Gong, V. G. Shore and T. M. Forte (1985). "Molecular pathways in the transformation of model discoidal lipoprotein complexes induced by lecithin:cholesterol acyltransferase." Biochim Biophys Acta **834**: 285-300.

Nilsson-Ehle, P., A. S. Garfinkel and M. C. Schotz (1980). "Lipolytic enzymes and plasma lipoprotein metabolism." Annu Rev Biochem **49**: 667-93.

Nissen, S. E., T. Tsunoda, E. M. Tuzcu, P. Schoenhagen, C. J. Cooper, M. Yasin, G. M. Eaton, M. A. Lauer, W. S. Sheldon, C. L. Grines, S. Halpern, T. Crowe, J. C. Blankenship and R. Kerensky (2003). "Effect of recombinant ApoA-I Milano on coronary atherosclerosis in patients with acute coronary syndromes: a randomized controlled trial." Jama **290**(17): 2292-300.

Okamoto, H., F. Yonemori, K. Wakitani, T. Minowa, K. Maeda and H. Shinkai (2000). "A cholesteryl ester transfer protein inhibitor attenuates atherosclerosis in rabbits." Nature **406**(6792): 203-7.

Oram, J. F. and A. M. Vaughan (2000). "ABCA1-mediated transport of cellular cholesterol and phospholipids to HDL apolipoproteins." Curr Opin Lipidol **11**: 253-260.

Oram, J. F. and S. Yokoyama (1996). "Apolipoprotein-mediated removal of cellular cholesterol and phospholipids." J Lipid Res **37**(12): 2473-91.

Osborne, J. (1986). "Delipidation of plasma lipoproteins." Methods Enzymol **128**: 213-222.

Osono, Y., L. A. Woollett, K. R. Marotti, G. W. Melchior and J. M. Dietschy (1996). "Centripetal cholesterol flux from extrahepatic organs to the liver is independent of the concentration of high density lipoprotein-cholesterol in plasma." Proc Natl Acad Sci U S A **93**(9): 4114-9.

Packman, C. H., S. I. Rosenfeld and J. P. Leddy (1985). "High-density lipoprotein and its apolipoproteins inhibit cytolytic activity of complement. Studies on the nature of inhibitory moiety." Biochim Biophys Acta **812**(1): 107-15.

Parthasarathy, S., J. Barnett and L. G. Fong (1990). "High-density lipoprotein inhibits the oxidative modification of low-density lipoprotein." Biochim Biophys Acta **1044**(2): 275-83.

Phillips, J. C., W. Wriggers, Z. Li, A. Jonas and K. Schulten (1997). "Predicting the structure of apolipoprotein A-I in reconstituted high-density lipoprotein disks." Biophys J **73**(5): 2337-46.

Picardo, M., J. B. Massey, D. E. Kuhn, A. M. Gotto, Jr., S. H. Gianturco and H. J. Pownall (1986). "Partially reassembled high density lipoproteins. Effects on cholesterol flux, synthesis, and esterification in normal human skin fibroblasts." Arteriosclerosis **6**(4): 434-41.

Piran, U. and R. J. Morin (1979). "A rapid radioassay procedure for plasma lecithin-cholesterol acyltransferase." J Lipid Res **20**(8): 1040-3.

Poston, R. N., D. O. Haskard, J. R. Coucher, N. P. Gall and R. R. Johnson-Tidey (1992). "Expression of intercellular adhesion molecule-1 in atherosclerotic plaques." Am J Pathol **140**(3): 665-73.

Pruzanski, W., E. Stefanski, F. C. de Beer, M. C. de Beer, P. Vadas, A. Ravandi and A. Kuksis (1998). "Lipoproteins are substrates for human secretory group IIA phospholipase A2: preferential hydrolysis of acute phase HDL." J Lipid Res **39**(11): 2150-60.

Quarfordt, S. H. and H. L. Hilderman (1970). "Quantitation of the in vitro free cholesterol exchange of human red cells and lipoproteins." J Lipid Res **11**(6): 528-35.

Rader, D. J., K. Ikewaki, N. Duverger, H. Schmidt, H. Pritchard, J. Frohlich, M. Clerc, M. F. Dumon, T. Fairwell, L. Zech and et al. (1994). "Markedly accelerated catabolism of apolipoprotein A-II (ApoA-II) and high density lipoproteins containing ApoA-II in classic lecithin: cholesterol acyltransferase deficiency and fish-eye disease." J Clin Invest **93**(1): 321-30.

Rainwater, D. L., D. W. Andres, A. L. Ford, F. Lowe, P. J. Blanche and R. M. Krauss (1992). "Production of polyacrylamide gradient gels for the electrophoretic resolution of lipoproteins." J Lipid Res **33**(12): 1876-81.

Rajaram, O. V. and P. J. Barter (1985). "Reactivity of human lipoproteins with purified lecithin: cholesterol acyltransferase during incubations in vitro." Biochim Biophys Acta **835**(1): 41-9.

Redgrave, T. G. and D. M. Small (1979). "Quantitation of the transfer of surface phospholipid of chylomicrons to the high density lipoprotein fraction during the catabolism of chylomicrons in the rat." J Clin Invest **64**(1): 162-71.

Report from the Committee of Principal Investigators (1978). "A co-operative trial in the primary prevention of ischaemic heart disease using clofibrate. Report from the Committee of Principal Investigators." Br Heart J **40**: 1069-1118.

Riepponen, P., J. Marniemi and T. Rautaoja (1987). "Immunoturbidimetric determination of apolipoproteins A-1 and B in serum." Scand J Clin Lab Invest **47**(7): 739-44.

Rittershaus, C., D. Miller, L. Thomas, M. Picard, C. Honan, C. Emmett, C. Pettey, H. Aldari, R. Hammond, D. Beattie and H. Marsh (2000). "Vaccine induced antibodies inhibit CETP activity in vivo and reduce aortic lesions in rabbit model of atherosclerosis." Arterioscler Thromb Vasc Biol **20**: 2106-2112.

Robins, S. J. and J. M. Fasulo (1997). "High density lipoproteins, but not other lipoproteins, provide a vehicle for sterol transport to bile." J Clin Invest **99**(3): 380-4.

Robins, S. J., J. M. Fasulo, R. Leduc and G. M. Patton (1989). "The transport of lipoprotein cholesterol into bile: a reassessment of kinetic studies in the experimental animal." Biochim Biophys Acta **1004**(3): 327-31.

Robinson, S. F. and S. H. Quarfordt (1978). "Chylomicron apoprotein alteration after plasma exposure." Biochim Biophys Acta **541**(4): 492-503.

Rogers, D. P., L. M. Roberts, J. Lebowitz, G. Datta, G. M. Anantharamaiah, J. A. Engler and C. G. Brouillette (1998a). "The lipid-free structure of apolipoprotein A-I: effects of amino-terminal deletions." Biochemistry **37**(34): 11714-25.

Rogers, D. P., L. M. Roberts, J. Lebowitz, J. A. Engler and C. G. Brouillette (1998b). "Structural analysis of apolipoprotein A-I: effects of amino- and carboxy-terminal deletions on the lipid-free structure." Biochemistry **37**(3): 945-55.

Rose, H. (1972). "Origin of cholesterol esters in the blood of cholesterol-fed rabbits: relative contributions of serum lecithin-cholesterol acyltransferase and hepatic ester synthesis." Biochim Biophys Acta **260**: 312-316.

Rosenfeld, S. I., C. H. Packman and J. P. Leddy (1983). "Inhibition of the lytic action of cell-bound terminal complement components by human high density lipoproteins and apoproteins." J Clin Invest **71**(4): 795-808.

Rosenson, R. S. and G. D. Lowe (1998). "Effects of lipids and lipoproteins on thrombosis and rheology." Atherosclerosis **140**(2): 271-80.

Rosseneu, M., P. Van Tornout, H. Caster and M. J. Lievens (1981). "Displacement of apo A-I by A-II in lipid-apoprotein complexes and human HDL." Acta Cardiol Suppl **27**: 11-29.

Rothblat, G. H., M. de la Llera-Moya, V. Atger, G. Kellner-Weibel, D. L. Williams and M. C. Phillips (1999). "Cell cholesterol efflux: integration of old and new observations provides new insights." J Lipid Res **40**(5): 781-96.

Rubin, E. M., R. M. Krauss, E. A. Spangler, J. G. Verstuyft and S. M. Clift (1991). "Inhibition of early atherogenesis in transgenic mice by human apolipoprotein AI." Nature **353**(6341): 265-7.

Rubins, H., S. Robins, D. Collins, C. Fye, J. Anderson, M. Elam, F. Faas, E. Linares, E. Schaefer, G. Schectman, T. Wilt and J. Wites (1999). "Gemfibrozil for the secondary prevention of coronary heart disease in men with low levels of high-density lipoprotein cholesterol." N Engl J Med **341**: 410-418.

Rust, S., M. Rosier, H. Funke, J. Real, Z. Amoura, J. C. Piette, J. F. Deleuze, H. B. Brewer, N. Duverger, P. Denefle and G. Assmann (1999). "Tangier disease is caused by mutations in the gene encoding ATP-binding cassette transporter 1." Nat Genet **22**(4): 352-5.

Rye, K.-A. (1990). "Interaction of apolipoprotein A-II with recombinant HDL containing egg phosphatidylcholine, unesterified cholesterol and apolipoprotein A-I." biochim Biophys Acta **1042**: 227-236.

Rye, K.-A. and M. Duong (2000). "Influence of phospholipid depletion on the size, structure, and remodeling of reconstituted high density lipoproteins." J Lipid Res **41**: 1640-1650.

Rye, K. A. and P. J. Barter (1994). "The influence of apolipoproteins on the structure and function of spheroidal, reconstituted high density lipoproteins." J Biol Chem **269**(14): 10298-10303.

Rye, K. A., M. A. Clay and P. J. Barter (1999). "Remodelling of high density lipoproteins by plasma factors." Atherosclerosis **145**(2): 227-238.

Rye, K. A., K. H. Garrety and P. J. Barter (1992). "Changes in the size of reconstituted high density lipoproteins during incubation with cholesteryl ester transfer protein: the role of apolipoproteins." J Lipid Res **33**(2): 215-24.

Rye, K. A., K. H. Garrety and P. J. Barter (1993). "Preparation and characterization of spheroidal, reconstituted high-density lipoproteins with apolipoprotein A-I only or with apolipoprotein A-I and A-II." Biochim Biophys Acta **1167**(3): 316-25.

Rye, K. A., N. J. Hime and P. J. Barter (1995). "The influence of cholesteryl ester transfer protein on the composition, size, and structure of spherical, reconstituted high density lipoproteins." J Biol Chem **270**(1): 189-196.

Rye, K. A., N. J. Hime and P. J. Barter (1997). "Evidence that cholesteryl ester transfer protein-mediated reductions in reconstituted high density lipoprotein size involve particle fusion." J Biol Chem **272**(7): 3953-3960.

Rye, K. A., M. Jauhiainen, P. J. Barter and C. Ehnholm (1998). "Triglyceride-enrichment of high density lipoproteins enhances their remodelling by phospholipid transfer protein." J Lipid Res **39**(3): 613-22.

Sacks, F., M. Pfeffer and L. Moye (1996). "The effect of pravastatin on coronary events after myocardial infarction in patients with average cholesterol levels." N Engl J Med **335**: 1001-1009.

Sanan, D. A., J. Fan, A. Bensadoun and J. M. Taylor (1997). "Hepatic lipase is abundant on both hepatocyte and endothelial cell surfaces in the liver." J Lipid Res **38**(5): 1002-13.

Santamarina-Fojo, S., C. Haudenschild and M. Amar (1998). "The role of hepatic lipase in lipoprotein metabolism and atherosclerosis." Curr Opin Lipidol **9**(3): 211-9.

Santamarina-Fojo, S., G. Lambert, J. Hoeg and H. Brewer Jr (2000). "Lecithin: cholesterol acyltransferase: role in lipid metabolism, reverse cholesterol transport and atherosclerosis." Curr Opin Lipidol **11**: 267-276.

Scandinavian Simvastatin Survival Study Group (1994). "Randomised trial of cholesterol lowering in 4444 patients with coronary heart disease: the Scandinavian Simvastatin Survival Study (4S)." Lancet **344**: 1383-1389.

Schaefer, E. J., C. B. Blum, R. I. Levy, L. L. Jenkins, P. Alaupovic, D. M. Foster and H. B. Brewer, Jr. (1978). "Metabolism of high-density lipoprotein apolipoproteins in Tangier disease." N Engl J Med **299**(17): 905-10.

Schwartz, C. C., M. Berman, Z. R. Vlahcevic, L. G. Halloran, D. H. Gregory and L. Swell (1978a). "Multicompartmental analysis of cholesterol metabolism in man. Characterization of the hepatic bile acid and biliary cholesterol precursor sites." J Clin Invest **61**(2): 408-23.

Schwartz, C. C., M. Berman, Z. R. Vlahcevic and L. Swell (1982). "Multicompartmental analysis of cholesterol metabolism in man. Quantitative kinetic evaluation of precursor sources and turnover of high density lipoprotein cholesterol esters." J Clin Invest **70**(4): 863-76.

Schwartz, C. C., L. G. Halloran, Z. R. Vlahcevic, D. H. Gregory and L. Swell (1978b). "Preferential utilization of free cholesterol from high-density lipoproteins for biliary cholesterol secretion in man." Science **200**(4337): 62-4.

Schwartz, C. C., Z. R. Vlahcevic, L. G. Halloran and L. Swell (1981). "An in vivo evaluation in man of the transfer of esterified cholesterol between lipoproteins and into the liver and bile." Biochim Biophys Acta **663**(1): 143-62.

Schwartz, C. C., L. A. Zech, J. M. VandenBroek and P. S. Cooper (1993). "Cholesterol kinetics in subjects with bile fistula. Positive relationship between size of the bile acid precursor pool and bile acid synthetic rate." J Clin Invest **91**(3): 923-38.

Scobey, M. W., F. L. Johnson and L. L. Rudel (1989). "Delivery of high-density lipoprotein free and esterified cholesterol to bile by the perfused monkey liver." Am J Physiol **257**(4 Pt 1): G644-52.

Segrest, J. P., D. W. Garber, C. G. Brouillette, S. C. Harvey and G. M. Anantharamaiah (1994). "The amphipathic alpha helix: a multifunctional structural motif in plasma apolipoproteins." Adv Protein Chem **45**: 303-69.

Segrest, J. P., R. L. Jackson, J. D. Morrisett and A. M. Gotto, Jr. (1974). "A molecular theory of lipid-protein interactions in the plasma lipoproteins." FEBS Lett **38**(3): 247-58.

Segrest, J. P., M. K. Jones, A. E. Klon, C. J. Sheldahl, M. Hellinger, H. De Loof and S. C. Harvey (1999). "A detailed molecular belt model for apolipoprotein A-I in discoidal high density lipoprotein." J Biol Chem **274**(45): 31755-8.

Segrest, J. P., L. Li, G. M. Anantharamaiah, S. C. Harvey, K. N. Liadaki and V. Zannis (2000). "Structure and function of apolipoprotein A-I and high-density lipoprotein." Curr Opin Lipidol **11**(2): 105-15.

Serfaty-Lacrosniere, C., F. Civeira, A. Lanzberg, P. Isaia, J. Berg, E. D. Janus, M. P. Smith, Jr., P. H. Pritchard, J. Frohlich, R. S. Lees and et al. (1994). "Homozygous Tangier disease and cardiovascular disease." Atherosclerosis **107**(1): 85-98.

Shah, P. K., J. Nilsson, S. Kaul, M. C. Fishbein, H. Ageland, A. Hamsten, J. Johansson, F. Karpe and B. Cercek (1998). "Effects of recombinant apolipoprotein A-I(Milano) on aortic atherosclerosis in apolipoprotein E-deficient mice." Circulation **97**(8): 780-5.

Shepherd, J., S. M. Cobbe, I. Ford, C. G. Isles, A. R. Lorimer, P. W. MacFarlane, J. H. McKillop and C. J. Packard (1995). "Prevention of coronary heart disease with pravastatin in men with hypercholesterolemia. West of Scotland Coronary Prevention Study Group." N Engl J Med **333**(20): 1301-7.

Shimokawa, H. (1999). "Primary endothelial dysfunction: atherosclerosis." J Mol Cell Cardiol **31**(1): 23-37.

Silver, D. L., N. Wang, X. Xiao and A. R. Tall (2001). "High density lipoprotein (HDL) particle uptake mediated by scavenger receptor class B type 1 results in selective

sorting of HDL cholesterol from protein and polarized cholesterol secretion." J Biol Chem **276**(27): 25287-93.

Smit, J. J., A. H. Schinkel, R. P. Oude Elferink, A. K. Groen, E. Wagenaar, L. van Deemter, C. A. Mol, R. Ottenhoff, N. M. van der Lugt, M. A. van Roon and et al. (1993). "Homozygous disruption of the murine mdr2 P-glycoprotein gene leads to a complete absence of phospholipid from bile and to liver disease." Cell **75**(3): 451-62.

Soma, M. R., E. Donetti, C. Parolini, C. R. Sirtori, R. Fumagalli and G. Franceschini (1995). "Recombinant apolipoprotein A-IMilano dimer inhibits carotid intimal thickening induced by perivascular manipulation in rabbits." Circ Res **76**(3): 405-11.

Spady, D. K., D. M. Kearney and H. H. Hobbs (1999). "Polyunsaturated fatty acids up-regulate hepatic scavenger receptor B1 (SR-BI) expression and HDL cholesteryl ester uptake in the hamster." J Lipid Res **40**(8): 1384-94.

Spady, D. K., L. A. Woollett, R. S. Meidell and H. H. Hobbs (1998). "Kinetic characteristics and regulation of HDL cholesteryl ester and apolipoprotein transport in the apoA-I-/- mouse." J Lipid Res **39**(7): 1483-92.

Sparks, D. L. and M. C. Phillips (1992). "Quantitative measurement of lipoprotein surface charge by agarose gel electrophoresis." J Lipid Res **33**(1): 123-30.

Speidel, M. T., F. M. Booyse, A. Abrams, M. A. Moore and B. H. Chung (1990). "Lipolyzed hypertriglyceridemic serum and triglyceride-rich lipoprotein cause lipid accumulation in and are cytotoxic to cultured human endothelial cells. High density lipoproteins inhibit this cytotoxicity." Thromb Res **58**(3): 251-64.

Spieker, L. E., I. Sudano, D. Hurlimann, P. G. Lerch, M. G. Lang, C. Binggeli, R. Corti, F. Ruschitzka, T. F. Luscher and G. Noll (2002). "High-density lipoprotein restores endothelial function in hypercholesterolemic men." Circulation **105**(12): 1399-402.

Stamler, J., D. Wentworth and J. D. Neaton (1986). "Is relationship between serum cholesterol and risk of premature death from coronary heart disease continuous and graded? Findings in 356,222 primary screenees of the Multiple Risk Factor Intervention Trial (MRFIT)." Jama **256**(20): 2823-8.

Stein, O., J. Vanderhoek and Y. Stein (1976). "Cholesterol content and sterol synthesis in human skin fibroblasts and rat aortic smooth muscle cells exposed to lipoprotein-depleted serum and high density apolipoprotein/phospholipid mixtures." Biochim Biophys Acta **431**(2): 347-58.

Stein, Y., Y. Dabach, G. Hollander, G. Halperin and O. Stein (1983). "Metabolism of HDL-cholesteryl ester in the rat, studied with a nonhydrolyzable analog, cholesteryl linoleyl ether." Biochim Biophys Acta **752**(1): 98-105.

Subbaiah, P. V., C. H. Chen, J. D. Bagdade and J. J. Albers (1985). "Substrate specificity of plasma lysolecithin acyltransferase and the molecular species of lecithin formed by the reaction." J Biol Chem **260**(9): 5308-14.

Suc, I., I. Escargueil-Blanc, M. Troly, R. Salvayre and A. Negre-Salvayre (1997). "HDL and ApoA prevent cell death of endothelial cells induced by oxidized LDL." Arterioscler Thromb Vasc Biol **17**(10): 2158-66.

Sugano, M. and N. Makino (1996). "Changes in plasma lipoprotein cholesterol levels by antisense oligodeoxynucleotides against cholesteryl ester transfer protein in cholesterol-fed rabbits." J Biol Chem **271**: 19080-19083.

Sugano, M., N. Makino, S. Sawada, S. Otsuka, M. Watanabe, H. Okamoto, M. Kamada and A. Mizushima (1998). "Effect of antisense oligonucleotides against cholesteryl ester transfer protein on the development of atherosclerosis in cholesterol-fed rabbits." J Biol Chem **273**: 5033-5036.

Sugano, M., K. Tsuchida and N. Makino (2000). "High-density lipoproteins protect endothelial cells from tumor necrosis factor-alpha-induced apoptosis." Biochem Biophys Res Commun **272**(3): 872-6.

Sugatani, J., M. Miwa, Y. Komiyama and S. Ito (1996). "High-density lipoprotein inhibits the synthesis of platelet-activating factor in human vascular endothelial cells." J Lipid Mediat Cell Signal **13**(1): 73-88.

Sultan, F., D. Lagrange, H. Jansen and S. Griglio (1990). "Inhibition of hepatic lipase activity impairs chylomicron remnant-removal in rats." Biochim Biophys Acta **1042**: 150-152.

Takahashi, S., Y. Kawarabayasi, T. Nakai, J. Sakai and T. Yamamoto (1992). "Rabbit very low density lipoprotein receptor: a low density lipoprotein receptor-like protein with distinct ligand specificity." Proc Natl Acad Sci U S A **89**(19): 9252-6.

Tall, A. (1993). "Plasma cholesteryl ester transfer protein." J Lipid Res **34**: 1255-1274.

Tall, A., X. Jiang, Y. Luo and D. Silver (2000). "1999 George Lyman Duff Memorial Lecture: lipid transfer protein, HDL metabolism and atherogenesis." Arterioscler Thromb Vasc Biol **20**: 1185-1188.

Tall, A. R., C. B. Blum, G. P. Forester and C. A. Nelson (1982). "Changes in the distribution and composition of plasma high density lipoproteins after ingestion of fat." J Biol Chem **257**(1): 198-207.

Tam, S. P. and W. C. Breckenridge (1983). "Apolipoprotein and lipid distribution between vesicles and HDL-like particles formed during lipolysis of human very low density lipoproteins by perfused rat heart." J Lipid Res **24**(10): 1343-57.

Tamagaki, T., S. Sawada, H. Imamura, Y. Tada, S. Yamasaki, A. Toratani, T. Sato, S. Komatsu, N. Akamatsu, M. Yamagami, K. Kobayashi, K. Kato, K. Yamamoto, K. Shirai, K. Yamada, T. Higaki, K. Nakagawa, H. Tsuji and M. Nakagawa (1996). "Effects of high-density lipoproteins on intracellular pH and proliferation of human vascular endothelial cells." Atherosclerosis **123**(1-2): 73-82.

The BIP Study Group (2000). "Secondary prevention by raising HDL cholesterol and reducing triglycerides in patients with coronary artery disease: The Bezafibrate Infarction Prevention (BIP) Study." Circulation **102**: 21-27.

Thompson, G. R. (1983). Plasma lipids and lipoproteins, and the hyperlipoproteinaemias. Biochemical Aspects of Human Disease. R. Elkeles and A. Tavill. Oxford, Blackwell**:** 85-123.

Thuren, T. (2000). "Hepatic lipase and HDL metabolism." Curr Opin Lipidol **11**(3): 277-83.

Tietge, U., C. Maugeais, S. Lund-Katz, D. Grass, F. deBeer and D. Rader (2002). "Human secretory phospholipase A2 mediates decreased plasma levels of HDL cholesterol and apoA-I in response to inflammation in human apoA-I transgenic mice." Arterioscler Thromb Vasc Biol **22**: 1213-1218.

Tilly-Kiesi, M., A. H. Lichtenstein, J. Joven, E. Vilella, M. C. Cheung, W. V. Carrasco, J. M. Ordovas, G. Dolnikowski and E. J. Schaefer (1997). "Impact of gender on the metabolism of apolipoprotein A-I in HDL subclasses LpAI and LpAI:AII in older subjects." Arterioscler Thromb Vasc Biol **17**(12): 3513-3518.

Tollefson, J., A. Liu and J. J. Albers (1988). "Regulation of plasma lipid transfer by the high-density lipoproteins." Am J Physiol **255**: E894-E902.

van der Wal, A. C., P. K. Das, A. J. Tigges and A. E. Becker (1992). "Adhesion molecules on the endothelium and mononuclear cells in human atherosclerotic lesions." Am J Pathol **141**(6): 1427-33.

Vanhoutte, P. M. (1997). "Endothelial dysfunction and atherosclerosis." Eur Heart J **18 Suppl E**: E19-29.

Varban, M. L., F. Rinninger, N. Wang, V. Fairchild-Huntress, J. H. Dunmore, Q. Fang, M. L. Gosselin, K. L. Dixon, J. D. Deeds, S. L. Acton, A. R. Tall and D. Huszar (1998). "Targeted mutation reveals a central role for SR-BI in hepatic selective uptake of high density lipoprotein cholesterol." Proc Natl Acad Sci U S A **95**(8): 4619-24.

Velez-Carrasco, W., A. H. Lichtenstein, Z. Li, G. G. Dolnikowski, S. Lamon-Fava, F. K. Welty and E. J. Schaefer (2000). "Apolipoprotein A-I and A-II kinetic parameters as assessed by endogenous labeling with [(2)H(3)]leucine in middle-aged and elderly men and women." Arterioscler Thromb Vasc Biol **20**(3): 801-806.

Verkade, H. J., R. J. Vonk and F. Kuipers (1995). "New insights into the mechanism of bile acid-induced biliary lipid secretion." Hepatology **21**(4): 1174-89.

von Eckardstein, A., Y. Huang, S. Wu, H. Funke, G. Noseda and G. Assmann (1995). "Reverse cholesterol transport in plasma of patients with different forms of familial HDL deficiency." Arterioscler Thromb Vasc Biol **15**(5): 691-703.

von Eckardstein, A., M. Jauhiainen, Y. Huang, J. Metso, C. Langer, P. Pussinen, S. Wu, C. Ehnholm and G. Assmann (1996). "Phospholipid transfer protein mediated conversion of high density lipoproteins generates pre beta 1-HDL." Biochim Biophys Acta **1301**(3): 255-262.

von Eckardstein, A., J. R. Nofer and G. Assmann (2001). "High density lipoproteins and arteriosclerosis. Role of cholesterol efflux and reverse cholesterol transport." Arterioscler Thromb Vasc Biol **21**(1): 13-27.

Waite, M., T. Y. Thuren, R. W. Wilcox, P. J. Sisson and G. L. Kucera (1991). "Purification and substrate specificity of rat hepatic lipase." Methods Enzymol **197**: 331-9.

Wang, C. S., H. Bass, R. Whitmer and W. J. McConathy (1993). "Effects of albumin and apolipoprotein C-II on the acyl-chain specificity of lipoprotein lipase catalysis." J Lipid Res **34**(12): 2091-8.

Wang, N., D. L. Silver, P. Costet and A. R. Tall (2000). "Specific binding of ApoA-I, enhanced cholesterol efflux, and altered plasma membrane morphology in cells expressing ABC1." J Biol Chem **275**(42): 33053-8.

Wang, N., W. Weng, J. L. Breslow and A. R. Tall (1996). "Scavenger receptor BI (SR-BI) is up-regulated in adrenal gland in apolipoprotein A-I and hepatic lipase knock-out mice as a response to depletion of cholesterol stores. In vivo evidence that SR-BI is a functional high density lipoprotein receptor under feedback control." J Biol Chem **271**(35): 21001-4.

Warren, R. J., D. L. Ebert, A. Mitchell and P. J. Barter (1991). "Rabbit hepatic lipase cDNA sequence: low activity is associated with low messenger RNA levels." J Lipid Res **32**(8): 1333-9.

Watson, A. D., J. A. Berliner, S. Y. Hama, B. N. La Du, K. F. Faull, A. M. Fogelman and M. Navab (1995). "Protective effect of high density lipoprotein associated paraoxonase. Inhibition of the biological activity of minimally oxidized low density lipoprotein." J Clin Invest **96**(6): 2882-91.

Weisweiler, P. (1987). "Isolation and quantitation of apolipoproteins A-I and A-II from human high-density lipoproteins by fast-protein liquid chromatography." Clin Chim Acta **169**: 249-254.

Werns, S. W., J. A. Walton, H. H. Hsia, E. G. Nabel, M. L. Sanz and B. Pitt (1989). "Evidence of endothelial dysfunction in angiographically normal coronary arteries of patients with coronary artery disease." Circulation **79**(2): 287-91.

Whitlock, M., T. Swenson, R. Ramakrishnan, M. Leonard, Y. Marcel, R. Milne and A. Tall (1989). "Monoclonal antibody inhibition of cholesteryl ester transfer protein activity

in the rabbit: effects on lipoprotein composition and high density lipoprotein cholesteryl ester metaboism." J Clin Invest **84**: 129-137.

Wiebusch, H., P. Cullen, J. S. Owen, D. Collins, P. S. Sharp, H. Funke and G. Assmann (1995). "Deficiency of lecithin:cholesterol acyltransferase due to compound heterozygosity of two novel mutations (Gly33Arg and 30 bp ins) in the LCAT gene." Hum Mol Genet **4**(1): 143-5.

Woollett, L. A. and D. K. Spady (1997). "Kinetic parameters for high density lipoprotein apoprotein AI and cholesteryl ester transport in the hamster." J Clin Invest **99**(7): 1704-13.

Xia, P., J. R. Gamble, K. A. Rye, L. Wang, C. S. Hii, P. Cockerill, Y. Khew-Goodall, A. G. Bert, P. J. Barter and M. A. Vadas (1998). "Tumor necrosis factor-alpha induces adhesion molecule expression through the sphingosine kinase pathway." Proc Natl Acad Sci U S A **95**(24): 14196-201.

Xia, P., M. A. Vadas, K. A. Rye, P. J. Barter and J. R. Gamble (1999). "High density lipoproteins (HDL) interrupt the sphingosine kinase signaling pathway. A possible mechanism for protection against atherosclerosis by HDL." J Biol Chem **274**(46): 33143-7.

Xiang, S. Q., K. Cianflone, D. Kalant and A. D. Sniderman (1999). "Differential binding of triglyceride-rich lipoproteins to lipoprotein lipase." J Lipid Res **40**(9): 1655-63.

Yen, F. T., C. J. Mann, L. M. Guermani, N. F. Hannouche, N. Hubert, C. A. Hornick, V. N. Bordeau, G. Agnani and B. E. Bihain (1994). "Identification of a lipolysis-stimulated receptor that is distinct from the LDL receptor and the LDL receptor-related protein." Biochemistry **33**(5): 1172-80.

Zechner, R. (1997). "The tissue-specific expression of lipoprotein lipase: implications for energy and lipoprotein metabolism." Curr Opin Lipidol **8**(2): 77-88.

Zhao, Y. and Y. L. Marcel (1996). "Serum albumin is a significant intermediate in cholesterol transfer between cells and lipoproteins." Biochemistry **35**(22): 7174-80.

Zhao, Y., D. L. Sparks and Y. L. Marcel (1996). "Effect of the apolipoprotein A-I and surface lipid composition of reconstituted discoidal HDL on cholesterol efflux from cultured fibroblasts." Biochemistry **35**(51): 16510-8.