INFORMATION TO USERS

This manuscript has been reproduced from the microfilm master. UMI films the text directly from the original or copy submitted. Thus, some thesis and dissertation copies are in typewriter face, while others may be from any type of computer printer.

The quality of this reproduction is dependent upon the quality of the copy submitted. Broken or indistinct print, colored or poor quality illustrations and photographs, print bleedthrough, substandard margins, and improper alignment can adversely affect reproduction.

In the unlikely event that the author did not send UMI a complete manuscript and there are missing pages, these will be noted. Also, if unauthorized copyright material had to be removed, a note will indicate the deletion.

Oversize materials (e.g., maps, drawings, charts) are reproduced by sectioning the original, beginning at the upper left-hand corner and continuing from left to right in equal sections with small overlaps. Each original is also photographed in one exposure and is included in reduced form at the back of the book.

Photographs included in the original manuscript have been reproduced xerographically in this copy. Higher quality 6" x 9" black and white photographic prints are available for any photographs or illustrations appearing in this copy for an additional charge. Contact UMI directly to order.

UMI®

Bell & Howell Information and Learning
300 North Zeeb Road, Ann Arbor, MI 48106-1346 USA
800-521-0600

Molecular Defects and Structure/Function Studies of Proteins in Reverse Cholesterol Transport

by

Evelyn Mei-lin Teh

Submitted in partial fulfillment of the requirements for the

Doctor of Philosophy Degree

at

Dalhousie University

Halifax, Nova Scotia

October, 1999

DALHOUSIE UNIVERSITY

FACULTY OF GRADUATE STUDIES

The undersigned hereby certify that they have read and recommend to the Faculty of Graduate Studies for acceptance a thesis entitled "Molecular Defects and Structure/ Function Studies of Proteins in Reverse Cholesterol Transport"

by Evelyn Mei-Lin Teh

in partial fulfillment of the requirements for the degree of Doctor of Philosophy.

Dated: October 7, 1999

External Examiner

Research Supervisor

Examining Committee

DALHOUSIE UNIVERSITY

DATE: October 13, 1999

AUTHOR: Evelyn Mei-lin Teh

TITLE: "Molecular Defects and Structure/function Studies of Proteins in Reverse Cholesterol Transport"

DEPARTMENT: Biochemistry and Molecular Biology

DEGREE: Ph. D. CONVOCATION: May YEAR: 2000

Permission is herewith granted to Dalhousie University to circulate and to have copied for non-commercial purposes, at its discretion, the above title upon the request of individuals or institutions.

SIGNATURE OF AUTHOR

Table of Contents

List of Figures

List of Tables

Abstract

One of the many mechanisms by which cholesterol homeostasis is maintained is through the reverse cholesterol transport (RCT) pathway. This pathway transports excess cholesterol from the periphery to the liver for bile acid synthesis and excretion, and maintains high density lipoprotein (HDL) concentration and composition. In humans, the enzyme lecithin:cholesterol acyltransferase (LCAT) and cholesteryl ester transfer protein (CETP) are key components of the RCT pathway. HDL particles play a major role in RCT by acting as the initial acceptors of cholesterol from the peripheral cells. Epidemiological studies have shown an inverse correlation between HDL concentration and premature coronary heart disease (CHD). Hence, genetic defects of the enzymes involved in RCT can greatly affect the composition and concentration of HDL particles.

LCAT and CETP deficiency syndromes are rare genetic disorders of HDL metabolism. Of particular interest are LCAT deficient patients with severe HDL deficiency who show no signs of premature CHD. In contrast, CETP-deficient patients have elevated HDL cholesterol levels but are not particularly protected from premature CHD. These findings are inconsistent with the general concept that increased HDL cholesterol is a negative risk factor for atherosclerosis. Novel mutations have been characterized in the human CETP and the LCAT genes. CETP mutations are prevalent mainly in Japanese populations. The CETP gene mutation in this study is the first in a Caucasian North American subject; this offers the opportunity to study CETP deficiency in a different genetic and environmental background. The CETP mutation was identified as a single nucleotide substitution (**C**GA → **T**GA) in exon 9 that resulted in the premature truncation of the CETP protein. A novel mutation was also identified in the LCAT gene from a French patient. The lack of LCAT activity is the result of a di-nucleotide deletion (TG) in exon 4 of the LCAT gene that leads to the premature truncation of the LCAT protein.

Structural and functional studies were performed to evaluate the significance of the N-terminal 80 residues of the mature LCAT protein. A rat-human chimera (R80H) containing the first 80 residues of the rat sequence attached to residues 81-416 of human LCAT was constructed and transiently expressed in COS-7 cells. A functional R80H chimeric protein was expressed, showing activity with both rHDL substrates (α-LCAT activity) and purified human LDL (β-LCAT activity). Furthermore, the α-LCAT activity was enhanced in the presence of human apoA-I. The β-LCAT activity also appeared to be enhanced. The 5 amino acid differences between the rat and human LCAT most likely disrupted the region involved in interfacial interaction of the enzyme such that the activity with the different substrates were enhanced. In order to test the hypothesis that the far N-terminus of LCAT may anchor the putative "lid" to the enzyme, sequential N-terminal deletion mutants were constructed. The results showed that the first 10 residues were not essential for secretion but are required for enzymatic function as both the α- and β-LCAT activity were abolished.

List of Abbreviations

BCA	Bicinchoninic acid
BSA	Bovine serum albumin
CE	Cholesteryl ester
CER	Cholesterol esterification rate
CETP	Cholesteryl ester transfer protein
CHD	Coronary heart disease
FED	Fish eye disease
FLD	Familial LCAT deficiency
HDL	High density lipoprotein
HL	Hepatic lipase
IDL	Intermediate density lipoprotein
LCAT	Lecithin:cholesterol acyltransferase
LDL	Low density lipoprotein
LPL	Lipoprotein lipase
Lp-X	Lipoprotein X
LRP	Low density lipoprotein receptor-related protein
PAGE	Polyacrylamide gel electrophoresis
POPC	1-palmitoyl-2-oleyl phosphatidylcholine
RCT	Reverse cholesterol transport
rHDL	Reconstituted high density lipoprotein
TG	Triglycerides
TLC	Thin layer chromatography

UC	Unesterified cholesterol
VLDL	Very low density lipoprotein
cDNA	Complementary DNA
DNA	Deoxyribonucleic acid
dATP	deoxyadenylate 5'-triphosphate
dGTP	deoxyguanylate 5'-triphosphate
dCTP	deoxycytidylate 5'-triphosphate
dTTP	deoxythymidylate 5'-triphosphate
A	Adenine
G	Guanine
T	Thymine
C	Cytosine
kb	Kilobase
kDa	Kilodalton
mRNA	Messenger RNA
PCR	Polymerase chain reaction
RFLP	Restriction fragment length polymorphism
RNA	Ribonucleic acid
Ala	Alanine (A)
Arg	Arginine (R)
Asn	Asparagine (N)
Asp	Aspartate (D)
Cys	Cysteine (C)

Gln	Glutamine (Q)
Glu	Glutamate (E)
Gly	Glycine (G)
His	Histidine (H)
Ile	Isoleucine (I)
Leu	Leucine (L)
Lys	Lysine (K)
Met	Methionine (M)
Phe	Phenylalanine (F)
Pro	Proline (P)
Ser	Serine (S)
Thr	Threonine (T)
Trp	Tryptophan (W)
Tyr	Tyrosine (Y)
Val	Valine (V)

Acknowledgements

I would like to begin by thanking my parents and family for all their confidence and support. I never would have made it this far in life without them. I would also like to thank Brent for always being here for me, for his love, patience, and constant encouragement. For always taking care of me especially during the past year, and for their generosity, I wish to extend my sincerest gratitude to Ernest and Gloria Conrad. I wish to acknowledge my good friends, Naomi, Joelius, Jason, and John for their friendship. I thank Joelius and Naomi for being there during the more difficult times.

I would also like to thank the members of my supervisory committee, Dr. Carl Breckenridge, Dr. Catherine Lazier, and Dr. Catherine Too for their guidance throughout my studies. I am especially grateful to Dr. Too and Dr. Roger McLeod for their helpful discussions and technical advice. I thank Dr. Lazier for believing in me and for her support throughout my undergraduate and graduate career.

I am grateful to Dr. Meng Tan, Dr. Pascale Benlian and to their patients for providing the blood samples for my research. I would like to thank Dr. John Parks and Dr. Jeffrey Chisholm at the Bowman Gray School of Medicine for the reagents for the LCAT experiments, and Dr. Ross Milne at the University of Ottawa for providing the monoclonal antibodies. For providing the antibodies for the CETP studies, I thank Dr. Ronald Clark and Dr. Mark Bamberger of Pfizer Inc. I also wish to thank Dr. Jeffrey Chisholm for the helpful discussions.

I am especially indebted to Nicola Vickaryous for her excellent technical assistance during the last few months of my research. I wish you every success in your graduate studies. I thank Rose Abraham and Jackie Froom for their technical assistance.

I wish to thank Bruce Stewart for his excellent technical assistance and patience throughout my four years in the Dolphin lab, and to all the people in the Department of Biochemistry and Molecular Biology for taking the time to help me with work and non-work related matters.

Lastly, for his much needed advice, guidance, and generosity, I would like to thank my supervisor, Dr. Peter Dolphin.

This research was supported by a grant from the Medical Research Council of Canada to Dr. Peter Dolphin.

1. Introduction

A) Overview

i) Lipoprotein and apolipoprotein structure and function

The transport of insoluble lipids in the aqueous environment of the plasma from their site of synthesis to their site of catabolism or deposition is achieved through the formation of macromolecular complexes of protein and lipid referred to as lipoproteins. All mature plasma lipoproteins have a similar structure consisting of a neutral lipid core surrounded by a surface monolayer of amphipathic lipids and a protein component termed the apolipoproteins. Lipoprotein particles are highly heterogeneous and are classified based on their particle size, electrophoretic mobility, and more traditionally their buoyant density. The four main classes of lipoprotein particles are the chylomicrons, very low density lipoprotein (VLDL), low density lipoprotein (LDL), and high density lipoprotein (HDL) (Table 1.1).

Apolipoproteins (apo) are the protein component of lipoproteins and some are essential for the structural integrity (e.g., apoB-100 and apoB-48). Other apolipoproteins act as lipolytic enzyme cofactors (e.g., apoC-II, apoA-I) or mediate receptor binding (e.g., apoE, apoB-100), and thus determine the metabolic fate of the lipoprotein with which they are associated (Dolphin, 1985). The 8 apolipoproteins that have a major role as metabolic programmers of lipoprotein metabolism are apoA-I, A-II, A-IV, B, C-I, C-II, C-III, and E (Table 1.2).

Structural studies of apolipoproteins have allowed the classification of the proteins into three major groups based on their characteristics. The first group consists of

Table 1.1 Classification and composition of plasma lipoproteins

Lipoprotein Species	Density Range**	Particle Diameter*	Electrophoretic Mobility in agarose	Associated apolipoproteins (circulating)	Protein	FC	PL	CE	TG
	g/ml	*nm*			*Weight % **				
Chylomicron	$d < 1.006$	75-1200	origin	B-48, Cs, E	2	< 1	5	< 1	93
Chylo remnants	$d < 1.019$		pre-β	B-48, E	8	10	4	4	74
VLDL	$d < 1.006$	30-80	pre-β	B-100,Cs, E	10	6	15	14	53
IDL	$1.006 < d < 1.019$	25-35	pre-β	B-100, E	18	7	22	23	31
LDL	$1.019 < d < 1.063$	18-25	β	B-100	25	9	21	42	4
HDL	$1.063 < d < 1.21$	5-12	α	A-I, A-II, A-IV, E, Cs, D	-	-	-	-	-
HDL_1	$1.050 < d < 1.063$		α	A-I, A-II, A-IV, Cs	32	8	36	23	2
HDL_2	$1.063 < d < 1.12$		α	A-I, A-II, A-IV, Cs	43	5	30	20	2
HDL_3	$1.12 < d < 1.21$		α	A-I, A-II, A-IV,	55	3	25	16	1
Pre-β_1 (VHDL)†	$1.21 < d < 1.25$	5-6	pre-β	A-I	47.5	7.6	44	0	0
Pre-β_2 (VHDL)	$1.21 < d < 1.25$		pre-β	A-I	21.5	5.7	73.8	0	0

Lipoprotein Species	Density Range**	Particle Diameter*	Electrophoretic Mobility in agarose	Associated apolipoproteins (circulating)	Protein	FC	PL	CE	TG
	g/ml	*nm*			*Weight % **				
Pre-β_3 (VHDL)	$1.21 < d < 1.25$		pre-β	A-I, LCAT, CETP and D[+]					
VHDL	$1.21 < d < 1.25$		γ	E					
Lp(a)	$1.027 < d < 1.10$	25-30	pre-β	-					

Data compiled from several sources: * from Davis and Vance (1996), ** from Fielding and Fielding (1996), [+] Francone et al. (1989), and [†] Castro and Fielding (1988). All the VHDL data was obtained from Castro and Fielding, (1988). IDL: intermediate density lipoprotein; LDL: low density lipoprotein; HDL: high density lipoprotein; VHDL: very high density lipoprotein. The chylomicrons are responsible for the transport of exogenous triglycerides while the chylomicron remnants are involved in the transport of exogenous cholesterol. Endogenous triglycerides are transported in the VLDL and the IDL moves the endogenous cholesterol. LDL transports cholesterol to all tissues and HDL particles mediate reverse cholesterol transport (Beisiegel, 1998).

soluble apolipoproteins that are members of a multigene family (Karathanasis, 1985; Lusis et al., 1986). There are seven such apolipoproteins (apoA-I, A-II, A-IV, C-I, C-II, C-III and E) that are characterized by repeats of amphipathic helices essential for phospholipid binding (Segrest et al., 1974; Li et al. 1988). The helices allow for interaction of the apolipoprotein with enzymes and receptors through clustering of charged residues (Lys, Arg) that interact with opposite charges on the enzyme or receptor protein. These apolipoproteins also have very little tertiary structure giving them the flexibility to bind onto lipoprotein surfaces and change in conformation as the lipoprotein load and unload its lipid core.

The second group of proteins contain the apo(a), and the insoluble protein apoB-100 and it's various truncated forms. In humans, the intestine secretes apoB-48 which is the product of the unique mRNA editing process that changes the glutamine codon at amino acid residue 2153 to a STOP codon leaving the apoB-48 with only 48% of the full-length apoB-100 (Kane, 1983; Chan, 1992). The apoB protein is not exchangeable between lipoproteins because of it's repeating hydrophobic proline-rich sequence that forms β-sheets embedded in the monolayer of the lipoprotein particle. The apoB-100 is responsible for the removal of LDL particles from the circulation by acting as a ligand for the LDL receptor. This is a well-defined receptor-mediated endocytosis pathway described by Brown and Goldstein (1986). In contrast, apoB-48 lacks the LDL receptor binding domain located towards the C-terminus of apoB-100.

Apolipoproteins classified in the third group are those that have little or no structural homology with either group one or group two apolipoproteins. These include apoD, H, I, and J.

Table 1.2: Major human plasma apolipoproteins

Apolipoproteins	Lipoprotein distribution	Function(s)	Site of synthesis
ApoA-I	All HDL classes	Cholesterol efflux; LCAT activation; HDL receptor ligand	Liver, intestine
ApoA-II	HDL_1, HDL_2, HDL_3	Inhibition of apoA-I activity; HDL receptor ligand	Liver
ApoB-48	Chylomicrons	Chylomicron secretion	Intestine
ApoB-100	VLDL, IDL, LDL	VLDL secretion; LDL receptor ligand	Liver
ApoC-I	VLDL, HDLs	LCAT activation; inhibits the hepatic uptake of TG-rich particles	Liver
ApoC-II	VLDL, HDLs	Activates LPL; LCAT; activation; inhibits the hepatic uptake of TG-rich particles	Liver
ApoC-III	VLDL, HDLs	Inhibits apoC-II activity; inhibits the uptake of TG-rich particles	Liver
ApoE	VLDL, IDL, HDL_1	Cholesterol efflux; LDL and LRP receptor ligand;	Ubiquitous (majority in liver, macrophages and steroidogenic tissues)

The major apolipoproteins and their functions in lipoprotein metabolism. Modified from Fielding and Fielding, (1996), and von Eckardstein et al. (1994).

ii.) Lipoprotein metabolism

The delivery of dietary cholesterol and triglycerides to the periphery for energy and storage is facilitated by chylomicrons that are synthesized by the intestine. Dietary fatty acids are absorbed into the enterocyte after hydrolysis by pancreatic lipase (E.C. 3.1.1.3) and re-esterified to triglycerides for chylomicron synthesis (Figure 1.1). Newly secreted chylomicrons are enriched in triglycerides and contain apoB48, an essential structural apolipoprotein, apoA-I, phospholipids and cholesterol. Once circulating in the plasma, chylomicrons acquire apoC-II, a necessary cofactor for lipoprotein lipase (LPL; E.C. 3.1.1.34), apoC-I, apoC-III and apoE in exchange for apoA-I from circulating HDL. LPL is bound to proteoglycans on the surface of endothelial cells of those tissues utilizing free fatty acid for energy production (oxidation or storage), and is the most important enzyme in plasma triglyceride metabolism. In addition, LPL can also bind to triglyceride-rich lipoproteins. With the help of apoC-II, triglycerides in the chylomicrons are hydrolyzed. Chylomicron remnants are generated after sufficient hydrolysis and the LPL is released with the remnants to the liver where apoE and LPL first bind to the proteoglycans and then to the receptors (Beisiegel et al., 1991; Willnow et al., 1994; Biesiegel, 1998). The receptors that are responsible for the uptake of the remnants are the LDL receptor and the LDL-receptor-related protein (LRP), both of which recognize apoE.

In VLDL, exogenous and endogenous lipids are assembled with a single molecule of apoB-100, and acquire the C apolipoproteins and apoE while in the plasma. In the circulation, VLDL are catabolized similarly to chylomicrons and the resultant remnants,

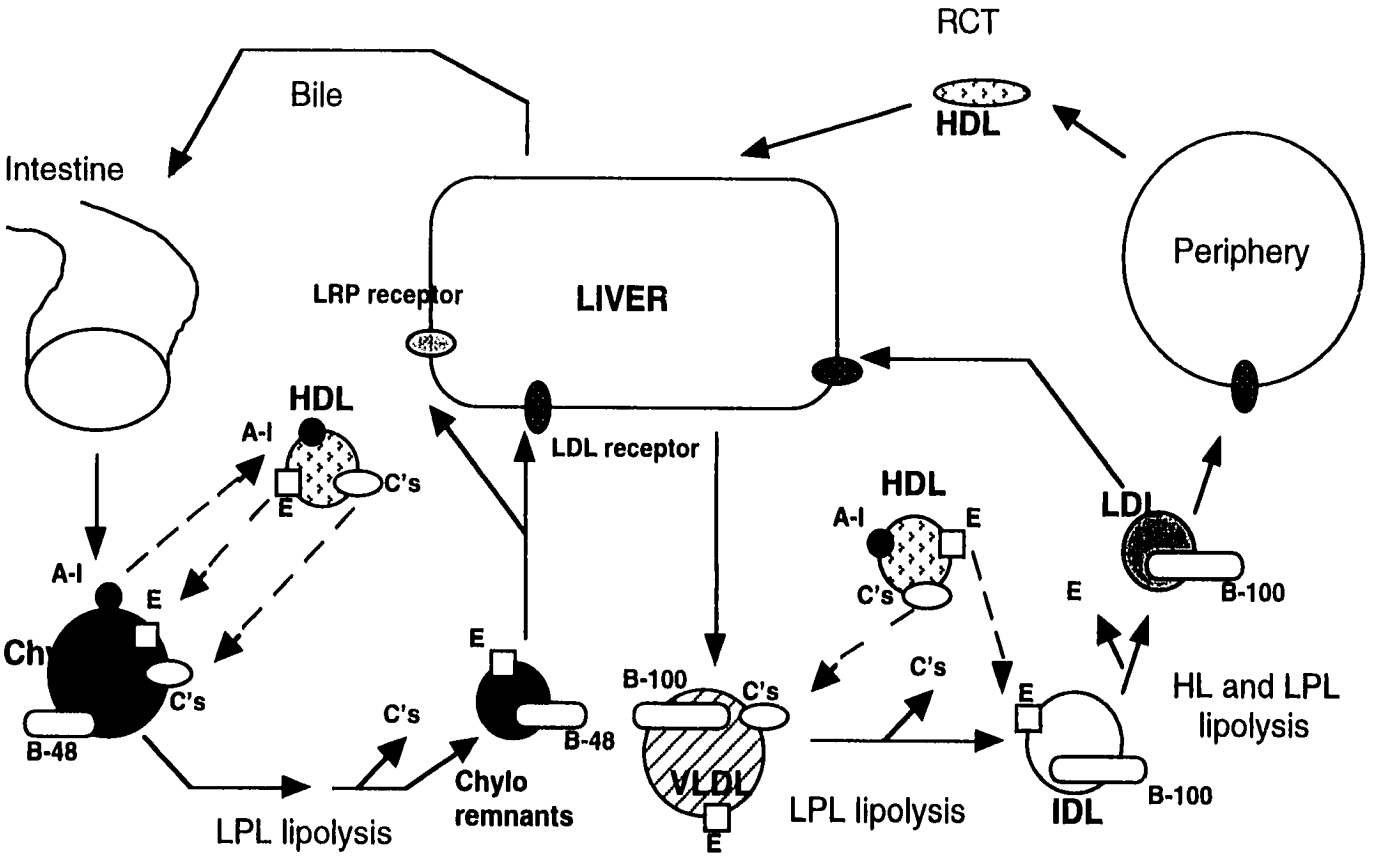


Figure 1.1: Lipoprotein metabolism. Chylomicrons (chylos) are secreted by the intestine with apoB-48 and apoA-I. Once in the circulation the exchangeable lipoproteins are redistributed as depicted by the dashed lines. The acquisition of apoC-II from circulating HDL by the chylos allows for triglyceride (TG) hydrolysis in the chylos by LPL. The resulting remnants are cleared by the LDL and/or LRP receptor. VLDL are secreted by the liver and contains apoB-100. VLDL remnants are cleared similarly to the chylos and can also be further catabolize to IDL and then LDL. The LDL is cleared from the plasma via the LDL receptor. LPL: lipoprotein lipase; RCT: reverse cholesterol transport; LRP: LDL receptor related protein. Adapted from Schnider, 1996.

the IDL, can either be taken up by liver via the LDL or LRP receptor or further catabolized to LDL. The majority of the IDL are catabolized to LDL through hydrolysis of triglycerides by both the LPL and hepatic lipase (HL; E.C. 3.1.1.34). As the VLDL is depleted of its triglycerides through lipolysis, it sequentially loses the C apolipoproteins and then apoE. ApoB-100 is then in the right conformation to act as a ligand for the LDL receptor. During their circulation, the triglycerides in chylomicrons, VLDL, and LDL are also exchanged for cholesteryl ester in HDL via the action of cholesteryl ester transfer protein (CETP).

LDL-cholesterol plays an important regulatory role in cellular cholesterol homeostasis. The uptake of LDL into cells is followed by lysosomal degradation of the particles and subsequent release of free cholesterol into the cytosol (Brown and Goldstein, 1986). The regulatory events associated with receptor-mediated endocytosis of the LDL-derived cholesterol include: 1) down-regulation of 3-hydroxy-3-methylglutaryl-CoA (HMG-CoA) synthase and HMG-CoA reductase, two key enzymes in cellular cholesterol biosynthesis; 2) the activation of acyl-CoA cholesterol acyltransferase (ACAT), a cholesterol-esterifying enzyme that allows the cell to store excess cholesterol in the form of cholesteryl ester droplets, and 3) the down-regulation of LDL receptor synthesis to prevent further cellular cholesterol uptake. Thus, expression of the LDL receptor can regulate plasma LDL levels.

Atherogenic lipoproteins can be derived from both the endogenous and exogenous pathways. It is now well established that elevated levels of circulating cholesterol-rich apoB-containing particles such as β-VLDL (VLDL remnants), IDL, and LDL are atherogenic. Evidence for this comes from the genetic diseases in human lipoprotein

metabolism and extensive studies in animal models, which have provided us with some insights on lipoprotein metabolism and atherogenesis. Prolonged circulation of the LDL particles particularly the small, dense LDL lead to their oxidative modification and subsequent internalization by macrophages by the means of the scavenger receptors on the cell surface (Khoo et al., 1988; Steinberg, 1997; Chapman et al., 1998). Cholesterol deposition in the macrophages leads to foam cell formation which contributes to the onset and progression of atherosclerosis.

B) Reverse Cholesterol Transport

i.) Overview

As the peripheral cells obtain their cholesterol from the uptake of VLDL and LDL and by local synthesis, an excess of cholesterol in non-steroidogenic tissues has to be removed and returned to the liver where it can be re-utilized or converted to bile acids for secretion. With the exception of the liver, intestine, and steroidogenic tissues, peripheral cells are unable to metabolize cholesterol. Glomset (1968) was the first to recognize the reverse cholesterol transport (RCT) pathway, which involves the active transport of cholesterol in its esterified form from the periphery to the liver. The esterification of cholesterol in the plasma is catalyzed by lecithin:cholesterol acyltransferase (LCAT; E.C. 2.3.1.43) and mediated by HDL.

RCT is a very specific and well-regulated process that is governed by the efflux, esterification, transfer, and clearance of cholesterol (Fielding and Fielding, 1996) (Figure 1.2). The first step in RCT involves the efflux of cholesterol, in its unesterified form, from the plasma membrane to acceptor HDL particles. Although all HDL can mediate

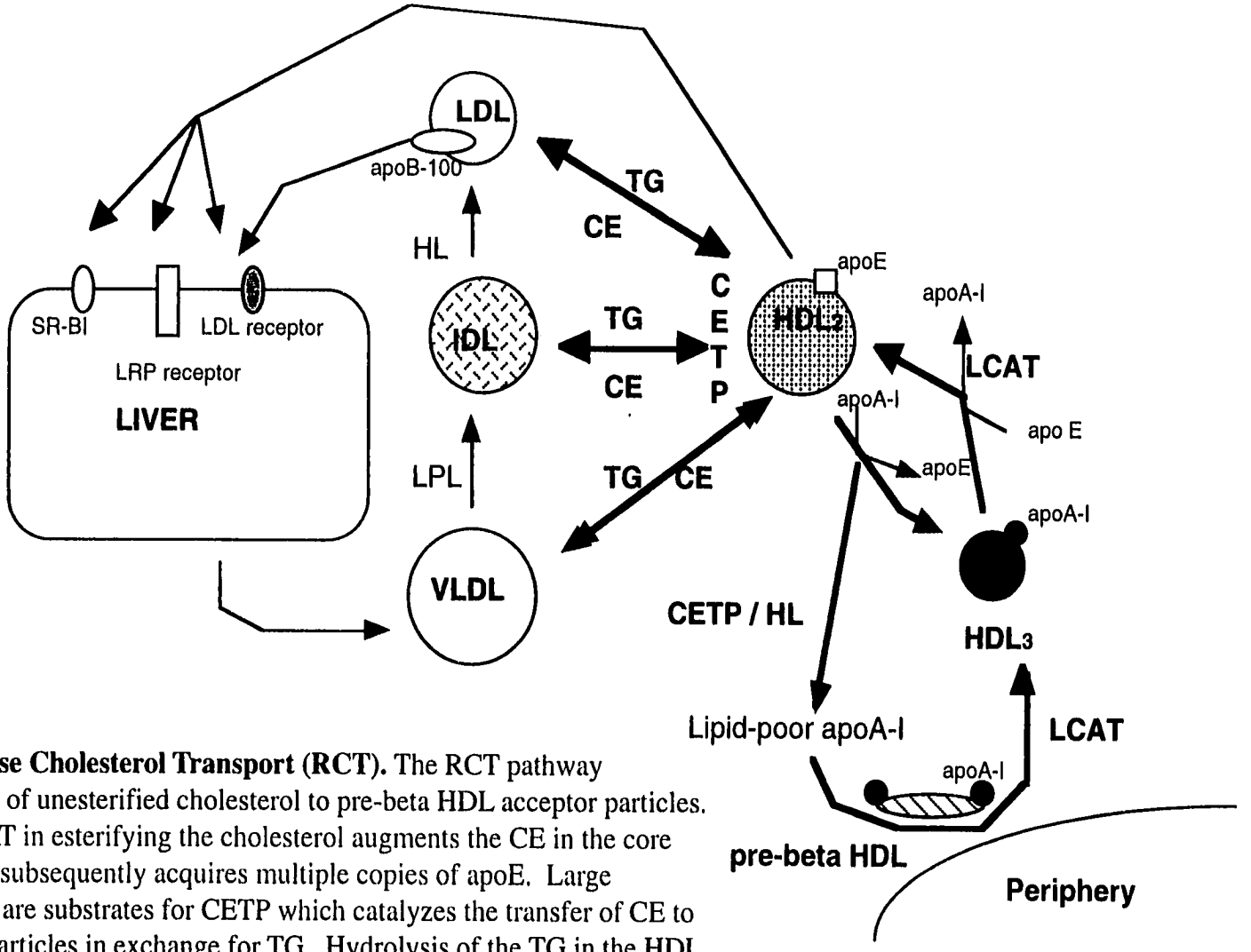


Figure 1.2: Reverse Cholesterol Transport (RCT). The RCT pathway involves the efflux of unesterified cholesterol to pre-beta HDL acceptor particles. The action of LCAT in esterifying the cholesterol augments the CE in the core of the HDL which subsequently acquires multiple copies of apoE. Large CE-enriched HDL are substrates for CETP which catalyzes the transfer of CE to apoB-containing particles in exchange for TG. Hydrolysis of the TG in the HDL regenerates pre-beta particles that mediate cholesterol efflux. Alternatively, the HDL-CE can be removed via the LDL receptor, the LRP receptor or selectively removed by the SR-BI. The bolded arrows depicts RCT in species with CETP. CE:cholesteryl esters; TG:triglycerides; LRP: LDL-related protein.

cholesterol efflux, a particular sub-fraction of HDL with pre-β mobility on agarose gels has been shown to have the highest efficiency in removing cholesterol from peripheral tissues (Castro and Fielding, 1988). These pre-β particles are associated with apoA-I, the main cofactor for LCAT. ApoA-II, apoA-IV, and apoE can also act as initial acceptors of cellular cholesterol. The efflux of cholesterol involves the desorption of individual cholesterol molecules through a concentration gradient provided by the esterification of cholesterol by LCAT, which moves cholesteryl ester into the core of acceptor particles allowing the surface phospholipids to acquire more cholesterol (Rothblat and Phillips, 1982). Lipid-poor apolipoproteins can interact with the plasma membrane and remove cholesterol and phospholipid. The reversible interaction of apoA-I with cell surface binding sites on the plasma membrane is able to stimulate the mobilization of intracellular cholesterol pools (Oram et al., 1996). Recently, the exchange of unesterified cholesterol through the binding of HDL particles to the SR-B1 (scavenger receptor Class I Type B) receptor has been demonstrated (Jian et al., 1998)

The SR-B1 receptor, a member of the scavenger receptor family, is expressed in the liver and steroidogenic tissues and has broad ligand specificity (Acton et al., 1996). It binds HDL with high affinity (Babitt et al, 1997) but does not internalize the lipoprotein (Krieger and Herz, 1994).

As the esterification of cholesterol to cholesteryl esters by LCAT drives the lipid to the core of HDL and thereby maintains a concentration gradient resulting in a net efflux of cholesterol from cells, the HDL undergoes remodelling from its discoidal pre-β form to spherical HDL_3 and subsequently to HDL_2. Between the transition from $HDL_3 \rightarrow HDL_2$, the enlarged cholesteryl ester-rich HDL_2 acquires multiple copies of apoE.

The tissue-derived cholesteryl ester in the HDL may then undergo three different fates. The acquisition of apoE is required for the uptake of HDL through the LDL and LRP receptor in the liver. It has also been shown that cholesteryl esters can also be selectively taken up by the SR-BI receptor without the internalization or degradation of the HDL protein (Acton et al., 1996; Glass et al., 1983). Finally, cholesteryl esters in the HDL particles can be transferred to apoB-containing lipoproteins such as the VLDL and LDL in the presence of cholesteryl ester transfer protein (CETP) activity. CETP catalyses the exchange of cholesteryl ester for triglycerides from the HDL particle to the apoB-containing particles. The triglycerides transferred to HDL are subsequently hydrolyzed by HL to regenerate pre-β HDL. The cholesteryl esters transferred to apoB-containing lipoproteins are removed by their normal metabolism through the LDL and LRP receptor as described previously.

The relative importance of the three different pathways varies in different species. Since mice and rats lack CETP activity (Ha and Barter, 1982), the predominant pathway for cholesteryl ester uptake is likely through the HDL particle. In contrast, the high CETP activity in humans allows for the uptake of cholesteryl ester by the liver through the LDL receptor.

Epidemiological studies (Gordon and Rifkind, 1989) have shown that increased levels of plasma HDL have a strong inverse correlation with premature coronary heart disease (CHD). However, a precise understanding of the causal mechanism(s) underlying the inverse relationship between HDL and CHD remains elusive. The putative anti-atherogenic roles of HDL and apoA-I arise from their observed ability to stimulate the efflux of cholesterol from peripheral cells and, after esterification by plasma

LCAT, facilitate the transfer of tissue-derived cholesterol to the liver via the reverse cholesterol transport pathway (Tall, 1990). HDL also contains antioxidants such as platlet-activating factor acetylhydrolase and paraoxanase (Mackness et al., 1993; Watson et al., 1995) activity that reduces the oxidation and subsequent uptake of LDL by macrophages and vascular tissues. Since HDL are intimately involved in RCT, an increase in HDL cholesterol is thought to reflect the effectiveness of this pathway or the efficiency of the metabolism of the other atherogenic lipoproteins such as VLDL and LDL. HDL levels are determined both by environmental and genetic factors. In fact, an estimated 50% of the variance in HDL levels between individuals are determined by their genes (von Eckardstein et al., 1994). Several environmental factors that are able to influence HDL levels act indirectly through the lipases and lipid transfer proteins (Tall, 1990).

ii.) Plasma proteins involved in RCT

The plasma proteins involved in the RCT pathway are obviously important for HDL remodelling and hence, influencing HDL speciation. LCAT plays an important role in the maturation of the HDL particles from their pre-β form to the α-migrating form. In addition, LCAT is pivotal in maintaining the RCT cycle by maintaining a cholesterol concentration gradient between HDL and peripheral tissues through cholesterol esterification (Fielding and Fielding, 1995). Hence, LCAT catalyzes the first committed step of cholesterol removal in the RCT pathway.

The remodelling of HDL particles changes their sizes and morphology, and are accompanied by of the loss or gain of core neutral lipids and phospholipids. While CETP

is responsible for all of the neutral lipids transfer activity and part of the facilitated phospholipid transfer activity in human plasma (Hesler et al., 1988; Yen et al., 1989), phospholipid transfer protein (PLTP) is important for transferring the majority of the phospholipids (Tollefson et al., 1988). Not only is CETP responsible for the proper catabolism of all lipoproteins (i.e., by exchanging neutral lipids), its activity has also been shown to regulate LCAT activity in CETP transgenic mice (Francone et al., 1996). Recently, CETP was shown to act in synergism with HL to promote and enhance the selective uptake of HDL cholesteryl esters by the SR-BI receptor (Collet et al., 1999). CETP is therefore, a key regulator for the return of cholesterol to the liver.

In addition to transferring phospholipids between lipoprotein classes, PLTP also transfers phospholipids within the HDL subclasses (Jauhiainen et al., 1993). PLTP can cause the formation of larger particles through the fusion of two HDL_3 particles with the concomitant dissociation of apoA-I. The apoA-I subsequently complexes with a small amount of phospholipids to form smaller particles which have pre-β mobility on agarose gels (Marques-Vidal et al., 1997), do not contain a core, are discoidal (Forte et al., 1995) and hence, are excellent substrates for LCAT. Together with CETP, PLTP can regulate the initial step in RCT through the regeneration of acceptor particles (von Eckardstein et al., 1996). Recently, Rye et al. (1998) showed that triglycerides enhanced the remodelling of HDL particles by PLTP to produce larger and smaller HDL species suggesting a synergistic role between CETP and PLTP in the RCT pathway.

The major role of HL in the RCT pathway is in the remodelling and catabolism of HDL by hydrolysing HDL triglycerides and phospholipids (Stahnke et al., 1987). More specifically, the large triglyceride-rich HDL_2 particles that are produced by CETP

activity become preferential substrates for HL (Patsch et al, 1984). About 90% of the human HL is expressed in the liver and is located on the sinusoidal epithelium where it fulfills its functions (Sanan et al., 1997). *In vitro* evidence suggests that HL may stimulate the uptake of HDL cholesteryl esters as well as unesterified cholesterol (Marques-Vidal et al., 1994) and that HL may work in concert with SR-BI to promote the selective uptake of cholesteryl esters from HDL (Ginsberg and Taskinen, 1997). As mentioned previously, Collet et al. (1999) recently showed that CETP and HL work in synergy to enhance the uptake of HDL cholesteryl ester by the SR-BI receptor.

iii.) Subclasses of HDL particles

Over the past decade, considerable effort has been put into improving the predictive value of HDL cholesterol by quantification of the HDL subclasses. Each HDL subclass is inter-convertible by the plasma proteins involved in RCT as mentioned previously, and each subclass has a distinct structure, function, and clinical significance.

HDL particles (7-13 nm; density *(d):* 1.063-1.20 mg/dl) function to remove excess lipid (cholesterol) deposited in peripheral tissues to the liver and steroidogenic tissues. Unlike the lower density particles, nascent HDL particles are discoidal rather than spherical and are composed of a phospholipid bilayer stabilized by their association with apolipoprotein A-I (apoA-I). HDL particles are formed both intracellularly (Howell and Palade, 1982) and extracellularly from the lipolysis of chylomicron and VLDL and subsequently interacting with lipid-poor apoA-I that is secreted by the liver and intestine (Winkler and Marsh, 1989). Nascent HDL particles derived from the intestine contain apoA-I and apoA-IV whereas hepatically derived nascent HDL particles consist of apoA-

I, apoA-II and apoE (Assmann et al., 1992). Within the HDL density range itself there is a mixture of HDL particles that differ in their size, hydrated density, apolipoprotein, and lipid composition (Alaupovic, 1985). These variations result in different functionality of each HDL species. For example, the lipid-poor particles are extremely effective acceptors of cholesterol and thus facilitate cholesterol removal from cells.

Besides its major role in mediating RCT, HDL particles also function as a reservoir for the loading and unloading of phospholipids and apolipoprotein (e.g., apoC's and apoE). The cholesterol on the surface of HDL can also freely exchange with cell membranes (Johnson et al., 1991) (Figure 1.1). The catabolism of HDL particles is largely dependent on their lipid and apolipoprotein content which dictates their intravascular processing. HDL particles also have naturally occurring lipophilic compounds that can protect LDL from oxidative damage (Banka, 1996). This coupled with its function in reverse cholesterol transport is thought to make HDL anti-atherogenic.

The HDL subclasses can be separated based upon density, charge, size, lipid and apolipoprotein content. Sequential precipitation or ultracentrifugation separates the HDL on the basis of their density. These methods produce two classes of HDL particles, the HDL_2 (*d:* 1.063-1.125 g/ml) and HDL_3 (*d:* 1.125-1.21 g/ml) (Patsch et al., 1980) (Table 1.1). Further resolution by size with non-denaturing polyacrylamide gradient gel electrophoresis (PAGGE) yields two HDL_2subclasses, HDL_{2a} and HDL_{2b}, and three subclasses for HDL_3, which are HDL_{3a}, HDL_{3b}, and HDL_{3c} (Blanche et al., 1981). Both the HDL_2 and HDL_3 particles are inter-convertible by enzymes (Figure 1.3). The conversion of cholesterol to cholesteryl ester through the action of LCAT and subsequent

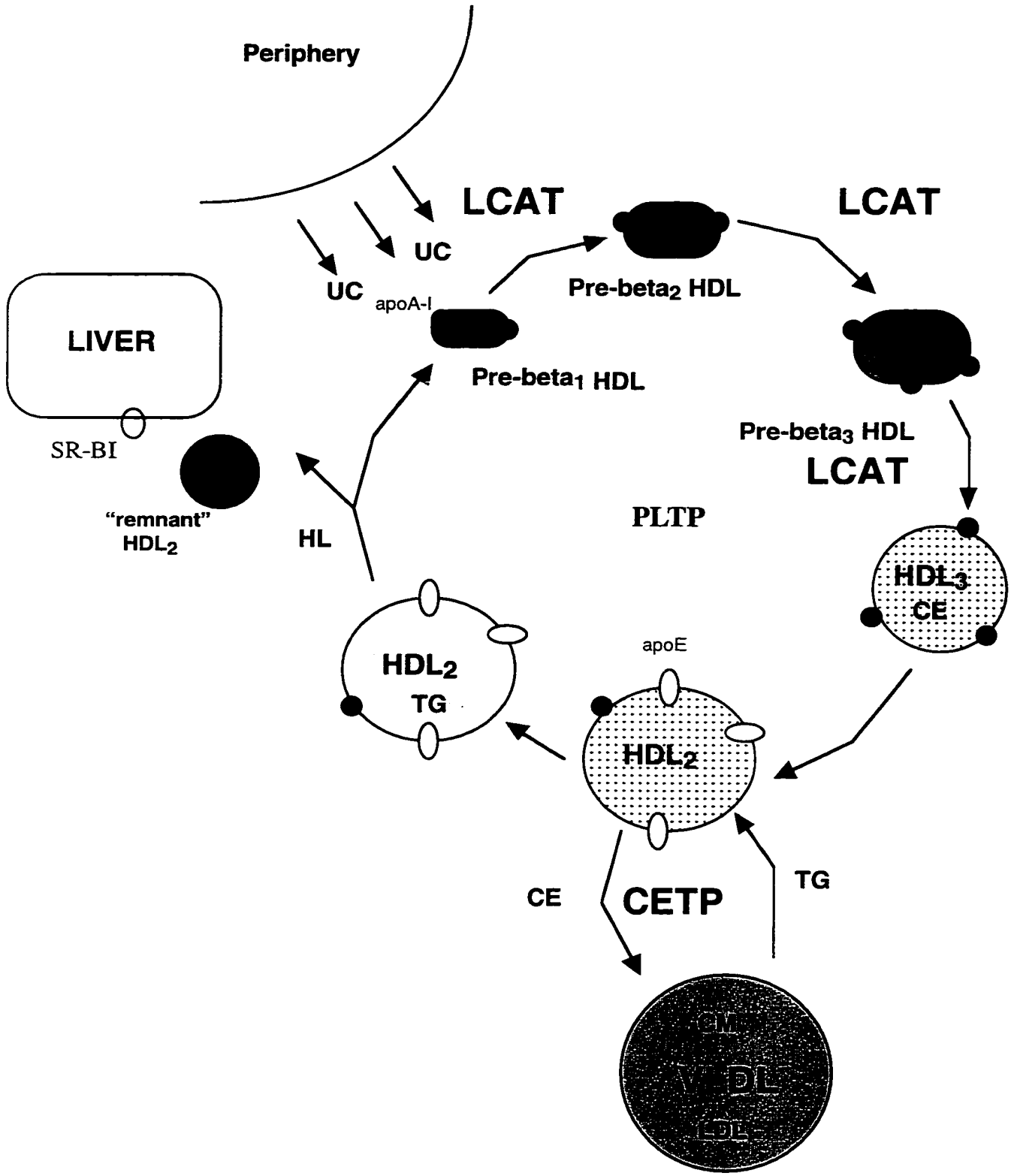


Figure 1.3: The proposed model of HDL inter-conversion. The formation of cholesteryl ester (CE) by the action of LCAT causes the enlargement of the HDL particles. The CE is exchanged for triglycerides (TG) with the apoB-containing lipoproteins under the action of CETP. The TG-rich HDL_2 undergoes lipolysis by HL forming pre-$beta_1$-HDL and remnants that are cleared by the liver. (Adapted from Barrans et al., 1996).

acquisition of phospholipids from the lipolysis of triglyceride-rich lipoproteins by PLTP converts HDL_3 → HDL_2 (Jonas, 1991; Jauhiainen et al., 1993; Tu et al., 1993). Conversely, the hetero-exchange of cholesteryl esters for triglycerides from the HDL_2 to the apoB-containing lipoproteins mediated by CETP followed by the HL-mediated hydrolysis of the HDL_2 triglycerides causes the conversion of HDL_2 → HDL_3 (Patsch et al., 1984; Tall, 1993). HDL in general have α-mobility upon agarose gel electrophoresis but at least 5% of the total plasma HDL has pre-β -mobility (Fielding and Fielding, 1980; Kunitake et al., 1985). These pre-β particles are also heterogenous and are the initial acceptor of cellular cholesterol (Castro and Fielding, 1988). Specifically, the pre-β_1 HDL particles, the smallest of the pre-β HDL, are discoidal and contain only apoA-I. In the presence of LCAT, pre-β_2 and pre-β_3 are sequentially formed from pre-β_1 (Barrans et al, 1996) (Figure 1.3). Pre-β_3 eventually becomes mature α-HDL (HDL_3 and HDL_2) through the continuous action of LCAT. Unlike pre-β particles, mature α-HDL particles contain both apo A-I and apoA-II and can accept cholesterol from LDL particles (Cheung and Wolf, 1989). Another fraction of HDL with γ-mobility that are also able to promote cellular cholesterol efflux are the apoE-only containing HDL (Castro and Fielding, 1988; Huang et al., 1994).

An additional method for separating HDL subclasses is by sequential immunoaffinity chromatography, which separates HDL subclasses according to their apolipoprotein content. The two major classes of HDL separated by this method are termed LpA-I and LpA-I:A-II. Other minor classes include LpA-IV, LpA-I:A-IV and LpE. Both the LpA-I and LpA-I:A-II particles are functionally and metabolically distinct (Fruchart et al., 1993). LpA-I represents the HDL subclass that only contains apoA-I,

whereas LpA-I:A-II particles contain both apoA-I and apoA-II. Both the liver and intestine produce LpA-I but LpA-I:A-II is secreted only by the liver (Brewer et al., 1991). The LpA-I but not LpA-I:A-II particles have the greatest antiatherogenic potential (Puchois et al., 1987; Parra et al., 1990; Dobiásová and Frohlich, 1994) because of their ability to promote cellular cholesterol efflux from Ob1771 adipocytes (Barbaras et al., 1987) and bovine aortic endothelial cells (Fruchart et al., 1993). However, studies from another group found that both LpA-I and LpA-I:A-II to be equally efficient in removing intracellular cholesterol from hepatoma, fibroblasts, endothelial, and smooth muscle cells from bovine aorta (Johnson et al., 1991; Oikawa et al.,1993). This suggests that cell type specificity may be involved in cellular cholesterol efflux. About 30-50% of the LpA-I particles are found in the HDL_{2b} sub-population (Dobiásová and Frohlich 1994). Studies have indicated that the lipids in the LpA-I and LpA-I:A-II particles are eliminated at different rates; LpA-I particles are removed by the liver and the cholesterol processed into bile more effectively than that from LpA-I:A-II particles (Pieters et al., 1993). Triglycerides in LpA-I:A-II particles are also better substrates for HL than triglycerides in LpA-I particles (Rader et al., 1991; Mowri et al., 1992).

iv) Purpose of the Study

The focus of this research is on the proteins that are involved in RCT, in particular, the LCAT enzyme and CETP. As previously mentioned the LCAT enzyme is responsible for the esterification of cholesterol to cholesteryl esters, and this reaction establishes a concentration gradient between the HDL and the periphery such that a net efflux of cholesterol can occur. Hence, the reaction catalyzed by LCAT is an important

step in the return of cholesterol to the liver. In humans, the action of CETP provides an effective pathway for the removal of the cholesteryl esters formed by LCAT. CETP transfers the cholesteryl esters in HDL to apoB-containing particles in exchange for triglycerides. This mechanism allows the cholesteryl esters to be removed and small HDL particles to be regenerated for effective cholesterol efflux.

The discovery of inborn errors in proteins of RCT provides a tool to study the role and mechanism of action of these proteins. A novel mutation was identified in the CETP gene in a Caucasian North American. In addition, a novel LCAT mutation resulting in familial LCAT deficiency will be described, along with preliminary results on the structure/function studies. These latter studies were designed to elucidate the structure and function of the far N-terminus of LCAT, and to contribute to the incomplete model of LCAT that was recently proposed (Peelman et al., 1998). Both an overview of CETP and LCAT enzyme will be presented in two subsequent chapters in which the results of the research are discussed.

2. Materials and Methods

A) Subjects

i.) CETP-deficient patient

The proband, who presented to her family physician with hypercholesterolemia, is a 57 year-old female resident of Nova Scotia with no Japanese ancestry. Her total plasma cholesterol was in excess of 9.4 mmol/l (Normal: 4.40 ± 0.57 mmol/) and she was placed on a lipid-lowering agent, Pravachol®, 40 mg once daily. Pharmacological intervention had little effect on her total plasma cholesterol and she was referred to Dr. Meng Tan (Camp Hill Medical Centre) for lipoprotein analysis which revealed an LDL-cholesterol (LDL-C) of 4.23 mmol/l (Normal: 2.90 ± 0.30 mmol/l), HDL cholesterol (HDL-C) of 4.9 mmol/l (Normal: 1.24 ± 0.28 mmol/l), and triglycerides of 1.4 mmol/l (Normal: 0.99 ± 0.28 mmol/l). She has never had a myocardial infarction and did not suffer from angina, dyspena, or claudication on exertion. She did not have diabetes mellitus nor thyroid, hepatic, or renal dysfunction and had no history of hypertension or xanthoma. Her family members tested all had normal plasma lipid values except for one sibling who was also hypercholesterolemic. Her other siblings and family members were not prepared to participate in this study. The subject's father died of heart disease of an unknown cause and her mother is presently aged 90 years. The patient was not on any lipid lowering medication for the duration of this study.

ii.) LCAT-deficient patient

The female proband, born in 1949 and residing near Paris, France consulted with Dr. Pascale Benlian with bilateral corneal opacity first detected at age 18. She received a

corneal transplant to her right eye in 1992 at age 43 and to the left eye in 1997. Analysis of her plasma showed low levels of total cholesterol (2.34 mmol/l; Normal: 4.40 ± 0.57 mmol/) with massive reductions in cholesteryl ester concentrations (0.21 mmol/l; Normal: 2.53 ± 0.10). She had low HDL cholesterol and very little HDL upon agarose gel electrophoresis of her plasma. No signs of proteinuria or renal insufficiency were present and her liver function, plasma glucose, uric acid and creatinine were all normal. She was slightly anemic (Hemoglobin: 11.6 ± 0.03 g/l; Normal: 14 ± 2 g/l). There were no symptoms of coronary heart disease as indicated by a negative stress test, ultrasound and echotomography.

B) Lipid and Lipoprotein Analysis

i.) Lipoprotein and apolipoprotein analysis

Plasma lipoproteins were isolated and subfractionated using discontinuous density gradient ultracentrifugation in an SW-41 rotor [Beckman] by a slight modification of the method of Chapman et al. (1981) as previously described by Guérin et al. (1994). When necessary, individual subfractions were dialyzed against normal saline for 24h at 4°C prior to analysis of their lipid and apolipoprotein content. Each subfraction was analyzed for lipid and protein content. The lipid components of lipoproteins, whether present in plasma or in fractions isolated by density gradient ultracentrifugation, were analyzed using either enzyme kits [Boerhringer Mannheim, Laval, Que.] for total cholesterol, unesterified cholesterol, triglycerides, and phospholipids or by the gas chromatographic total lipid profiling technique of Kuksis *et al.* (1978). Total protein was quantified by a sodium dodecyl sulfate-modified Lowry procedure (Markwell et al., 1978) using bovine

serum albumin [Sigma, St. Louis, MO] as a standard. Apolipoproteins A-I, A-II, B, C-II, C-III, and E were measured by electroimmunoassay (EIA) as previously described by Dolphin et al. (1984).

ii.) Determination of cholesteryl ester and phospholipid fatty acid composition

The lipid classes present within individual lipoprotein fractions separated by density gradient ultracentrifugation were extracted using the method of Folch et al. (1957). The lipoprotein fractions used to represent the lipoprotein classes were selected based upon the lipoprotein profile. Fractions 1 and 2 were used to represent VLDL, fractions 5-9 for LDL and fractions 12-22 for HDL in the fatty acid composition determination of the CETP deficient individual. A 200 µl aliquot from each fraction was used. The isolated phospholipids and cholesteryl esters were immediately transmethylated using the method of Morrison and Smith (1964) and the fatty acid methyl esters were analyzed by gas chromatography using a 30 m X 0.25 mm Supelco SP-2330 capillary column. Fatty acid methyl ester standards were obtained from Supelco Inc., Bellefonte, PA.

iii.) Agarose Gel Electrophoresis

Agarose gel electrophoresis was performed on lipid fractions of $d > 1.006$ g/ml as described by Johanson (1972) with a slight modification (Maguire and Breckenridge, 1975). Samples were diluted with running buffer (80 mmol/l TRIS, 24 mmol/l Tricine) and run at 300V for 45-60 min. The gel was fixed in 10% acetic acid, dried, and stained with Sudan Black (Fisher Scientific) for 15 min.

C) Determination of plasma CETP mass and activity

Plasma CETP mass was determined using the double antibody sandwich immunoradiometric assay of Clark et al. (1995). The capture monoclonal antibody was 2F8 and the detection monoclonal antibody was ^{125}I-labeled 2E7. All reagents, including CETP standards, were most generously provided by Drs. Clark and Bamberger of the Department of Cardiovascular and Metabolic Disease, Central Research Division of Pfizer Inc., Groton, CT.

Reciprocal lipid transfer mediated by CETP was measured in the proband and normal individuals after incubation of 3 ml aliquots of plasma at either 4°C or 37°C for 5h. All incubations contained 1 mM phenylmethylsulfonyl fluoride [Sigma] to inhibit plasma LCAT activity (Jauhiainen and Dolphin, 1986). After incubation, the plasma samples were fractionated into VLDL and LDL + HDL by a single ultracentrifugation at *d:* 1.006 g/ml for 17h at 40,000 rpm using a Beckman 50.3Ti rotor and L5-50 ultracentrifuge [Beckman]. The lipid mass composition of each fraction was then quantified enzymatically as described previously.

D) DNA analysis

i.) Isolation of genomic DNA

Genomic DNA was purified from white blood cells according to Miller et al. (1988) with some modifications. Whole blood was collected in disodium ethylenediaminetetraacetate dihydrate (EDTA) vacutainer tubes and centrifuged to separate the buffy coat containing white blood cells. Five volumes of ammonium chloride (NH_4Cl):Tris [0.15 M NH_4Cl; 0.017 M Tris, pH 7.6] solution that was pre-

warmed to 37°C was added and the mixture was incubated at 37°C to lyse the red blood cells. The mixture was centrifuged at 2000 rpm for 10 min in a swinging bucket TJ-6 centrifuge [Beckman] to pellet the nucleated cells. The supernatant was removed and the pelleted cells were washed 3X with 0.9% w/v sodium chloride. The white blood cells were subsequently resuspended in 10 ml of NaCl:EDTA [4.38 g NaCl. 8.9 g EDTA, pH 8.0 in 1 L of Milli-Q H_2O], and lysed with 500 µl 20% sodium dodecyl sulphate (SDS) and 100 µl Proteinase K (20 mg/ml). The mixture was incubated overnight at 56°C. DNA extraction was performed by adding 5 M NaCl to the lysate followed by manual shaking to mix and pelleting by centrifugation for 15 min at 2500 rpm. The supernatant was then transferred to another tube for DNA precipitation where 2 volumes of 100% ethanol was added and gently inverted. The DNA was washed with 75% ethanol followed by 100% ethanol and allowed to air dry. The dried pellet was resuspended in 500 µl of sterile Milli-Q H_2O and stored at -20°C.

Concentration of the DNA was determined by spectrophotometry at wavelengths of 260 nm and 280 nm (Sambrook et al., 1989). The readings at 260 nm was used to calculate the concentration of the nucleic acid using the following equation:

Concentration of DNA = (OD_{260}) * (50 µg/ml) * (dilution factor)

An OD of 1 corresponds to approximately 50 µg/ml for double stranded DNA. The ratio OD_{260}/OD_{280} provides an estimate of the purity of the nucleic acid with an optimal value of 1.8. The ratio obtained for the DNA isolated from whole blood was 1.8 (Sambrook et al., 1989).

ii.) Polymerase chain reaction (PCR), cloning, and sequencing

In vitro amplifications of genomic DNA were performed by PCR using paired intronic oligonucleotide primers. Primers used for the amplification of all 16 exons of the CETP gene are listed (Appendix I). To obtain gene fragments containing all 6 exons and exon/intron junctions of the LCAT gene, PCR amplifications of the coding regions were performed using the paired oligonucleotides previously published by Guérin et al. (1997). All reactions were performed in a total volume of 50 µl containing 200 µM dNTPs (dCTP, dATP, dGTP, dTTP), 2 µM primers, 1 X PCR buffer [Gibco BRL, Mississauga, Ont.], 1.5 mM $MgCl_2$, 1% dimethyl sulfoxide (DMSO), 1U *Thermus Aquaticus* (Taq) polymerase [Gibco BRL, Burlington, Ont.], and 0.1-0.2 µg of template DNA. All amplifications for the CETP gene consisted of 30 cycles of denaturation at 94°C for 30s, annealing at 55°C for 1 min, and extension at 74°C for 3 min followed by a 10 min extension at 70°C. The PCR amplifications of the LCAT gene consisted of 30 cycles of denaturation at 94°C for 30s, annealing at 56°C for 15s, and extension at 72°C for 30s followed by a 10 min extension at 70°C as previously published by Guérin et al. (1997).

PCR products were separated by 1.0% agarose gel electrophoresis, the gel band isolated, and purified using the Sephaglas™ BandPrep Kit [Pharmacia Amersham Biotech, Uppsala, Sweden]. Purified gene fragments were cloned using the TA cloning vector, pCR 2.1 [Invitrogen, San Diego, CA] and transformed into electro-competent *E.coli* DH5α cells. Sequencing of double stranded templates was performed by dideoxy chain termination using the T7 Sequencing™ Kit [Pharmacia Amersham Biotech]. Sequencing of both strands employed previously described primers for both the CETP

and LCAT gene and/or the M13 universal primers. In addition to having sequenced separately amplified gene fragments, direct sequencing of the PCR products was also performed on the region containing the mutation to determine and confirm the genotype of the proband.

iii.) Primer-mediated restriction enzyme analysis of the gene mutations

In the CETP gene mutation, a restriction fragment length polymorphism (RFLP) site for *Mae*III [Pharmacia Amersham Biotech] was created in the presence of a mutation by using a 26 bp-mismatched primer 5'-CTACCTTGGCCAGCGAGTGGAAGAGT-3' together with an 18-bp forward primer 5'-GACACACAGGGTCCAGCCAG-3' to yield a 168-bp PCR product (Figure 2.1). Briefly, [^{32}P]-α-dCTP [DuPont, Missisauga, Ont.] labelled PCR products from both the proband and the control subjects were digested with 1 μl of *Mae*III for 1h at 55°C as recommended by the manufacturer [Gibco BRL]. The restriction digests were resolved by 12% non-denaturing polyacrylamide gel electrophoresis (ND-PAGE) and visualized by exposure to Kodak X-ray film [Interscience Inc., Markham, Ont.]. A 138 bp and a 29 bp fragment were generated in the DNA sequences containing the mutation.

A similar approach was used for the LCAT gene mutation. The 53 bp mismatched reverse primer 5'-TCCGCAGAGACACTCACCGGGCTCCAGCCGCCAGTCATAGGGGGCGGCGTGCG-3', designed to introduce an *Fsp*1 restriction enzyme site in the region of the mutation, was used to produce a 155 bp PCR product with the previously described forward primer for exon 4 (Guérin et al., 1997) (Figure 2.2). The digest was performed for 1h at 37°C

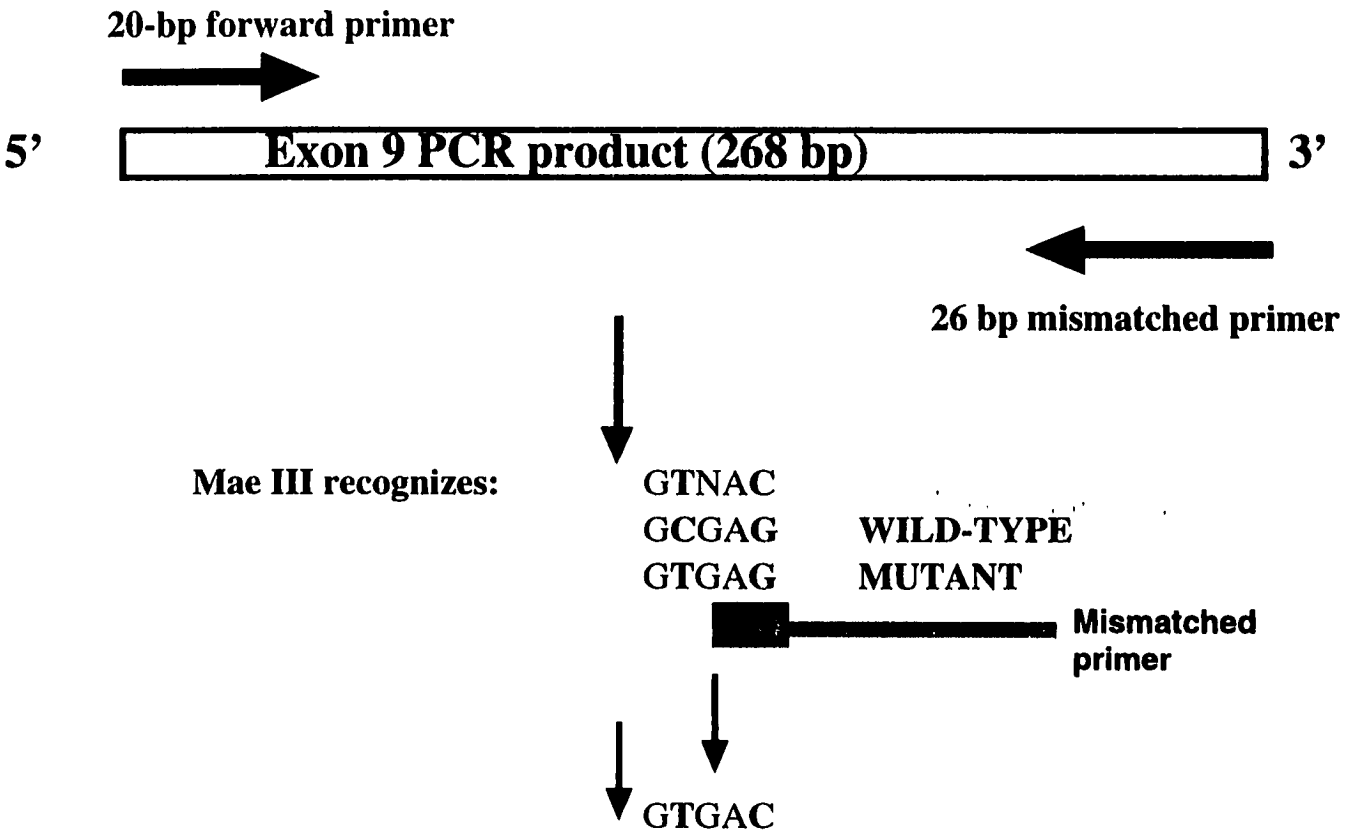


Figure 2.1: Restriction fragment length polymorphism (RFLP) design for rapid screening of the exon 9 mutation in the CETP gene. The mismtached primer changes a single nucleotide (C--> G) such that the C--> T transition in the CETP gene can be recognized by the restriction enzyme MaeIII.

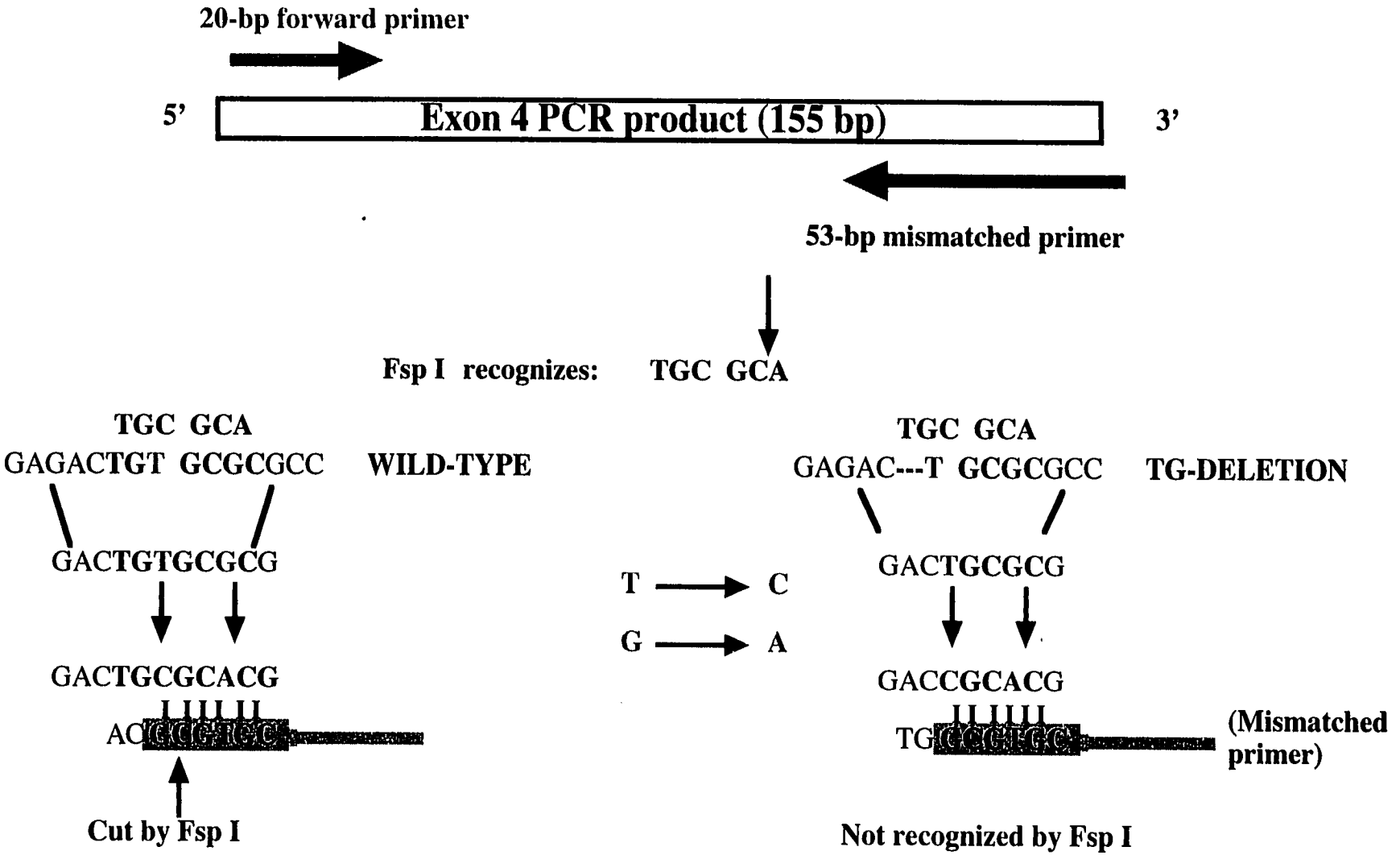


Figure 2.2: Restriction fragment length polymorphism (RFLP) design for rapid diagnosis of the exon 4 di-nucleotide deletion in the LCAT gene. By using the mismatched primer, the nucleotide sequence is changed such that an FspI site is introduced into the wild-type sequence but not the sequence with the di(TG)-deletion.

with 1U of *Fsp*1 as recommended by the manufacturer [Gibco BRL]. A 103 bp and 52 bp fragment were generated in wild-type sequences but not in the digestion of the LCAT deficient proband's PCR fragment.

E) Structural studies on the LCAT protein

i.) Cell culture

African Green monkey kidney (COS-7) cells (ATCC) were maintained in Dulbecco's minimal essential medium (DMEM) [Gibco BRL], pH 7.4, supplemented with 10% heat-inactivated fetal bovine serum (FBS) [Gibco BRL], 100 U/ml penicillin (pen) and 100 µg/ml of streptomycin (strep) [Gibco BRL]. Cells were incubated at 37° C in 5% CO_2 atmosphere. COS-7 cells were routinely passaged 1:3 or 1:5 and were maintained in T75 flasks [Sarstedt, Que.] while experimental cells were cultured in 100 mm culture dishes [Falcon]. Confluent cells were washed once with 1 X PBS (8 g NaCl, 0.1 g KCl, 0.72 g Na_2HPO_4, 0.12 g KH_2PO_4, pH 7.4) and stripped from the T75 flask with 1 X trypsin-EDTA [Gibco BRL]. For each experiment, cells were seeded 1:5 the day before transfection. Near confluent cells (at least 70%) were transfected with 6 µg of plasmid DNA using FuGENE™ 6 [Roche Diagnostics, Laval, Que.]. For a single dish transfection, 12 µl of FuGENE™ 6 was added to 188 µl of serum-free DMEM and incubated for 5 min at room temperature. The mixture was then added to the DNA and incubated for another 15 min at room temperature. After 15 min, the DNA-FuGENE™ 6 mixture was added dropwise to the culture dish. After 24h, the cells were washed twice with 1 X PBS and the media was replaced with 15 ml of serum-free OPTI-MEM [Gibco BRL] to reduce the serum background. Cells were incubated for another 48h before

media collection. At 48h the media were collected, centrifuged at 500g for 5 min at 4°C and immediately stored at -20°C until ready for use. COS-7 cells were harvested for RNA, DNA, and protein using the **TRI**ZOL® reagent [Gibco BRL]. Total RNA concentration was quantified by spectrophotometry at A_{260} and A_{280}. The RNA concentration was calculated as follow: A_{260} x 40 µg/ml where an OD of 1 is approximately 40 µg/ml (Sambrook et al., 1989). The ratio at A_{260}/A_{280} was also calculated to assess for RNA purity.

ii.) Generation of LCAT mutants by PCR, cloning and sequencing

Site-directed mutagenesis of the human LCAT cDNA was performed by overlap extension using PCR (Ho et al., 1989; Lowe 1997; Wong et al. 1997). This method employs two or more PCR reactions to generate overlapping fragments that together will make up the entire coding sequence. The first PCR reaction uses complementary primers that have the change or mutation encoded, and flanking primers at each end of the sequence. Deletion mutants were created using complementary primers (Appendix III) that excluded the sequences for deletion. The two separate PCR products were then combined in a second PCR reaction to obtain the full-length product. The same method was used to construct the rat-human chimera (Figure 2.3) (Appendix II). The pCMV5 vector, pCMV5-human LCAT and pCMV5-rat LCAT was most generously provided by Dr. John Parks, Dept. of Comparative Medicine, The Bowman Gray School of Medicine, Wake Forest University, Winston-Salem, NC.

All PCR reactions contained 60 ng of template DNA, pCMV5-human LCAT or pCMV5-rat LCAT, 1 µmol/L of each primer, 200 µmol/L of dNTPs, 1% (v/v) DMSO, 5

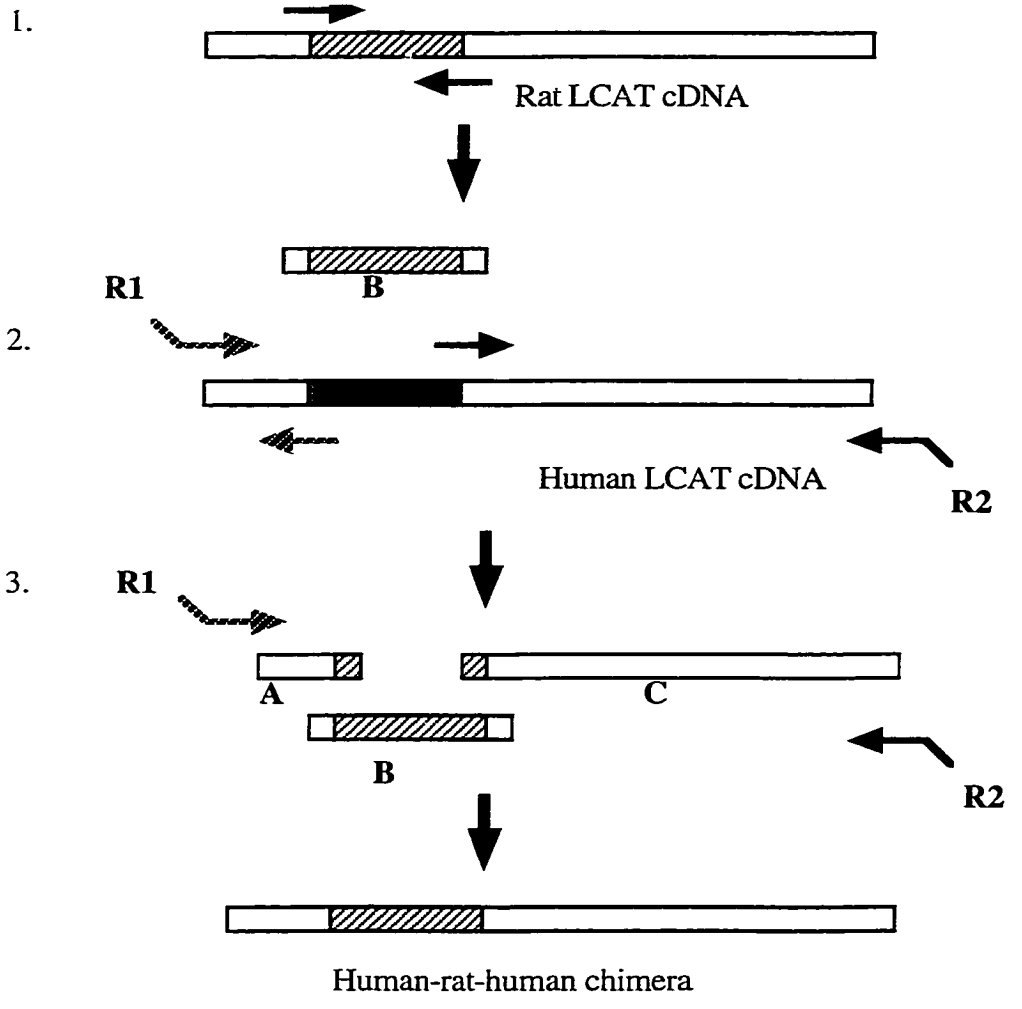


Figure 2.3: PCR was used as a tool to construct the human-rat-human (HRH) chimera. The first step (1) involved the amplification of the rat sequence using specific primers that gave the desired region (hatched) flanked by the human LCAT sequence. In step 2, the human LCAT cDNA was amplified for the remaining regions that was required for a full-length cDNA sequence. These fragments contained the complementary rat sequence at the ends that would overlap with the PCR products from the first step. Finally, the fragments from the three different PCR reactions were added together to form the full-length chimera with the primers R1 and R2 that encoded the necessary unique restriciton enzyme sites *EcoR*1 and *BamHI* for cloning.

µl of 10 X Vent buffer, 1U of $Vent_R$ polymerase [New England Biolabs, Beverly, MA.], and sterile Milli-Q water to a final volume of 50 µl. The reaction mixes were denatured at 94°C for 30s, annealing at either 55° or 58°C (depending on the primer used) for 1 min and extension at 74°C for 1.5 min followed by a final extension at 70°C for 10 min. The PCR products were separated on 1% agarose gel electrophoresis and purified using the Sephaglas™ Band Prep Kit [Amersham Pharmacia Biotech]. The purified products were used for a second round of PCR to generate a full-length human LCAT cDNA using specific flanking primers R1 5'-CGTCA*GAATTC*TCGAGCTCGTCG-3' and R2 5'-CACCCGG*GGATCC*TTATTCAGGAGGCGGGGGCTCTG-3' containing the *EcoR*I and *BamH*I restriction enzyme sites. These sites were used for cloning the human LCAT cDNA into the pCMV5 vector. The cloning sites for the rat LCAT cDNA were *EcoR*I and *Hind*III. The conditions for the PCR were as described above.

A 20 µl aliquot of the PCR product was digested simultaneously with 3 µl of *Eco*RI (20U/µl) and 3 µl *Bam*HI (20U/µl) [New England Biolabs] containing 3 µl of 10 X NEBuffer *EcoR1* buffer [New England Biolabs] and sterile Milli-Q water in a final volume of 30 µl at 37°C for 2h. Digested products were separated by 1% agarose gel electrophoresis, excised, and purified as described. The purified products were cloned into the pCMV5 vector (kindly provided by Dr. John Parks) (Figure 2.4) with T4 DNA ligase [New England Biolabs] and transformed into electro-competent *E.coli* DH5α cells. Positive clones were identified by PCR screening using the R1 and R2 primers described earlier. Positive clones were subsequently grown in Luria Bertani (LB) broth containing 100 ug/ml of ampicillin [Sigma] and purified using the Ultraclean™ Mini plasmid Prep Kit [Mo Bio Laboratories, Solana Beach, CA.] and sequence using the T7 Sequencing™

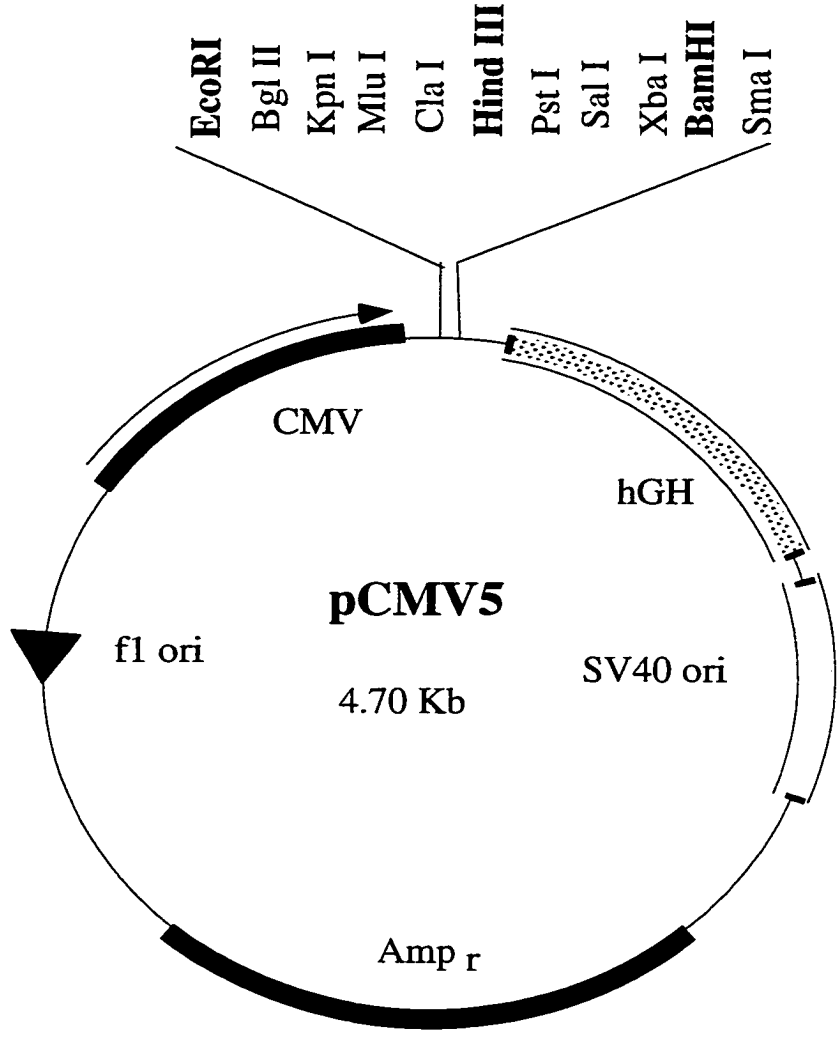


Figure 2.4: Schematic representation of the plasmid pCMV5. The pCMV5 plasmid is a mammalian vector that works extremely well in SV40 transformed simian COS cells (Thomsen et al., 1984). The pCMV5 vector contains the unique restriction sites (EcoRI, BamHI and HindIII) in the polylinker region required for cloning the human and rat LCAT cDNA.

Kit [Pharmacia Amersham Biotech]. Once the mutation was confirmed the clones were sent for complete bidirectional automated sequencing by the ABI prism sequencer (National Research Council sequencing facility, Halifax, Nova Scotia) to discern any errors. The Quantum Prep® Maxi plasmid prep kit [BioRad] was used to process larger amounts of plasmids, which were subsequently used for transfection.

iii.) LCAT activity assays with reconstituted HDL and isolated human LDL as substrates

LCAT activity was measured using two different substrates: reconstituted HDL (rHDL) and [^{3}H]-labelled LDL. rHDL particles were prepared by the sodium cholate dialysis method (Matz and Jonas, 1982). The rHDL complexes consisted of 1-pamitoyl-2-olelyl phosphatidylcholine (POPC), cholesterol and human (or rat) apoA-I at a molar ratio of 80:10:1 and were prepared as follows. Human and rat apoA-I were isolated from human plasma by preparative isoelectrofocusing (Dolphin, 1980). A trace amount of [1α, 2α-^{3}H]-cholesterol (1μCi/μl) [Amersham Pharmacia Biotech] was added to the POPC/cholesterol mixture and dried under nitrogen. To this, 1.0 mg of apoA-I resuspended in 1 ml of TBS, pH 8.0 [4.84g Tris-base, 32.76g NaCl, 1.49g EDTA and 0.4g NaN_3] (the standard buffer used in all subsequent experiments) was added to the dried lipid mixture. An aliquot of 48 μl of sodium cholate (312 mg/ml) was added to the mixture to emulsify the lipid. An additional 205 μl of TBS was added to the mixture and the entire contents were incubated at 37°C for 1h on a mechanical inverter. The resulting rHDL substrate was dialysed 6 X 2h against 1 L of TBS, pH 8.0 with at least one overnight dialysis. After dialysis, the rHDL particles were analyzed for cholesterol and phospholipid composition by gas liquid chromatography as described earlier and protein

concentration was determined by the BCA [Pierce] protein assay using bovine serum albumin [Sigma] as a standard. The rHDL stock was stored at 4°C under nitrogen for up to a month. The rHDL was diluted with TBS, pH 8.0 to 8 nmol of cholesterol/100 µl for LCAT assays.

For the assay of β-LCAT activity, human LDL was isolated from fresh plasma by sequential ultracentrifugation between the densities of *d:* 1.006 - 1.063 g/ml. Densities were adjusted using sodium bromide (NaBr) and ultracentrifugation was performed at 40,000 rpm for 18h and subsequently at 40, 000 rpm for 21h in 50.3Ti rotor and L5-50 centrifuge [Beckman]. The isolated LDL was dialysed overnight against TBS and stored at 4°C in 0.5 mM EDTA for up to a month. Cholesterol and phospholipid composition was determined by gas liquid chromatography as described previously. Protein concentration was determined by the BCA method [Pierce] as described previously. The appropriate volumes of purified LDL was radiolabelled with [1α, 2α-^{3}H]-cholesterol (1µCi/µl) [Amersham Pharmacia Biotech] at 4°C overnight (Stokke and Norum, 1971) and diluted to a concentration of 12 nmol of cholesterol /100 µl before the assays.

Each LCAT reaction consisted of 150 µl of TBS, 100 µl of rHDL as substrate, 125 µl of 2% essentially fatty acid free BSA [Sigma], 25 µl of 100 mM β-mercaptoethanol and 100 µl of culture medium as the LCAT source in a final volume of 500 µl. The reaction was allowed to proceed for 1h at 37°C in a shaking water bath and subsequently stopped by the addition of 2 ml of ethanol. Immediately after, the lipids were extracted with 4 ml of hexane, dried under nitrogen and resuspended in 200 µl of chloroform [BDH, Toronto, Ont.]. Cholesterol and cholesteryl esters were separated by silica-H thin layer chromatography [Gelman Sciences] developed with hexane:diethyl

ether:glacial acetic acid (90:10:1, v/v). Radioactivity was quantitated by Beckman LS 3801 liquid scintillation counter. LCAT activity with the rHDL was calculated as follow:

Activity (nmol CE formed / ml / h)

$$\frac{(\textbf{DPM}\ \text{CE spot} - \textbf{DPM}\ \text{CE blank}) * 10\ [10 \times 100\ \mu\text{l sample} = 1\ \text{ml}] * 1\text{h}}{(\textbf{DPM}\ \text{of UC spot} + \text{CE spot}) / (8\ \text{nmol UC in } 100\ \mu\text{l substrate})}$$

UC represents unesterified cholesterol.

LCAT activity assays with human LDL as substrates were performed essentially in the same way except the reactions were allowed to proceed for 6h at 37°C. The percent of cholesterol esterification due to LCAT activity with LDL substrates was calculated as nmol CE formed/ml/h as follow:

%CER (nmol / ml / h)

$$\frac{\dfrac{\textbf{DPM}\ CE_{37°C}}{\text{Total}\ \textbf{DPM}_{37°C}} - \dfrac{\textbf{DPM}\ CE_{4°C}}{\text{Total}\ \textbf{DPM}_{4°C}} * 100\ (\%) * 1\ \text{nmol UC} * 5\ [5 \times 200\ \mu\text{l sample} = 1\ \text{ml}]}{6\text{h}}$$

iv.) Western blot analysis

Western blot analysis was performed to detect the presence of LCAT protein in the culture medium and cell lysates. A 20 µl aliquot of the culture medium or 15 µl of cell lysate was resolved by 10% SDS-polyacrylamide gel electrophoresis (PAGE) with a 5% stacking gel at 40 mA until the dye front just migrates out of the gel. The proteins were subsequently transferred to a Hybond-P:polyvinylidene difluoride (PVDF) [Amersham Pharmacia Biotech] membrane in 1 X transfer buffer [3.03 g Tris, 14.4 g

glycine, 200 ml methanol in a final volume of 1 L, pH 8.3] at 100 V for 90 min with cooling using the BioRad Mini-Protean II Transfer apparatus. The membranes were subsequently blocked with BLOTTO [TBS: 2.42 g Tris, 8 g NaCl, pH 7.6, in 1L of distilled H_2O, 0.1% Tween 20 (v/v), 5% (w/v) powdered skim milk] and reacted with the monoclonal mouse anti-human LCAT 5D4 antibody (kindly provided by Dr. Ross Milne, Lipoprotein and Atheroscleorosis Group, University of Ottawa Heart Institute, Ottawa, Ont.) (Marcel et al., 1987) for 1h at room temperature. Sheep anti-mouse IgG conjugated to horseradish peroxidase (HRP) was used as the secondary antibody. The protein bands were visualized using the enhanced Chemiluminescence (ECL) kit [Amersham Pharmacia Biotech] and exposing the blots to X-ray film [Laboratory Scientific, Inc.].

v.) Northern Blot analysis

RNA was isolated using the **TRI**ZOL® reagent [Gibco BRL] following the manufacturer's protocol and quantitated by spectrophotometry at A_{260}. Northern blot hybridization was performed following the separation of 20 µg of total RNA on a 1% formaldehyde-agarose gel (2.5 g agarose, 45 ml formaldehyde [BDH], and 12.5 ml of 20 X MOPS (3-[N-morpholino]porpanesulfonic acid) buffer [Fisher Scientific] in a final volume of 250 ml). The RNA was subsequently transferred to Hybond N [Amersham Pharmacia Biotech] and UV- crosslinked to the membranes using the UV-Stratalinker [Stratagene, LaJolla, CA] for 1 min and stored at -20°C until ready for hybridization with the human LCAT cDNA.

The full-length human LCAT cDNA was excised from pCMV5 with *Eco*RI and *Bam*HI for 2h at 37°C, separated on a 0.8% agarose gel, excised with a razor blade and

subsequently purified using the Sephaglas™ Band Prep Kit [Amersham Pharmacia Biotech]. The cDNA was labelled using the Rediprime DNA labelling system [Amersham Pharmacia Biotech]. Briefly, the LCAT cDNA was diluted to 25 ng in a total of 45 µl with distilled water. The cDNA was denatured by heating to 100°C for 5 min and cooled rapidly on ice to prevent re-annealing. The human LCAT cDNA and a 5 µl aliquot of [^{32}P]-dCTP (Amersham Pharmacia Biotech] was added to a Rediprime tube containing a buffered solution of dATP, dGTP, and dTTP, exonuclease free Klenow enzyme and random primers (9-mers). Labelling of the cDNA was performed at 37°C for 20 min and the reaction was terminated with the addition of 5 µl of 0.2 M EDTA. The labelled cDNA was purified from the unincorporated nucleotides using the NucTrap® Probe Purification column [Stratagene]. The column was pre–wet with 70 µl of 1 X STE (100 mM NaCl, 20 mM Tris-HCl, pH 7.5, 10 mM EDTA) before loading 55 µl of the labelling reaction containing the human LCAT cDNA. The column was rinsed once with 15 µl of 1 X STE followed by an additional 70 µl of 1 X STE. Following purification, the labelled cDNA was used as a probe for Northern blot hybridization.

Before hybridization with the radiolabelled human LCAT cDNA, the Northern blots were pre-wet in 2 X SSC (20 X SSC: 175.3 g NaCl, 88.2 g Na Citrate in 1 L, pH 7.0) and incubated overnight at 42°C in the pre-hybridization solution (50% formamide, 5 X SSPE, 2 X Denhardt's reagent, 0.1% SDS). The pre-hybridization solution was discarded before the addition of the labelled cDNA and hybridization was allowed to proceed overnight at 42°C. The blots were subsequently rinsed twice in 2 X SSC, 0.1% SDS and washed with 1 X SSC, 0.1% SDS at 50°C for 20 min, followed by a second wash with 1 X SSC, 0.1% SDS at 55 °C for another 20 min, and a final wash with 0.5 X

SSC, 0.1% SDS at 55°C for 30 min. The blots were exposed to X-ray film [Laboratory Scientific, Inc.], scanned and analyzed using the NIH Image program.

DNA isolated from the transiently transfected COS-7 cells was amplified by PCR using the specific primers RT-1 5'-CTGCCGACCATGTTGAGATG-3' and RT-2 5'-GTACCTGCACACACTGGTGC targetted to the 3'-end of the human LCAT gene. The PCR was performed as previously described.

3. Cholesteryl Ester Transfer Protein

A) Perspectives

During their transit in the plasma compartment, all of the lipid constituents and some apolipoproteins of plasma lipoproteins are subject to transfers and exchanges between the various lipoprotein classes. Whereas the transfer of unesterified cholesterol is spontaneous, the transfer of phospholipids, triglycerides and cholesteryl esters require the presence of a protein facilitator (Albers et al., 1984; Rye and Barter, 1992). The plasma protein that facilitates the transfer of all the neutral lipids (triglycerides and cholesteryl esters) and some of the phospholipids is the cholesteryl ester transfer protein (CETP) (Hesler et al., 1988; Yen et al., 1989). Given that the majority of the plasma cholesteryl esters are formed on the HDL via the activity of LCAT, CETP is responsible for the redistribution of the cholesteryl esters to other lipoprotein fractions. In the light of epidemiological studies which have shown that high levels of HDL are a negative risk factor for coronary heart disease and a high concentration of LDL cholesterol is atherogenic, the study of CETP-mediated lipid transfer is of great importance.

CETP has a central role in reverse cholesterol transport (Figure 3.1). Through the LCAT reaction, cholesteryl esters are generated on HDL and become substrates for CETP. In species that display CETP activity such as humans, the cholesteryl ester enriched HDL is removed by several routes. In the presence of CETP, the cholesteryl esters in the HDL are exchanged for triglycerides present in the triglyceride-rich lipoproteins. The cholesteryl esters acquired by the apoB-containing particles (VLDL and LDL) are removed by the liver via the LDL-receptor or the remnant receptor during

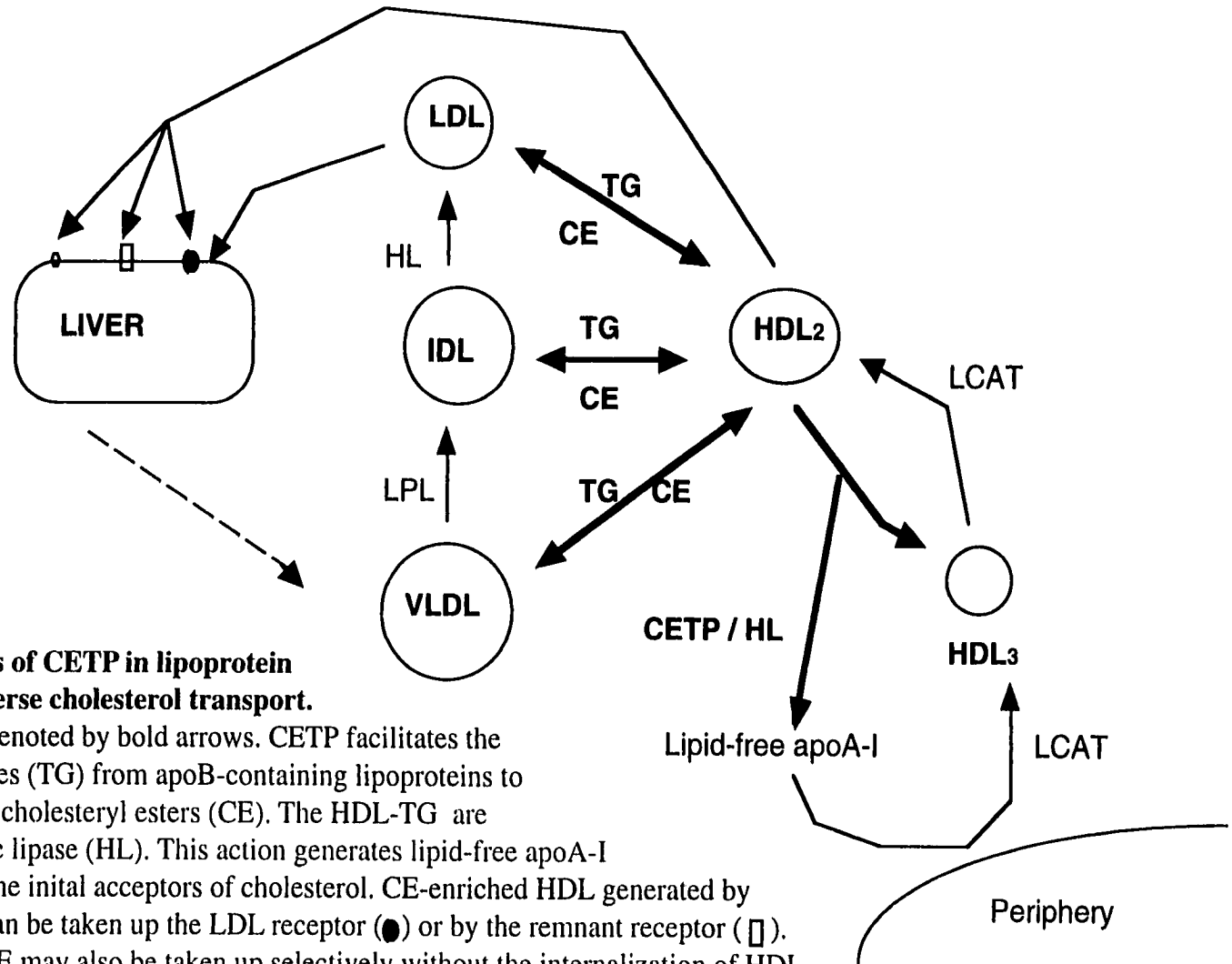


Figure 3.1: The roles of CETP in lipoprotein metabolism and reverse cholesterol transport.
CETP functions are denoted by bold arrows. CETP facilitates the transfer of triglycerides (TG) from apoB-containing lipoproteins to HDL in exchange for cholesteryl esters (CE). The HDL-TG are hydrolysed by hepatic lipase (HL). This action generates lipid-free apoA-I that is thought to be the inital acceptors of cholesterol. CE-enriched HDL generated by the action of LCAT can be taken up the LDL receptor (●) or by the remnant receptor (▯). Additionally, HDL-CE may also be taken up selectively without the internalization of HDL via the hepatic SR-BI receptor (o).

their normal metabolism. The triglycerides acquired by the HDL become substrates for hepatic lipase. The hydrolysis of triglycerides in the HDL by hepatic lipase promotes the formation of HDL that are excellent mediators of cholesterol efflux (Castro and Fielding, 1988; Kunitake et al., 1992), and subsequently become optimal substrates for LCAT (Francone et al., 1989). Thus, CETP activity enhances the reverse cholesterol transport pathway by increasing the rate of plasma cholesteryl ester removal by the liver (Jiang et al., 1993). The increased rate of cholesteryl ester removal from HDL also stimulates cholesteryl ester formation by LCAT (Francone et al., 1996; Olivera et al., 1997).

Alternatively, in species lacking CETP or in CETP deficiency, HDL cholesteryl esters are predominantly removed directly by the liver through the LDL receptor recognizing apoE that is acquired in multiple copies during the enlargement of HDL (i.e. cholesteryl ester accumulation due to the action of LCAT). In addition, the HDL cholesterol can be selectively removed without the degradation of the HDL protein by the SR-BI receptor. Recently, the synergistic remodelling of HDL by CETP and HL have been shown to enhance the uptake of HDL cholesteryl esters by cells expressing the SR-BI receptor (Collet et al., 1999).

B) The CETP gene and protein

The human CETP gene is located on the long arm of chromosome 16 in the region of 16q12-21, close to the LCAT gene locus (Lusis et al., 1987) and is present as a single copy (Agellon et al, 1990). The CETP gene consists of 16 exons and spans 25 kb of the genomic DNA (Agellon et al., 1990) (Figure 3.2). The exon sizes range from 32 bp (exon 13) to 250 bp (exon 16). The genomic organization of CETP is similar to that

of the human lipopolysaccharide binding protein (LPB), neutrophil bactericidal permeability increasing protein (BPI), and the phospholipid transfer protein (PLTP) suggesting that they may share similar functions (Hubacek et al., 1997). CETP shares a 20% sequence identity to PLTP and has the same exon / intron organization (Day et al., 1994). The 5' proximal promoter region consists of the classical TATA box, an SP1 binding site (Agellon et al., 1990) and a C/EBP site (-242 to –229 bp) (Agellon et al., 1992). Unlike LCAT, the CETP gene is highly responsive to dietary cholesterol (Quinet et al., 1990; Jiang et al., 1991), corticosteroids and lipopolysaccharides (Masucci-Magoulas et al., 1995). Studies in transgenic mice have shown that the diet-induced increase in CETP mRNA and increase in plasma CETP activity is a result of CETP gene transcription (Jiang et al., 1992). The CETP minigene linked to the natural flanking sequence of human CETP showed a 4 to10-fold increase in liver CETP mRNA and a 2.5-fold increase in plasma CETP activity in response to a high fat, high cholesterol diet. Furthermore, transgenic mice crossed with apoE or LDL receptor knock-out mice also showed an increase in plasma CETP after cholesterol feeding with a parallel increase in hepatic mRNA levels (Masucci-Magoulas et al., 1996). Therefore, CETP is responsive to both diet-induced and endogenous hypercholesterolemia (Jiang et al., 1992; Masucci-Magoulas et al., 1996). Accordingly, a positive sterol response element containing a tandem repeat of sequence known to mediate sterol down regulation similar to that found in HMG-CoA reductase and the LDL receptor genes was identified by Olivera et al. (1996) to be between –370 and -138 in the 5'-untranslated region of CETP. However, in contrast to the HMG-CoA reductase and LDL receptor genes that are down regulated in response to cholesterol, the CETP gene is up regulated in the presence of increased sterol

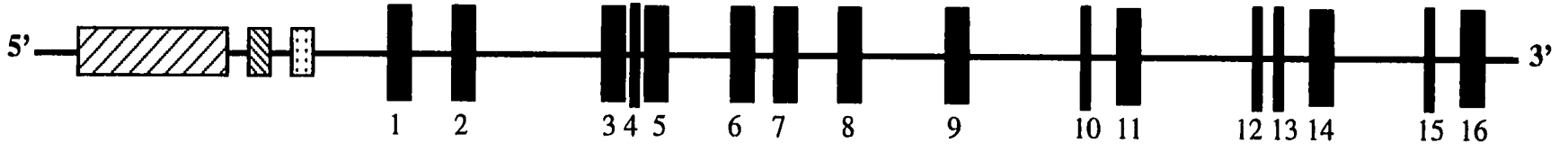


Figure 3.2: Schematic representation of the human cholesteryl ester transfer protein (CETP) gene. CETP contains 16 exons separated by 15 introns. The exons are represented by the black boxes. The dotted box represents the classical TATA box and the stripped box (▨) represents the Sp1 binding site. The left-hand stripped box denotes the 5' regulatory region between -361 to -134. Regions betwen -242 to -229 contains the C/EBP binding site, region -361 to -138 contains the CRE, and the binding site for RARE is found between -165 to -134.
CRE: cholesterol response element; RARE: Retinoic acid receptor element.

concentrations. Recently, a similar region, termed the cholesterol response element (CRE) (between –361 to –138), was shown to be trans-activated by the YY1 (Red25), SREBP-1a and to a lesser extent by SREBP-2 (Gauthier et al., 1999). In addition to this, the region between –165 to –134 was found to have the consensus retinoic acid receptor element (RARE) (Jeoung et al., 1999). Tissue-specific elements were also identified for the liver and spleen (-3.4kb to –570), intestine (-570 to –370), and the adrenals (-370 to –138) (Olivera et al., 1996). Furthermore, the orphan nuclear hormone receptor apolipoprotein A-I regulatory protein-I (ARP-I) and its homologue Ear-3/COUP-TF, when over-expressed, could either activate or repress transcriptional activity of the CETP gene depending on the architecture of the promoter region (Gaudet and Ginsburg, 1995). Clearly, future studies are required to define the complex transcriptional regulation of the CETP gene by cholesterol, hormones and other factors.

In humans, the CETP mRNA is expressed predominantly in the liver, spleen, and adipocytes with lower levels in the small intestine, adrenals, kidneys, and heart (Drayna et al., 1987; Jiang et al., 1991). Two forms of transcript, the full length CETP mRNA with all 16 exons and an exon 9-deleted transcript are present in all tissues expressing CETP (Inazu et al, 1992). Exon 9 encodes 60 amino acids in the central portion of the protein. The exon 9-deleted transcript forms an intracellular complex with the full length CETP inhibiting its secretion (Quinet et al., 1993) and thus is not detected in the plasma (Inazu et al., 1992; Quinet et al., 1993). Cell culture studies showed that the alternatively spliced CETP is poorly secreted and lacks lipid transfer activity (Inazu et al, 1992; Tall, 1993). Transgenic mice were also shown to express both forms of CETP with a preferential accumulation of the full-length CETP mRNA during development and in

response to hypercholesterolemia (Yang et al., 1996). Dessi et al. (1997) also found the two forms of CETP expressed in Caco-2 (intestinal) cells but when challenged with oleate, the full-length CETP was preferentially expressed. The function of the exon 9-deleted transcript is unclear and may have a potential post-transcriptional role in regulating CETP secretion (Lagrost, 1994; Tall, 1995).

The cloning of the CETP cDNA revealed a 476 amino acid sequence with a hydrophobic 17 residue prepeptide and has a high content of hydrophobic residues scattered throughout (Drayna et al., 1987). The CETP protein is also N-linked glycosylated at residues 88, 240, 341, and 396 (Drayna et al., 1987). The calculated mass of the protein is approximately 53 kDa and the mature CETP protein appears as a doublet with an apparent molecular weight between 66 to 74 kDa in SDS polyacrylamide gels (Hesler et al., 1987). The doublet represents two isoforms of CETP, with or without glycosylation at Asn-341 (Stevenson et al., 1993). Glycosylation at position 88 appeared essential for activity (Stevenson et al., 1993) and CETP without the carbohydrate chain at Asn-341 had a 40 % higher specific activity than the fully glycosylated form (Swenson et al., 1987; Tall, 1993). The CETP cDNA from the rabbit (Nagashima et al., 1988), cynomolgous monkey (Pape et al., 1991) and a partial hamster sequence (residues 188-476) (Jiang et al., 1991) have been cloned and sequenced.

Although the CETP cDNA shows no homology to the apolipoproteins cDNAs, there is a conserved pentapeptide (Val-Leu-Thr-Leu-Ala) within the signal sequence of human CETP, lipoprotein lipase, apoA-I and apoA-IV (Agellon et al., 1990) not found in any signal sequence of other proteins. The authors suggest this sequence may have a

conserved function that may be related to translational or post-translational regulation of the expression of these genes.

C) CETP Structure and Function

Several approaches have been used to define the functional regions of CETP. These include a limited digestion with proteases, linker scanning insertion mutagenesis, the production and epitope analysis of CETP monoclonal antibodies by site-directed mutagenesis and deletion mutants, and chemical modifications.

The limited proteolysis approach was undertaken to identify a domain of CETP that may independently facilitate neutral lipid transfer (Hesler et al., 1989). The digestion with various proteases resulted in active fragments that could not be dissociated under various reducing and dissociating conditions. This led the authors to suggest that CETP possess a distinct and highly stable tertiary structure which is required for cholesteryl ester transfer activity. The linker insertion scanning mutagenesis studies (Wang et al., 1991) produced some mutants that had relatively normal cholesteryl ester transfer activity. However, inserting residues in regions 48-53, 165, and 373-379 resulted in reduced triglyceride and cholesteryl ester transfer activity. While CETP is quite tolerant to local structural disturbances, alteration of the structure in regions that are important for function will impair activity. This study also suggests that a similar structure is required for both neutral lipid transfer activities (Wang et al, 1991).

Studies employing monoclonal antibodies have been the most informative. A CETP monoclonal antibody was found to neutralize all cholesteryl ester and triglyceride transfer activities in human plasma suggesting that CETP is responsible for all the neutral

lipid and some of the phospholipid transfer activity (Hesler et al., 1988). The epitope for this neutralizing monoclonal antibody, TP2, was localized to the C-terminal 26 residues of CETP (Swenson et al., 1989). Furthermore, Fab fragments from TP2 were also able to inhibit neutral lipid transfer activity and the binding of neutral lipids to CETP suggesting that TP2 inhibited lipid transfer by blocking the interaction of lipid with CETP. Interestingly, while lipid transfer activity was inhibited by the TP2 Fab, the binding of CETP to plasma lipoproteins, particularly VLDL and LDL, and phospholipid vesicles was enhanced. The binding of TP2 Fab may cause a conformational change in CETP that enhances the binding to lipoproteins and vesicles and the TP2 Fab may mimic the binding of neutral lipids to CETP (Swenson et al., 1989; Tall, 1993).

In order to evaluate the involvement of the C-terminal 26 residues in lipid transfer activities, C-terminal deletions and site-directed mutagenesis were performed. Wang et al. (1992) were able to further localize the epitope of TP2 to be between residues 463 and 475. The deletion mutants were well secreted but had reduced cholesteryl ester transfer activity. Furthermore, the deletion mutant Δ470-475 had reduced cholesteryl ester and triglyceride transfer activities but an increased phoshoplipid transfer activity. The fact that phospholipid transfer activity is intact suggests that the loss of the C-terminal residues (Δ470-475) does not affect the binding of CETP to lipoproteins and the loss of cholesteryl ester and triglyceride transfer activities is not a result of misfolding of CETP (Wang et al, 1992; Tall, 1993).

Point mutagenesis within the residues of 463 to 475 revealed that the amino acids required for TP2 binding were different from those required for cholesteryl ester transfer activity (Wang et al., 1993). Accordingly, the residues involved in cholesteryl ester

transfer were hydrophobic residues whereas those required for TP2 binding appeared to be hydrophilic or charged. In view of the secondary structure prediction of an amphiphatic α-helix within this region, it is conceivable that TP2 binds to the hydrophilic face of the helix while the region necessary for cholesteryl ester transfer is located on the hydrophobic face of the helix (Wang et al., 1993). It was suggested that the binding of TP2 might immobilize the flexible C-terminal tail of CETP preventing cholesteryl ester transfer (Wang et al., 1992; Tall, 1993). A proposed putative hinge sequence at residue 462-464 (Gly-Phe-Pro) may provide this flexibility (Wang et al., 1992).

Au-Young and Fielding (1992) have also characterized three large C-terminal deletion mutants (of 26, 48, and 66 residues) that had impaired binding to lipoproteins and were inactive. As these deletions all contain the Phe-Leu-Leu-Leu (residues 454–457) that are also found in BPI, LBP and several lipid metabolizing enzymes including LCAT, the authors hypothesized that these residues might be necessary for lipoprotein binding and for the effective catalysis of neutral lipid transfer. However, substitution of Phe-454 → Ala resulted in only a slight reduction of cholesteryl ester transfer activity (Wang et al, 1992). The general hydrophobicity in the C-terminal region thus appears to be important for function rather than specific residues (Tall, 1993). Since the binding of CETP to lipoprotein substrates is influenced by the negative charge on lipoproteins (Sammett and Tall, 1985) and involves hydrophobic and ionic interactions (Nishida et al., 1993), Jiang et al. (1995) performed site directed mutagenesis on key positively charged residues within CETP. The substitution of Lys-233 or Arg-259 with neutral ofracidic residues led to a markedly reduced lipid transfer activity. The Lys-233 → Ala mutant of CETP was shown to be defective in binding to HDL. The authors proposed that both the

Lys-233 and Arg-259 might be the region that is involved in associating with the lipoprotein surface. Later work by Roy et al. (1996) using monoclonal antibodies showed that the region between residues 261-331 and 367-409 did not influence cholesteryl ester transfer activity. The monoclonal antibodies that partially neutralized lipid transfer activity (between residues 184-260) result from the proximity of this region to Lys-233 and Arg-259 and thus may interfere with the binding of CETP to lipoproteins.

Morton and Zilversmit (1982) showed that CETP activity is inhibited by p-chloromercuriphenylsulphonate, a hydrophobic cysteine-modifying reagent. This led Connolly et al. (1996a) to study the effects of other cysteine-modifying reagent and the authors found that CETP can only be inactivated by hydrophobic cysteine-modifying reagents such as p-chloromercuriphenylsulphonate suggesting that CETP contains essential free cysteine(s) residing in a hydrophobic environment within the protein. Recently, Kotake et al. (1997) investigated the requirement for the conserved N-terminal Cys residue found in humans, rabbits, and cynomolgous monkeys by removing the N-terminal Cys. Although the lack of a free Cys did not impair neutral lipid transfer activity, the modification with p-chloromercuriphenylsulfonic selectively inhibited triglyceride transfer activity. These results suggest that this free cysteine residue may be close to an active site that can determine the selectivity for neutral lipids. More interestingly, Melchoir et al. (1995) were able to show that cholesteryl ester and triglyceride transfer by cynomolgous monkey CETP could be separately inhibited suggesting that two independent sites for neutral lipid transfer exist. Whereas monoclonal antibodies made against purified cynomolgous monkey CETP inhibited triglyceride transfer with variable effect on cholesteryl ester transfer, 6-cholomecuric

cholesterol inhibited cholesteryl ester transfer with minimal inhibition of triglyceride transfer. Fukasawa et al. (1992) also demonstrated, using anti-human monoclonal antibodies, that triglyceride transfer can be inhibited without any effects on cholesteryl ester transfer.

Recently, the crystal structure of BPI, which shares structural similarity with CETP, was solved (Beamer et al., 1997) (Figure 3.3). This structure was shown to contain 2 main domains: the N- and C-terminal domain. Both these domains consist of a "barrel" formed by two α helices and a highly twisted anti-parallel β-sheet. The two domains are joined by a smaller anti-parallel β-sheet formed by residues from the beginning and end of each domain (Bruce et al., 1998b). The BPI structure also has an unusual "boomerang" shape and most unexpectedly, two-lipid binding sites (Beamer et al., 1997) (Figure 3.3). Based on the sequence similarity shared by BPI and CETP, the structure of CETP was modelled and proposed to have a similar overall protein fold and the two domain architecture of BPI (Bruce et al., 1998b). Both hydrodynamic and circular dichroism measurements were in agreement with the modelled CETP structure (Connolly et al., 1996b). Consistent with the studies by Fukusawa et al. (1992) and Melchoir et al. (1995), two separate lipid binding sites may be present in CETP. Furthermore, both the lipid-binding pockets may have a cysteine residue located in the interior such that their modification results in steric hindrance of lipid binding (Kotake et al., 1997; Bruce et al., 1998b). These residues were assigned to Cys-17 in the N-terminal pocket and Cy-325 in the C-terminal pocket (Bruce et al., 1998b). In addition, the two conserved cysteines in the gene family that form a disulphide bridge in BPI and are essential for the function of BPI has been ascribed to Cys-143 and Cys-184 of CETP

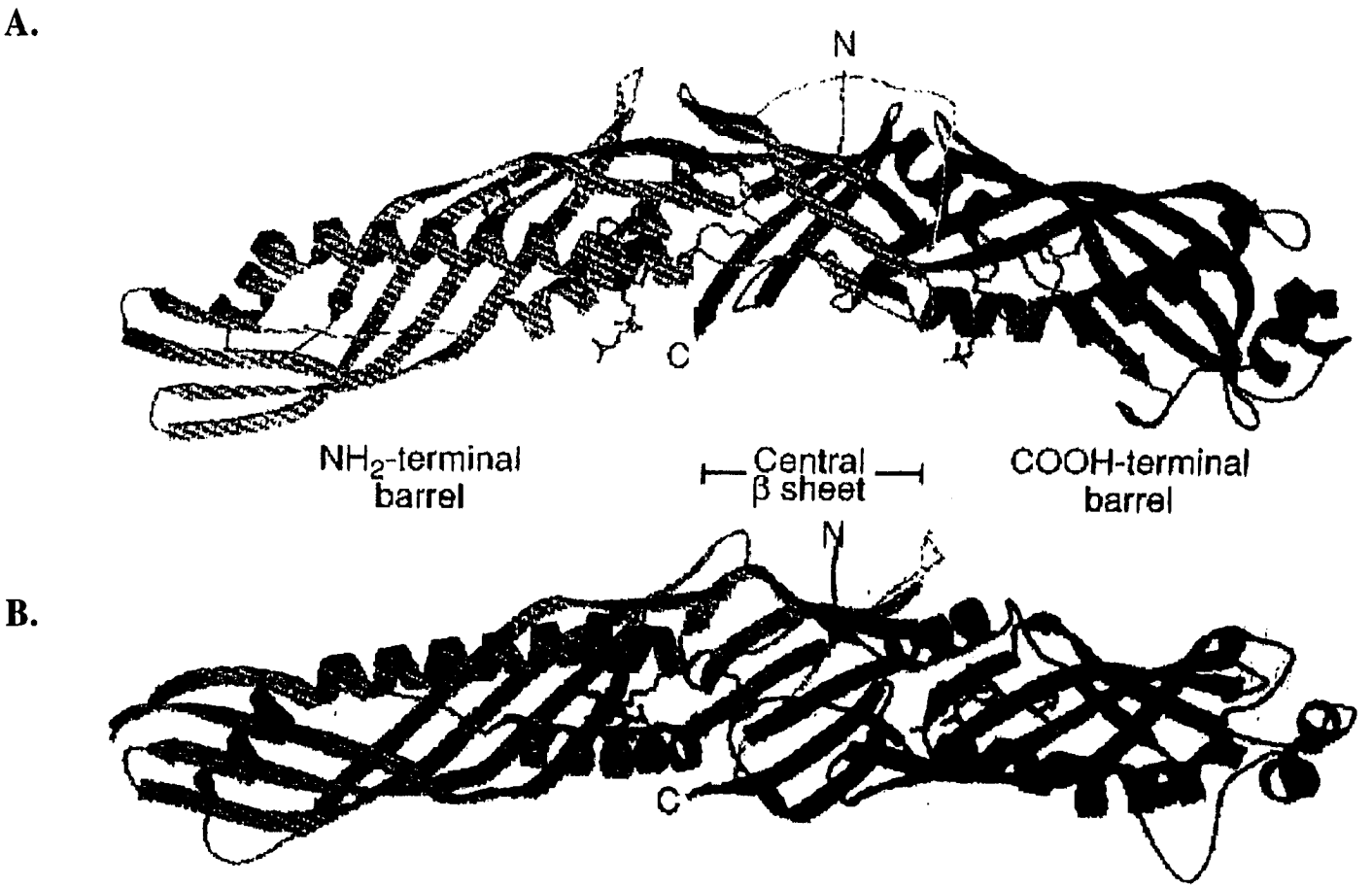


Figure 3.3: The crystal structure of Bactericidal/Permeability-increasing protein (BPI). This ribbon diagram depicts the two domains of BPI. The ball and stick figures represent the to two bound phosphatidylcholines. B. View after a 70° rotation of A. along the long axis of the molecule. From Beamer et al., 1997.

(Horwitz et al., 1996). Bruce et al. (1998b) also proposed that the C-terminal amphiphatic helix lies over the opening of the lipid binding pocket in the N-terminal domain with its hydrophobic side facing the interior pocket in its compact solution structure (closed conformation). This N-terminal pocket is more likely specialized for the transfer of neutral lipids whereas the C-terminal pocket might be involved in the transfer of phospholipids (Bruce et al., 1998b).

Although site-directed mutagenesis by Jiang et al. (1995) suggested that Lys-237 and Arg-259 might be involved in lipoprotein binding, the predicted model of CETP suggest otherwise since these residues are not located on the concave side of the protein (Bruce et al., 1998b).

The proposed mechanism of lipid transfer in light of this model may involve the binding of CETP to lipoproteins by residues on the concave side of the protein (in the open conformation) which causes the disordering of the phospholipid monolayer. Once a ligand is bound, the protein dissociates and forms a compact structure (closed conformation) until it encounters another lipoprotein (Bruce et al., 1998b). This would suggest that the mechanism of lipid transfer favours the carrier model rather than the ternary complex model of lipid transfer.

More recently, the structure of PLTP was proposed based on the crystal structure of BPI (Huuskonnen et al., 1999). This structure shows that PLTP also has two lipid binding sites. Site-directed mutagenesis was used to confirm functionality of the predicted essential residues.

D) Regulation of CETP

Plasma CETP activity is regulated by many factors. As mentioned previously, there are various transcriptional regulatory elements in the promoter region of the CETP gene that up regulate transcription in response to cholesterol. There is also the potential for post-translational regulation by the alternatively spliced exon 9-deleted transcript. The activity of CETP in the plasma is obviously influenced by its concentration. However, in a normolipidemic subject the amount of CETP is usually not rate-limiting. Hence in the plasma, it is more probable that CETP is regulated by the relative concentration of lipoprotein substrates and their composition, by CETP inhibitors, lipolytic enzymes, and dyslipidemias.

In humans, the majority of the CETP is associated with HDL and to a lesser extent with LDL and VLDL (Pattnaik and Zilversmit; 1979; Morton, 1985). The reciprocal transfer and net exchange of cholesteryl esters and triglycerides amongst the plasma lipoproteins are dependent on the relative abundance of donor and acceptor particles and their lipid composition, which can be influenced by the occurrence of dyslipidemia. For example, an increase in triglyceride-rich lipoproteins increases the net mass transfer of cholesteryl esters from HDL to these particles (Tall et al., 1986; Dousset et al., 1988; Mann et al., 1991; Guérin et al., 1994). Hypertriglyceridemic mouse models expressing human CETP also exhibited an increase cholesteryl ester transfer to apoB-containing lipoproteins (Hayek et al., 1993).

The binding of CETP to lipoproteins is also influence by the charge on the lipoproteins. An increase in negative charges increases CETP binding. Sammet and Tall (1985) showed that lipolysis of VLDL by endothelial lipases increased the binding of

CETP to VLDL as a result of enrichment of VLDL with negatively charged fatty acids, which subsequently increases the rate of cholesteryl ester transfer from HDL to VLDL. Furthermore, chemical modification or addition of anionic detergents to increase the negative charge of LDL enhanced the binding of CETP to these particles (Nishida et al., 1993). Lipolysis also increases the binding of CETP to HDL as a result of phospholipid and fatty acid enrichment (Tall, 1993). Increased transfer of cholesteryl ester from HDL to other lipoprotein fractions is also observed when HDL is treated with hepatic lipase (Clay et al., 1990).

The enrichment of lipoproteins with cholesterol and phospholipids also stimulates cholesteryl ester transfer (Tall et al., 1986; Morton, 1988; Morton and Steinbrunner, 1990). *In vitro* studies showed that an increase in the cholesterol content on the surface of lipoproteins decreased the ability of VLDL and LDL but not HDL to donate cholesteryl esters. Although there is no specific cofactor for CETP, various apolipoproteins appear to influence CETP activity. ApoA-I, apoA-II, apoE, and apoC-III were all found to stimulate neutral lipid transfer by CETP *in vitro* (Ohnishi and Yokoyama, 1993). Sparks et al. (1989) demonstrated that an increase in the apoA-II / apoA-I ratio in recombinant HDL increased the maximal CETP-mediated transfer rate. In contrast, Rye et al. (1992) showed that particles containing apoA-I and apoA-II impaired the ability of CETP to reduce the size of reconstituted HDL. Furthermore, the substitution of apoA-II for apoA-I in isolated HDL progressively reduced the ability of the HDL to serve as a donor or acceptor in cholesteryl ester transfer reactions (Lagrost et al., 1994). These results appear to show that apoA-II might be an inhibitor of CETP. However, when CETP transgenic mice were bred with transgenic mice expressing human

apoA-II, reductions in HDL and increases in VLDL cholesteryl esters were observed suggesting that apoA-II does not inhibit CETP (Zhong et al., 1994).

Lipid transfer inhibitor proteins (LTIP) that inhibit the activity of CETP have been reported and isolated from human (Morton and Zilversmit, 1981; Son and Zilversmit, 1984; Nishide et al, 1989), baboon (Kushwaha et al., 1993), and hog plasma (Cho et al., 1998). Recently, Wang et al. (1999) cloned a CETP inhibitor that was shown to be identical to apolipoprotein F. Inhibitor proteins that inhibit transfer of cholesteryl ester, triglyceride and phospholipid exchanges by CETP isolated from human plasma have an apparent molecular weight ranging from 29 to 35 kDa. The inhibition of lipid transfer by LTIP can be overcome by increased concentrations of lipoproteins suggesting that the inhibitor is substrate associated and not the result of a direct interaction with CETP (Son and Zilversmit, 1984). It was later shown by Morton and Green (1994) that the LTIP was associated with LDL and had a substrate selective effect on cholesteryl ester transfer. Increasing the levels of LTIP in plasma increased the mass transfer of lipids between HDL and VLDL as LTIP selectively inhibits the ability of LDL to act as an acceptor of cholesteryl ester. More recent studies by Serdyuk and Morton (1999) revealed that the preference for HDL cholesteryl ester transfer to VLDL is not caused by the increased association of CETP with HDL but rather by the presence of LTIP on LDL. The effectiveness of lipid transfer activity in the plasma seems to be determined by both the CETP and LTIP activity. These studies have also led to the suggestion that low CETP activity observed in species such as rodents and pigs may be related to the inhibition of CETP activity by LTIP.

E) Transgenic animals

Earlier work in determining the physiological significance of plasma CETP was performed by injecting purified human CETP into rats, which normally lack significant plasma CETP activity, and studying the change in the lipoprotein profile (Ha et al., 1985). The results showed a total loss of the large, cholesteryl ester and apoE-rich HDL (HDL_1) fraction normally predominant in the rat plasma with concomitant increase in cholesteryl ester content of the VLDL. In addition, triglyceride enriched HDL was also observed. The development of CETP transgenic mice has provided further insights into the role of CETP in both lipoprotein metabolism and atherogenesis. The over-expression of human CETP in mice (1 to 5-fold greater than in normolipidemic human plasma) causes a 30-40% decrease in HDL cholesterol levels and apoE-rich HDL (Dinchuk et al., 1995). HDL size and apoA-I content was also decreased while LDL cholesterol content and size was modestly increased. Expression of human CETP at levels comparable to human plasma, on the other hand, also showed a marked decrease (20-30%) in HDL cholesterol levels (Agellon et al., 1991) but no significant changes in the total cholesterol content of VLDL and LDL, or changes in HDL size or apoA-I content were observed. There was, however, a decrease in the cholesterol/cholesteryl ester ratio in the plasma and all lipoprotein fractions of the transgenic mouse plasma suggesting a stimulation of cholesterol esterification.

In two separate studies (Hayek et al., 1992; Francone et al., 1996), where CETP transgenic mice were crossed with human apoA-I transgenic mice, HDL cholesterol and apoA-I levels were dramatically reduced. The co-expression of CETP with human apoA-I (HuAICETPTg) resulted in the human-like speciation of HDL in the mouse plasma.

The formation of distinctive HDL_2 and HDL_3 subclasses was apparent (Hayek et al., 1992). Francone et al. (1996) also observed an increase in pre-β HDL fractions (HDL_{3a} and HDL_{3c}) at 1.4 and 2.2-fold respectively. Furthermore, HDL containing human apoA-I had a higher affinity for CETP than mouse HDL and were better substrates for human CETP (Hayek et al, 1992). Francone et al. (1996) reported that the HuAICETPTg mice also had a greater ability to promote cholesterol efflux from fibroblasts than plasma from HuAITg mice. In addition, CETP over-expression also induced a 1.4-fold increase in plasma LCAT activity.

In order to study the effects of hypertriglyceridemia on CETP expression, CETP transgenic mice were bred with mice over-expressing human apoC-III that were previously shown to be hypertriglyceridemic (Hayek et al., 1993). In the presence of hypertriglyceridemia, CETP caused a dramatic reduction in HDL cholesterol, apoA-I, and HDL particle size. The decrease in both the HDL cholesteryl ester and apoA-I was attributed to an increase in fractional catabolic rate as indicated by turnover studies. These results suggest that in hypertriglyceridemic individuals, the decrease in HDL cholesterol levels is associated with an increase in CETP activity (Tall, 1995).

The increase in VLDL and LDL cholesterol with a concomitant increase in apoB levels observed in transgenic mice over-expressing human CETP is a result of the down-regulation of hepatic LDL receptors (Jiang et al., 1993). An increase in cholesterol and cholesteryl ester content in the liver and a reduction in HMG-CoA reductase mRNA was also apparent, which suggest that the expression of CETP enhanced the return of cholesterol to the liver (i.e. enhanced RCT) (Tall, 1995).

F) Genetic CETP Deficiency

CETP gene mutations result in decreased plasma CETP levels and increased HDL (hyperalphalipoproteinemia). In 1985, Koizumi et al. described two Japanese siblings with hyperalphalipoproteinemia that exhibited no cholesteryl ester transfer activity. Subsequently, a point mutation (G → A) in the 5'-splice donor site of intron 14 of the CETP gene was discovered in these patients (Brown et al., 1989). Since, several other unique mutations have been described in the Japanese (Takahashi et al., 1993; Gotoda et al., 1993; Inazu et al., 1994; Sakai et al., 1996; Arai et al., 1996) and European (Funke et al., 1994) populations (Table 3.1). Genetic CETP deficiency is quite prevalent in the Japanese population. Both the intron 14(A) (Brown et al, 1989) and Asp442 → Gly mutations (Takahashi et al., 1993) are common in hyperalphalipoproteinemic individuals (Inazu et al., 1994) especially in Omagari City (Hirano et al., 1997). The heterozygote frequency for the Int14(A) and Asp-442 → Gly mutations are 2% and 7% respectively (Hirano et al., 1993; Inazu et al., 1990; Inazu et al., 1994). The intron 14 defects (Brown et al., 1989; Inazu et al., 1990) and the Asp-442 → Gly mutation that is partially expressed appear to be genetically dominant.

The phenotypic expression of CETP deficiency include elevated total plasma cholesterol with markedly increased HDL cholesterol and apoA-I levels (Table 3.2). Large HDL species enriched in apoE also accumulate in the HDL and LDL density ranges. Consequently, both apoA-I and apoE levels are elevated. An increased HDL_2 / HDL_3 ratio is often observed. HDL are enriched in cholesteryl esters and poor in triglycerides compared to control individuals. Conversely, the cholesteryl ester content in the VLDL and LDL are decreased but enriched in triglycerides. Furthermore, the LDL

Table 3.1: Genetic CETP deficiency

Mutation	Location	Type	Effect	References
Δ C (German)	Exon 2	Ala-38 → STOP (frameshift)	CETP act. = 0	Funke et al., 1994
G → T (Japanese)	Exon 6	Gly-181 → STOP	CETP act. = 0	Arai et al., 1996
C → A (Japanese)	Exon 10	Gln-309 → STOP	CETP act. = 0	Gotoda et al., 1993
T → G (Japanese)	Intron 10	splice donor site mutation (+2)	CETP act. =0	Sakai et al., 1996
G → C (German)	Exon 12	Ala-373 → Pro	HDL ↑	Funke et al., 1994
G → A (Japanese)	Intron 14	splice junction site mutation (+1)	CETP act. = 0	Inazu et al., 1990
Insert T (Japanese)	Intron 14	splice junction site mutation (+3)	CETP act. = 0	Inazu et al., 1994
A → G (Japanese)	Exon 15	Asp-442 → Gly	CETP act. ↓	Inazu et al., 1994
G → A (German)	Exon 15	Arg-451 → Gln	HDL ↑	Funke et al., 1994
A → G	Exon 14	Ile-405 → Val (polymorphism)*	HDL ↑	Funke et al., 1994

Reported mutations in the human CETP gene. * represents a polymorphic site. Elevated HDL cholesterol concentrations are significantly associated with this polymorphism (Funke et al., 1994). Hypertriglyceridemic men homozygous for this polymorphism had a higher prevalence for coronary heart disease (Bruce et al., 1998a). Modified from Teh et al., 1998.

Table 3.2: Lipid values in CETP deficiency

	CETP Deficiency		
Lipids	Homozygotes	Heterozygotes	Controls
		mmol/l	
Total cholesterol	7.01 + 0.83*	5.04 ± 1.14* 5.59 ± 1.08† 5.87 ± 0.86#	4.45 ± 0.59* 4.40 ± 0.44† 4.40 ± 0.57#
HDL cholesterol	4.24 + 1.01*	1.17 ± 0.39* 2.64 ± 0.72† 1.74 ± 0.70#	1.16 ± 0.26* 1.24 ± 0.28† 1.24 ± 0.21#
LDL cholesterol	1.99 + 0.80*	2.87 ± 1.11* 2.81 ± 0.45#	2.77 ± 0.42* 2.90 ± 0.30#
Triglycerides	1.69 + 1.34*	1.07 ± 0.59* 1.10 ± 0.52† 1.03 ± 0.47#	0.98 ± 0.34* 0.87 ± 0.29† 0.99± 0.28#
		g/l	
ApoA-I	2.13 + 0.47*	1.49 ± 0.43* 2.12 ± 0.33† 1.50 ± 0.31#	1.17 ± 0.20* 1.34 ± 0.16† 1.34 ± 0.13#
ApoB	0.54 + 0.14*	0.66 ± 0.20* 0.81 ± 0.20† 0.85 ± 0.31#	0.89 ± 0.13* 0.83 ± 0.16† 0.82 ± 0.12#
CETP mass (mg/l)	0	1.4 ± 0.3*	2.2 ± 0.6*
CETP activity (units)	0	8.5 ± 2.7†	20.4 ± 2.1†

Literature values (* Inazu et al., 1990; † Hirano et al., 1993; # Hirano et al., 1997) for the plasma parameters observed in CETP deficiency compared to controls.

cholesterol and apoB levels in homozygotes for the intron 14 splicing defect are decreased and the LDL is polydisperse with speciation into four or five distinctly size subspecies (Yamashita et al., 1988; Sakai et al., 1991). Interestingly, no reduction of LDL cholesterol and apoB are seen in the Asp-442 → Gly mutation that have detectable CETP activity (Inazu et al., 1994) suggesting that changes in LDL occurs only in the complete absence of CETP (Tall, 1995).

G) Objective of the Study

The objective of this study was to completely characterize the metabolic defect(s) in a patient who presented with hypercholesterolemia and was unresponsive to drug treatment. Initial lipid analyses revealed that the cause of hypercholesterolemia was associated with elevated levels of HDL cholesterol (hyperalphalipoproteinemia).

H) Results

Lipoprotein and apolipoprotein analysis

The plasma lipid values of the proband while still on Pravachol® (40 mg daily) are shown in Table 3.3. Her total plasma cholesterol (7.3 mmol/l; Normal: 4.4 ± 0.57 mmol/l), cholesteryl esters (5.14 mmol/l; Normal: 2.53 ± 0.10) and phospholipids (4.60 mmol/l; Normal: 1.83 ± 0.08) were markedly elevated. She was also mildly hypertriglyceridemic (1.59 mmol/l; Normal: 0.8-1.0 mmol/l). Her plasma apoA-I levels was 2-fold elevated and her apoE level was almost 3-fold increased compared to control subjects. Her apoB levels are near normal compared to control subjects. The increases in total plasma cholesterol and phospholipids were accounted for by the very pronounced

Table 3.3: Plasma lipids and apolipoproteins in CETP deficiency

Lipid	Whole Plasma	VLDL	LDL	HDL
		mmol/l		
Total cholesterol	7.34	0.24	2.92	4.38
Free cholesterol	2.20	0.20	1.10	1.00
Cholesteryl esters	5.14	0.04	1.82	3.38
Triglycerides	1.59	1.11	0.59	<0.01
Phospholipids	4.60	0.28	1.32	30.5
Apolipoproteins	*mg/dl*			
ApoA-I	256.0			
ApoB	94.0			
ApoE	14.4			

All lipid values were determined enzymatically as described in Methods. Plasma apolipoproteins were quantified by electroimmunoassay (Dolphin et al., 1984). Lipoprotiens were isolated by sequential ultracentrifugation at the following densities: VLDL, *d:* 1.006 g/ml; LDL, *d:* 1.006-1.063 g/ml; HDL, *d:* 1.063-1.2. g/ml. From Teh et al., 1998.

increase in HDL lipid mass. Her plasma LCAT activity was significantly lower (29 nmol cholesteryl ester formed / ml plasma per h) than the control (46.4 nmol cholesteryl ester formed / ml plasma per h). The low LCAT activity is probably a result of decreased substrate efficiency (large HDL_2) that subsequently accounted for the increased ratio of cholesterol to cholesteryl ester when compared to normal subjects. Analysis of the proband's lipoprotein lipid composition after isolation by density gradient ultracentrifugation is shown in Table 3.4. The weight % content of both the VLDL and LDL showed a marked decrease in their content of cholesteryl esters (Normal VLDL: 14%; LDL: 42%) with an increase in triglyceride content (Normal VLDL: 53%; LDL: 4%). The reverse pattern was observed within the HDL fraction where both the HDL_2 and HDL_3 subspecies had an elevated content of cholesteryl esters (Normal HDL_2: 20%; HDL_3: 16%) and a decreased triglyceride content (Normal HDL_2: 2%; HDL_3: 1%) when compared to normal subjects. Significantly, the relative content of HDL phospholipids is decreased, which indicates the presence of a higher proportion of larger HDL particles than normal.

Figure 3.4 represents the plasma lipids of the proband and a control female subject. The normal lipoprotein distribution of the control subject shows VLDL in fractions 1 and 2 ($d < 1.019$ g/ml), LDL represented in fractions 5-12 (*d:* 1.024-1.063 g/ml) with the peak in fraction 7 and the HDL in fractions 12-24 (*d:* 1.058-1.190 g/ml). The peak in HDL is seen in fraction 17. The lipoprotein distribution of the CETP deficient proband on the other hand, was skewed to a lower density range. Most significantly, the HDL fraction was elevated and represented the predominant plasma lipoprotein species. The majority of the HDL was in the HDL_2 density range (fractions

Table 3.4: Lipoprotein composition in CETP deficiency

Lipid	VLDL	LDL	HDL_2	HDL_3
		wt %		
Cholesterol	9.3	14.4	10.4	6.1
Cholesteryl esters	1.8	37.2	40.2	45.7
Triglycerides	67.3	18.9	3.3	2.5
Phospholipids	21.6	29.5	46.1	45.6
CE/TG mass	0.027	1.97	12.34	18.43
C/PL mass	0.43	0.49	0.23	0.13

Lipoproteins were isolated by density gradient ultracentrifugation as described in Methods and the lipids were quantified by gas chromatographic total lipid profiling (Kuksis et al., 1978). From Teh et al., 1998.

10-17; *d:* 1.044-1.112 g/ml). The plasma apolipoproteins distribution of both the proband and the control subject are shown in Figure 3.5. ApoB is associated with the lower density lipoproteins and hence their normal distribution is predominantly in the fractions 1-12. Fraction 7 of the lipid distribution coincides with the peak in apoB while the peak for HDL in fraction 17 coincides with the apoA-I peak. ApoE was detected in fraction 1-5 (*d:* 1.006-1.026 g/ml) and 11-18 (*d:* 1.050-1.121 g/ml) in the density range normally occupied by HDL_2. The apoA-I peak for the CETP deficient subject had also shifted to a lower density range and was present in fraction 13 (*d:* 1.068 g/ml). Similarly, the apoE had shifted from fractions 14-15 (*d:* 1.072-1.091 g/ml) in the control subject to fraction 11 (*d:* 1.054 g/ml) in the CETP deficient subject. These data are consistent with the previously reported hyperalphalipoproteinemia in CETP deficiency that showed an increased presence of a higher proportion of large HDL_2-like particles. The molar ratios of cholesteryl ester to apoB for the apoB-rich lipoprotein fractions isolated by ultracentrifugation (Figure 3.4 and 3.5) from the proband and a normal female subject are shown in Table 3.5. The cholesteryl ester / apoB ratio for the VLDL and IDL fractions of the CETP-deficient subject was much lower than that observed for the normal female. In contrast, the LDL cholesteryl esters / apoB ratio from both the proband and the normal were essentially equivalent. These data suggest that the VLDL and not the LDL cholesteryl esters / apoB ratio was markedly affected by an absence of CETP.

CETP activity assay and mass determination

The ability of the proband's plasma to catalyse the reciprocal transfer of cholesteryl esters and triglycerides between lipoprotein species were evaluated by comparing post-incubation values at 4 °C with those obtained after incubation at 37 °C.

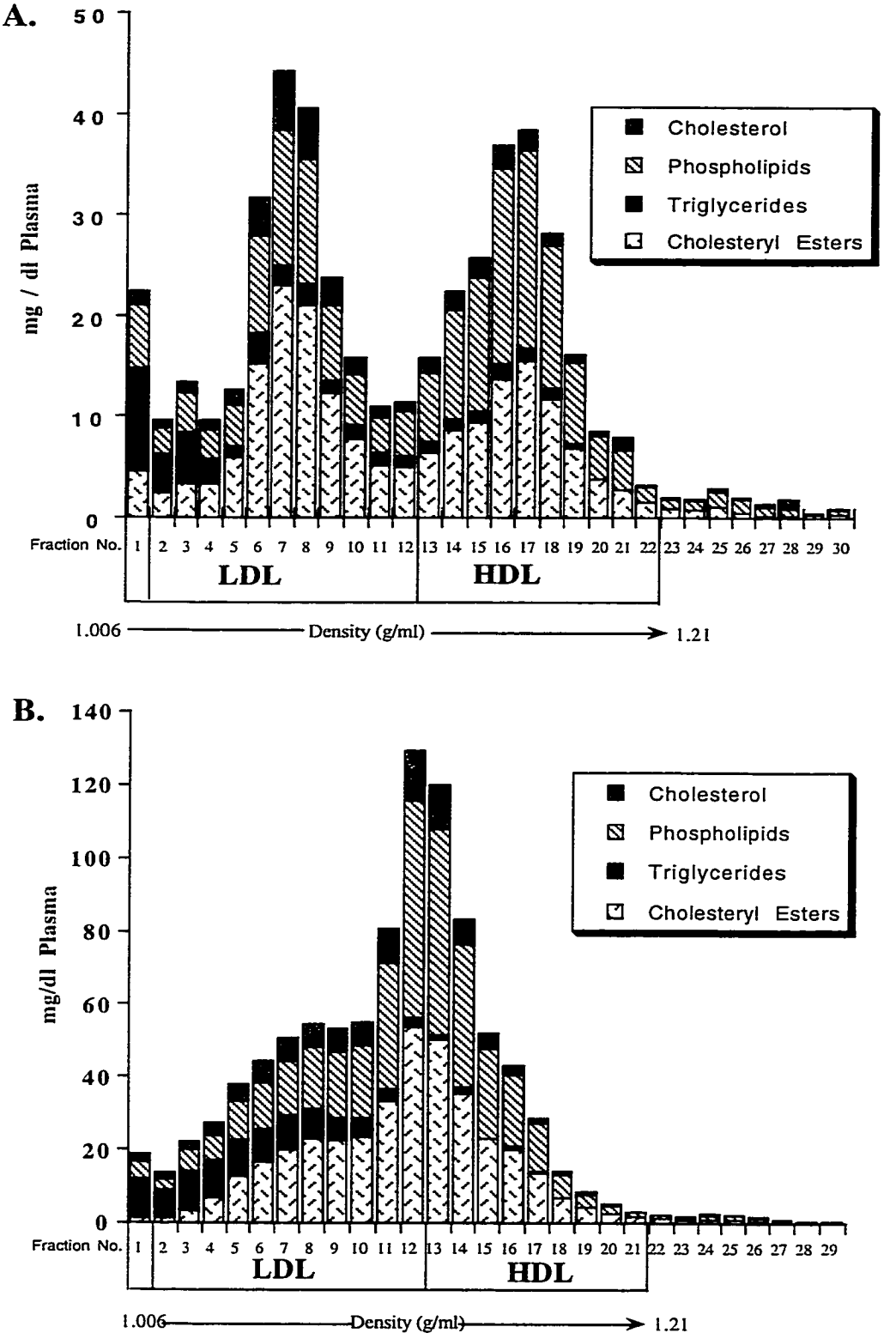


Figure 3.4: Plasma lipid distribution of a normolipidemic individual (A) and the CETP deficient deficient proband (B) fractionated by discontinuous density gradient ultracentrifugation. From Teh et al., 1998.

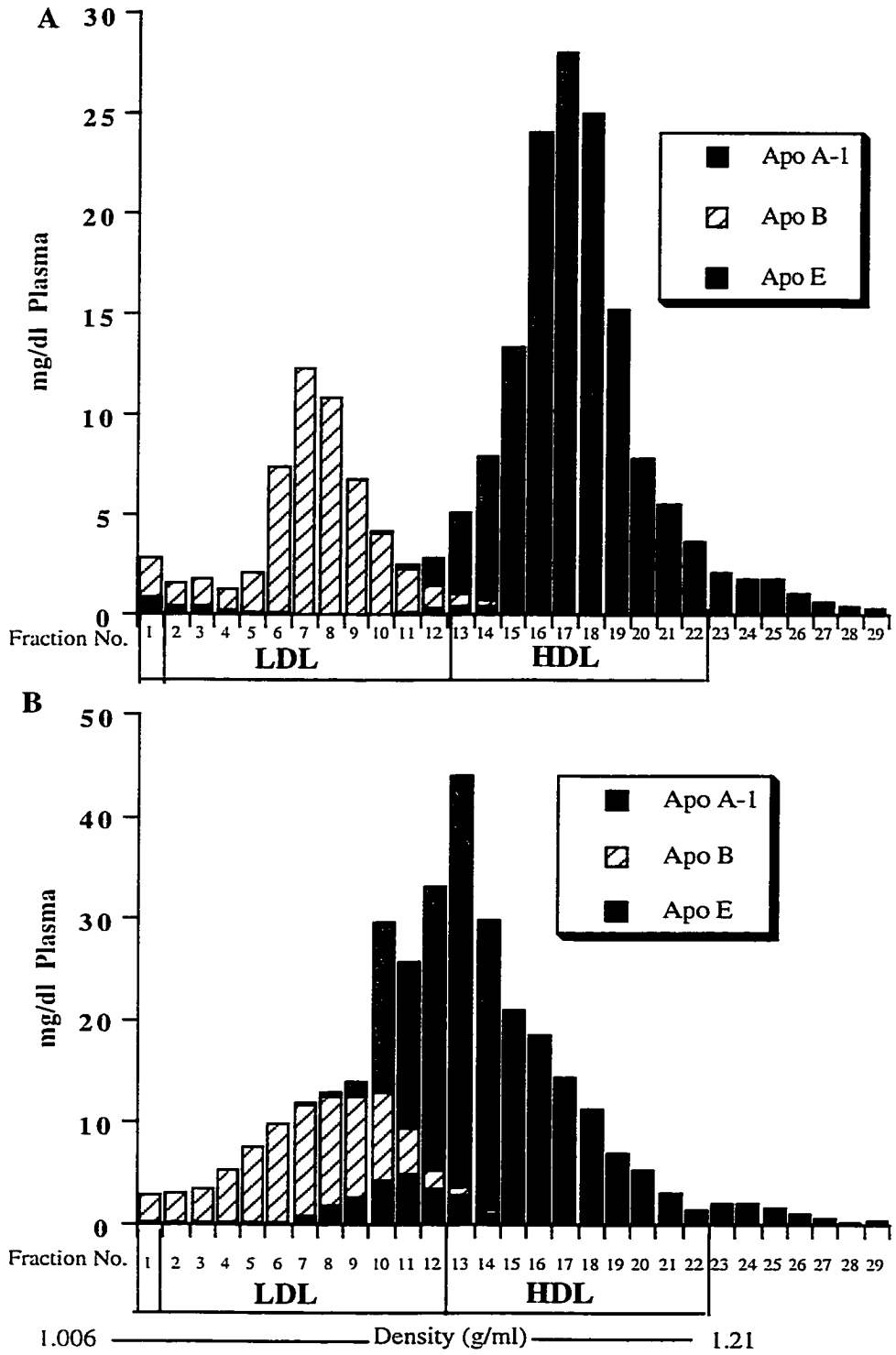


Figure 3.5: Plasma apolipoprotein distribution of the (A) a control subject and (B) proband. The apolipoproteins were quantitated by electroimmunoassay by the method of Dolphin et al. (1984) and total protein was measured by the Lowry method (Markwell et al., 1978). From Teh et al., 1998.

Table 3.5: Molar proportions of cholesteryl ester and apoB in apoB-rich fractions from density gradient ultracentrifugation.

Density gradient Fraction		CE/apoB molar ratio CETP-deficient subject	Control female subject
1	VLDL	330	1825
2		307	1668
3		739	1920
4		1015	2479
5		1362	2353
6		1345	1636
7		1427	1489
8		1707	1535
9		1813	1448

Molar proportions of cholesteryl ester to apoB were calculated from the lipid and apolipoprotein analyses of the density gradient ultracentrifugal fractions of the CETP-deficient subject and the normal control female individual in Figs. 3.4 and 3.5. VLDL is present in fractions 1 and 2, IDL in fractions 3 and 4, and LDL in fractions 5-9. The major apoB-containing fraction was fraction 7 in both individuals. From Teh et al., 1998.

While the control subject's VLDL showed the expected augmentation in cholesteryl ester and a decrease in triglyceride content with reciprocal changes in the LDL + HDL fraction, no augmentation of VLDL total or cholesteryl ester or significant decrease in VLDL triglycerides were observed for the proband (Table 3.7). These results are consistent with an absence of CETP-mediated reciprocal transfer of cholesteryl esters and triglycerides in the plasma of the proband.

In order to evaluate whether the CETP protein was present or absent from the plasma of the proband, a radioimmunometric, double antibody sandwich assay utilizing monoclonal antibodies that had been previously shown to inhibit human plasma CETP activity was performed (Clark et al., 1995). The assay was performed using plasma from three controls diluted 1 in 40–50 dilution as recommended and undiluted plasma from the proband. The values for CETP mass obtained from the controls ranged from 1.45 to 3.26 mg/ml and fell within the previously reported normal range (Clark et al., 1995). There was no detectable mass in the undiluted plasma of the proband indicating that at least one of the epitopes recognized by the primary monoclonal antibody utilized in this assay was absent.

Analysis of the cholesteryl ester and phospholipid fatty acid composition

There are two sources from which cholesteryl esters can be derived: intracellular acyl-CoA:cholesterol acyltransferase (ACAT) or plasma LCAT. Human LCAT exhibits a preference for the transesterification of linoleic acid (C18:2) from the sn-2 position of phosphatidylcholine to cholesterol forming cholesteryl linoleate. In contrast, hepatic ACAT prefers to utilize the CoA esters of stearic (C18:0) and oleic acid (C18:1). In the absence of CETP-mediated lipid transfer it would be expected that the VLDL would

contain cholesteryl esters predominantly derived through the esterification of cholesterol by hepatic ACAT, whereas the LDL and HDL, both of which are substrates (to varying degrees) for LCAT would contain a significant proportion of LCAT-derived cholesteryl esters. In the absence of exchange, the origin of plasma cholesteryl ester can be determined. The fatty acid compositions of the cholesteryl esters and phospholipids isolated from the major lipoprotein classes present in the proband and control plasmas are shown in Table 3.6. Owing to pronounced shift in hydrated density of the HDL in the CETP-deficient subject, care was taken to exclude fractions containing significant amounts of apoA-I from the LDL fraction when preparing lipoprotein fractions for fatty acid analysis. Therefore, fractions 1 and 2 (d < 1.019 g/ml) constituted the VLDL, fractions 5-9 (d: 1.023-1.044 g/ml) constituted the LDL, and fractions 12-22 (d: 1.058-1.167 g/ml) constituted the HDL fraction used for analyses. Almost 50% of the cholesteryl ester present in the VLDL of the patient is cholesteryl stearate (C18:0), which is in marked contrast to the control subject VLDL where cholesteryl linoleate (C18:2) predominates, followed by cholesteryl oleate (C18:1) and cholesteryl palmitate (C16:0). Hence, the cholesteryl ester 18:1 / 18:2 ratio is 2.36 in the patient's VLDL compared to the controls 0.58 $\pm$ 0.08. In contrast, there was no difference in the fatty acid composition of both the LDL and HDL from the control subjects and the proband, where the predominant cholesteryl ester fatty acids were cholesteryl linoleate (C18:2) and cholesteryl oleate (C18:1), and therefore the cholesteryl ester 18:1 / 18:2 were similar. These ratios are represented graphically in Figure 3.6. However, the proportion of cholesteryl stearate (C18:0) was significantly reduced in the patient's LDL and HDL when compared to VLDL. Furthermore, the phospholipid fatty acid composition present

Table 3.6: Lipoprotein cholesteryl ester and phospholipid fatty acid compositions

	VLDL		LDL		HDL	
Fatty Acid	Control	Patient	Control	Patient	Control	Patient
			wt %			
Cholesteryl ester fatty acids						
16:0	14.1 ± 2.0	19.4	12.7 ± 3.6	14.5	15.8 ± 1.4	17.3
16:1	3.1 ± 2.3	0.0	4.0 ± 0.8	4.0	5.6 ± 2.4	3.8
18:0	11.6 ± 8.8	59.8	3.9 ± 1.7	4.5	5.3 ± 1.9	2.0
18:1	19.3 ± 5.6	9.9	22.3 ± 4.6	21.7	27.9 ± 6.1	29.4
18:2	33.6 ± 7.4	4.2	41.7 ± 5.6	48.6	41.6 ± 4.4	43.0
18:3	8.1 ± 3.4	4.0	1.8 ± 0.5	3.1	3.8 ± 2.0	1.2
20:4	3.3 ± 1.6	2.7	2.8 ± 1.2	3.7	4.4 ± 0.5	3.2
18:1/18:2	0.58 ± 0.08	2.36	0.57 ± 0.06	0.45	0.68 ± 0.2	0.68
Phospholipid fatty acids						
16:0	37.2 ± 3.5	30.2	38.5 ± 1.8	35.4	38.6 ± 4.0	32.8
16:1	0.9 ± 1.1	0.0	1.9 ± 0.2	0.0	1.4 ± 0.9	1.7
18:0	17.1 ± 1.8	26.2	15.6 ± 1.0	15.8	15.5 ± 1.9	14.5
18:1	17.2 ± 2.4	15.5	17.2 ± 2.0	19.7	17.4 ± 2.7	20.5
18:2	21.2 ± 0.8	6.3	21.4 ± 1.8	21.9	20.7 ± 0.8	22.1
18:3	0.0 ± 0.0	0.0	0.2 ± 0.2	0.0	0.0 ± 0.0	0.0
20:4	6.3 ± 1.6	21.8	5.4 ± 1.5	7.2	6.5 ± 1.5	8.4
18:1/18:2	0.82 ± 0.13	2.47	0.83 ± 0.16	0.90	0.84 ± 0.14	0.93

Lipoprotein fractions were isolated from plasma by density gradient ultracentrifugation. Fractions 1 and 2 (d < 1.019 g/ml) (Figure 3.4) constituted the VLDL, fractions 5-9 (d: 1.023-1.044 g/ml) constituted the LDL fraction, and fractions 12-22 (d: 1.058-1.167 g/ml) constituted the HDL fraction and were used for the analyses. Lipids were extracted from the lipoprotein fractions by the method of Folch et al. (1957), separated by thin-layer chromatography as described in Methods. The isolated cholesteryl esters and phospholipids were transmethylated (Morrison and Smith, 1964) and analyzed by gas chromatography. Control values were obtained as described (n=6). From Teh et al., 1998.

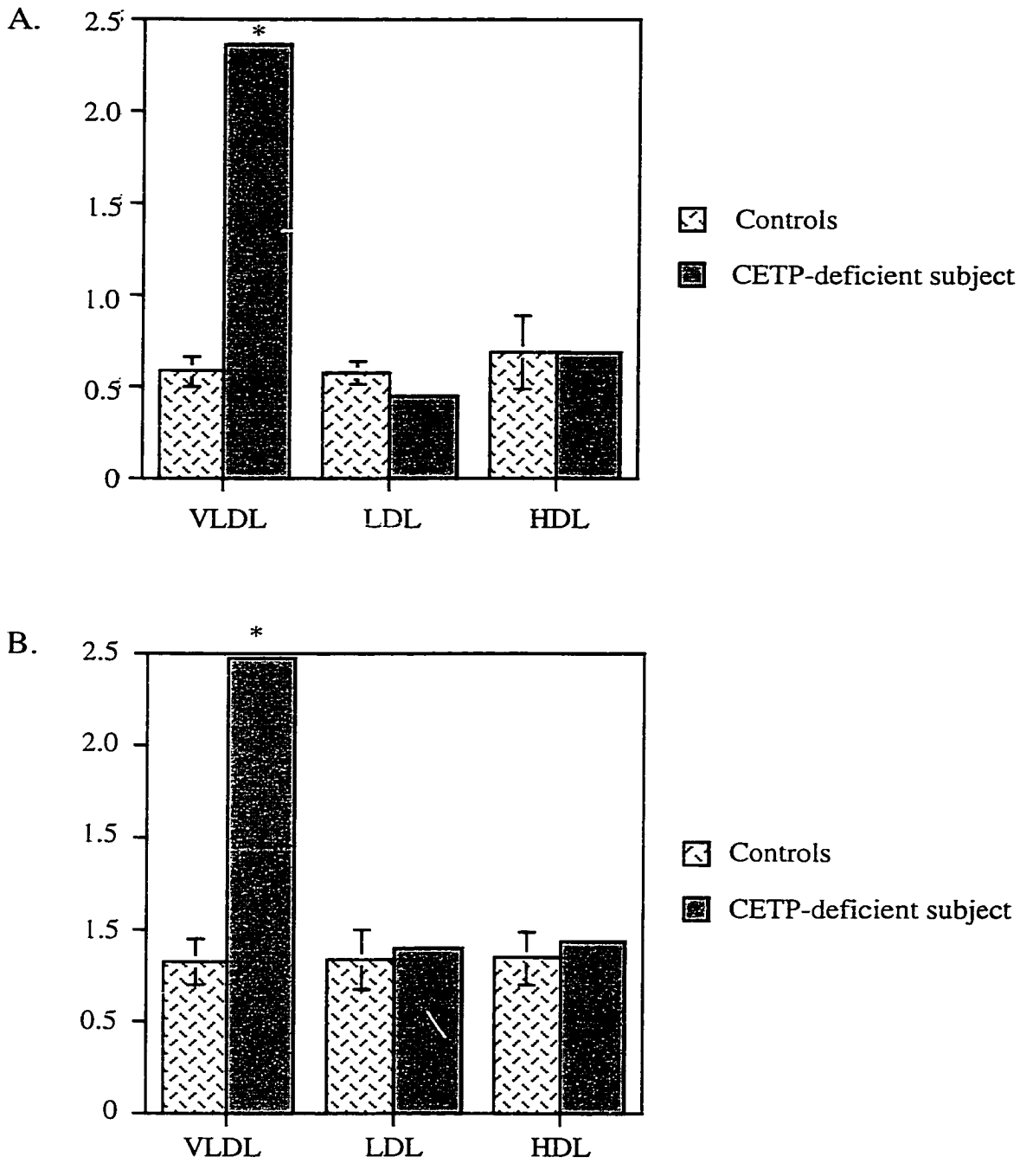


Figure 3.6: The C18:1 / C18:2 ratio of the fatty acid component from A. cholesteryl esters and B. phospholipids for each lipoprotein fraction from controls and the CETP-deficient subjects. The 18:1 / 18:2 fatty acid ratio from the cholesteryl esters and phospholipids of the VLDL was the only fraction that showed a significant difference as denoted by (*).

Table 3.7: Lipid transfer activity between lipoproteins in control and CETP-deficient plasmas

	Control				CETP-deficient			
				mmol/l				
Lipid	VLDL		LDL+HDL		VLDL		LDL+HDL	
	4°C	37°C	4°C	37°C	4°C	37°C	4°C	37°C
Total cholesterol	0.71	0.79	3.45	3.15	0.26	0.28	7.71	7.05
Cholesterol	0.40	0.39	0.89	0.86	0.24	0.26	2.25	2.05
Cholesteryl esters	0.31	0.40	2.58	2.29	0.02	0.02	5.46	5.00
Triglycerides	2.44	2.04	0.32	0.48	1.03	0.98	0.59	0.57

All incubations were performed at the indicated temperatures for 5h. The lipid mass was quantified using enzyme kits.

within the VLDL of the proband showed a marked decrease in linoleic (C18:2) and arachidonic acid (C20:4) compared to controls. In contrast, no differences in phospholipid fatty acid composition between the LDL and HDL fractions were observed. The fatty acid composition of the phospholipids within the lipoproteins of the controls were not significantly different with palmitate (C16:0), linoleate (18:2), oleate (18:1), and stearate (18:0) as the major species.

Sequence analysis of genomic DNA

The results above provided strong evidence for the possibility that the proband had a genetic mutation in the CETP gene. The previously reported mutations in the human CETP gene are shown in Table 3.1. Both the G → A substitution at intron 14(+1) and the single A → G substitution in exon 15 are relatively common in the Japanese population. Based on the known mutations at exon/intron 14, exon 15, 2, 6, 10, and 12, the proband's exons were amplified by PCR and either sequenced or digested with specific restriction enzymes as previously reported. Failing to find a mutation(s), all exons and intron/exon boundaries were subsequently amplified by PCR, cloned and sequenced. A single C → T substitution was identified at nucleotide 836 in exon 9 that replaces the Arg-268 with a nonsense codon (Figure 3.7). Bidirectional sequencing of three clones from two separate PCR products of exon 9 from the proband all showed the single C → T substitution. In addition, direct sequencing of the PCR product from exon 9 was also performed to determine the genotype. The results showed that the proband was homozygous for this mutation. The entire coding region including the exon/intron junctions of the proband's CETP gene was sequenced and showed no additional mutations.

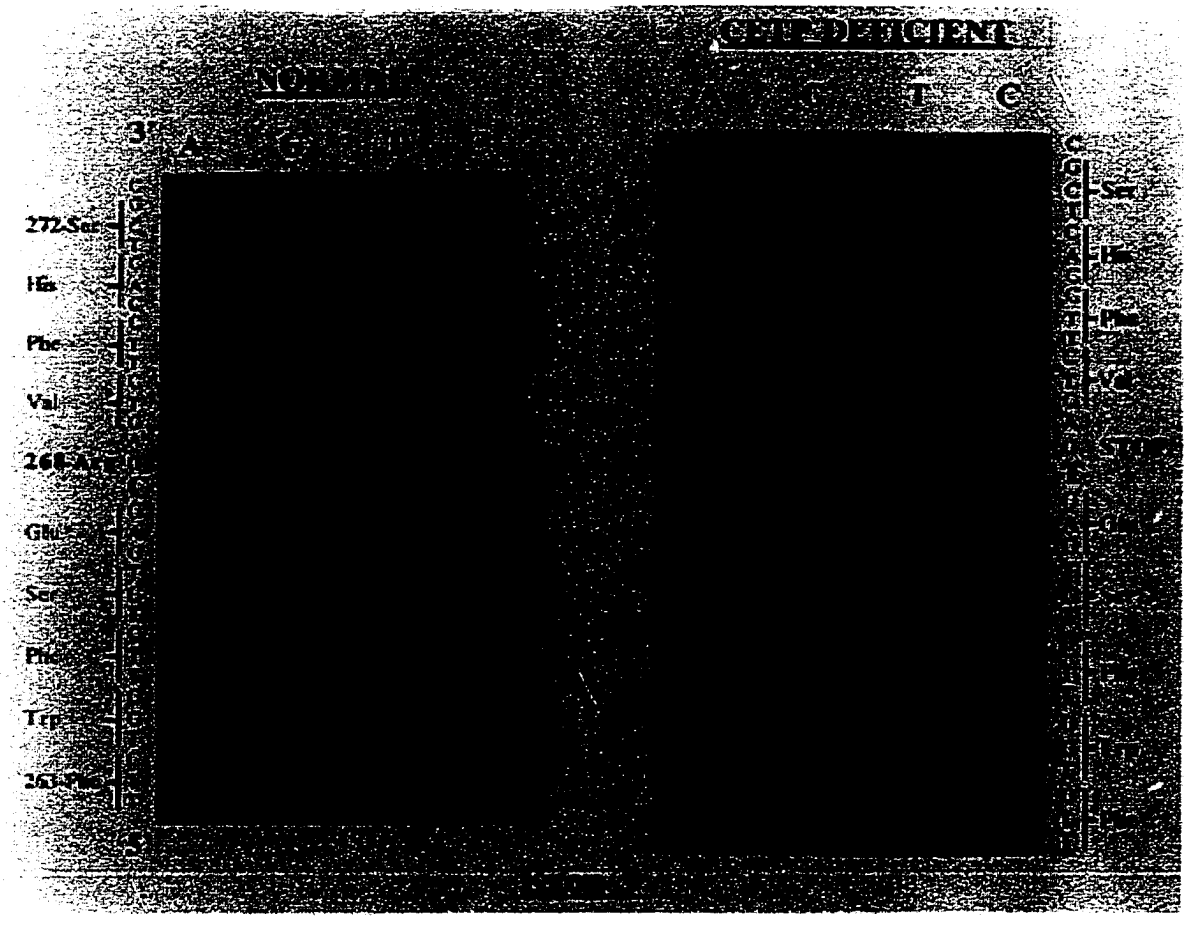


Figure 3.7: DNA seqence of exon 9 of the CETP gene. A C-->T substituition identified at nucleotide 836 introduced a nonsense codon at position 268 of the protein. From Teh et al., 1998.

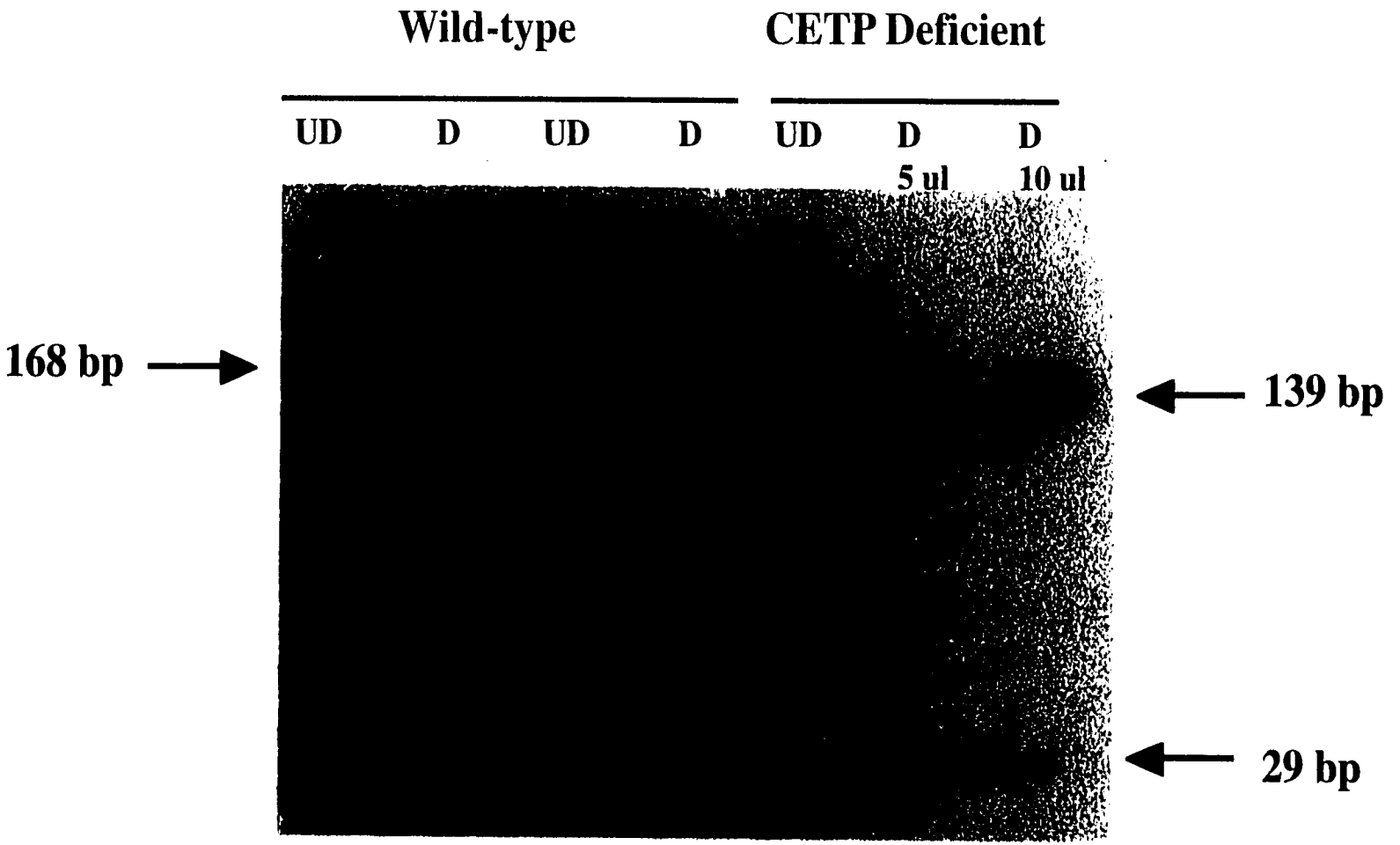


Figure 3.8: Rapid diagnosis of the exon 9 CETP gene mutation using MaeIII. Both the wild-type and mutant sequences were amplified using a mismatched primer that produced a recognition site for MaeIII in the mutant sequence. The digested mutant sequence produced two samller fragments at 139 bp and 29 bp from the 168 bp product (see Methods, Figure 2.1). (UD: undigested; D: digested). From Teh et al., 1998.

The C → T substitution did not result in a loss or gain of a unique restriction site that would allow for rapid screening. Thus, a site was introduced by PCR using a 26 bp-mismatched primer (see Methods, Figure 2.1) that resulted in a restriction site for *Mae*III in the presence of the C → T mutation. The results of the digestions are shown in Figure 3.8. The 168 bp PCR product of exon 9 from the proband was cleaved by *Mae*III into two fragments of 139 bp and 29 bp but no cleavage was observed in the controls. This result also confirms that the proband is homozygous for the mutation as there was no evidence of an uncut 168 bp band.

I) Discussion

The proband investigated in this study is a 57 year-old Caucasian female Nova Scotian without Japanese ancestry who presented with hypercholesterolemia later shown to be due to a marked hyperalphalipoproteinemia (Table 3.3). In concert with other reports of CETP deficiency (Inazu et al., 1990; Arai et al., 1996) the proband's apoA-I levels were increased two-fold and her plasma apoE levels were three-fold elevated. The proband's apoB levels were near normal at 94 mg/dl (Normal: 100 + 10 mg/dl) and her LDL cholesterol was within the normal range when compared to controls. This is in contrast with other reports of Japanese individuals homozygous for this genetic defect (Brown et al., 1989; Inazu et al., 1990; Ikewaki et al., 1995; Arai et al., 1996) where apoB (60-70 mg/dl) and LDL cholesterol levels (1.99 ± 0.8 mmol/l) were low (Inazu et al., 1990). This decrease has been shown to be caused by the up-regulation of LDL receptors leading to an enhanced LDL catabolic rate (Ikewaki et al., 1995). LDL turnover studies were not conducted in the proband but it is possible that the

hypercatabolism of LDL in the Japanese individuals may be polygenic in nature and not totally dependent on the absence of plasma CETP activity (Teh et al., 1998). The presence of HDL enriched in cholesteryl esters and poor in triglycerides with VLDL and LDL rich in triglycerides but cholesteryl ester poor are indicative of a lack of reciprocal transfer and are consistent with the previous findings (Inazu et al., 1990) in homozygous CETP deficiency. In addition, the aberrant lipoprotein and apolipoprotein ultracentrifuge profiles (Figure 3.4 and 3.5) are similar to the previously reported gel filtration studies of plasma from CETP-deficient homozygotes (Koizumi et al., 1985; Bisgaier et al., 1991).

Analysis of the fatty acid composition of the lipoprotein cholesteryl esters and phospholipids were performed to evaluate the possible origins of individual cholesteryl esters. Bisgaier et al. (1991) reported that the majority of cholesteryl ester in the VLDL and LDL of CETP-deficient subjects arises from the action of intracellular (presumably mostly hepatic) acyl CoA:cholesterol acyltransferase (ACAT) and that LDL is utilized as an LCAT substrate only when the preferred substrate, HDL, is exhausted. Using similar analyses, the fatty acid composition of the lipoprotein cholesteryl esters and the C18:1 / C18:2 ratio were determined (Table 3.6). These results are in contrast to those previously reported by Bisgaier et al. (1991) and only partially substantiate the hypothesis. In the control individuals, the predominant cholesteryl ester fatty acids present in all the major lipoproteins were C18:2 > C18:1 > C16:0 with very similar relative proportions, and there was no significant difference between in the C18:1 / C18:2 ratio. It has been previously shown *in vitro* that plasma VLDL is qualitatively the best acceptor of HDL cholesteryl ester in normolipidemic individuals and the transfer of HDL cholesteryl ester to the apoB-containing lipoproteins is proportional to their relative concentrations in

plasma (Guérin et al., 1994). Furthermore, in addition to catalyzing a net mass transfer of cholesteryl ester, CETP also mediates cholesteryl ester exchange between the lipoproteins (Guérin et al., 1994). Hence with time, a complete equilibration of cholesteryl ester species between lipoproteins would occur and is indeed observed in Table 3.6. This was not apparent in the data reported by Bisgaier et al., (1991) particularly with respect to C18:0 which ranged from 6.4 $\pm$ 0.4 to 19.7 $\pm$ 4.6% of cholesteryl ester fatty acid across the lipoprotein spectrum and approximated the lower value in the *d:* 1.050-1.063 g/ml fraction. The predominant cholesteryl ester fatty acids in the VLDL of the proband were C16:0 and C18:0 with all other fatty acids present at less than 10% of the total. In the absence of CETP, it appears that the cholesteryl esters of ACAT origin are cholesteryl palmitate (C16:0) and cholesteryl stearate (C18:0). In contrast, the proband's LDL and HDL cholesteryl ester fatty acid content were very similar to that of the control fractions (C18:2 > C18:1 > C16:0) with relatively minor contributions from C18:0 (2.0-4.5%). These results suggest that the cholesteryl esters present in the HDL and LDL fraction of the proband are derived from the action of LCAT on both HDL and LDL.

The phospholipid fatty acid composition in the controls were quite similar in all the lipoprotein fractions and were present as C16:0 > C18:2 > C18:1 $\geq$ C18:0. Although previous studies have noted significant differences in phospholipid molecular species among lipoprotein fractions from plasma that were analyzed immediately after phlebotomy and in blood samples taken from human umbilical cord (Breckenridge et al., 1984), the samples used in this study were from adults and were not analyzed immediately. It would be expected that a full equilibration of the phospholipid molecular

species by PLTP and CETP in the controls have occurred. The phospholipid fatty acid composition of the proband's HDL and LDL was very similar to those of the control fractions but significantly different from the VLDL. Given the high content of C18:1 and C18:2, which tend to occupy the sn-2 position of phosphatidylcholine and are preferred substrates for LCAT, it is not surprising that the cholesteryl esters in the LDL and HDL are predominantly cholesteryl linoleate (C18:1) and cholesteryl oleate (C18:2). The proband's VLDL phospholipid fatty acid composition on the other hand, was reduced in C18:2 but had significantly elevated C20:4. While dietary factors may have influenced her VLDL phospholipid composition, it is evident that a full equilibration of the phospholipids among the plasma in the CETP-deficient subject had not occurred. This could be due to the absence of CETP activity that is responsible for at least some of the phospholipid transfer activity in human plasma (Hesler et al., 1988).

Since the VLDL isolated from plasma is modified by CETP, the amount of cholesteryl ester synthesized and secreted by the human liver remains an area of controversy. The absence of CETP, however, could potentially provide an estimate of cholesteryl esters secreted in the VLDL. The proband had very low amounts of cholesteryl ester as VLDL lipid mass and her cholesteryl ester / apoB molar ratio in her VLDL and IDL fraction was significantly lower compared to a control female subject (Table 3.5). Thus, it appears that the amount of cholesteryl esters secreted in the VLDL from this proband is relatively low. The cholesteryl ester / apoB ratio further indicates that a considerable amount of cholesteryl ester from the HDL (approximately 1500 moles of cholesteryl ester per particle) would have to be transferred to VLDL in order to acquire a composition comparable to that of the plasma VLDL from the control subject.

Interestingly, the LDL fractions from the proband have cholesteryl ester / apoB molar ratios similar to those of equivalent fractions in the control. If the VLDL from the CETP-deficient subject had been catabolized to LDL without transfer of cholesteryl esters from HDL, it would be anticipated that the cholesteryl ester / apoB ratio would remain at 300-400 rather than the observed 1700-1800. The obvious explanation would be that LCAT shows significant activity with LDL as a substrate and as a result, the amount of cholesteryl esters in the LDL particle would be increased relative to the VLDL particle. This is consistent with the data obtained from the cholesteryl ester fatty acid composition of the LDL where the LDL cholesteryl esters appeared to be derived from LCAT whereas the VLDL cholesteryl esters are derived from the hepatic ACAT activity. Hence, it appears that these results are consistent with the concept that the cholesteryl ester content of nascent VLDL is low and that VLDL are major recipients of cholesteryl esters transferred from HDL. Furthermore, in the absence of CETP, the cholesteryl ester content of the LDL may be augmented by the action of LCAT. However, it is possible that the increase in the cholesteryl ester / apoB molar ratio in the LDL fraction (fractions 8 and 9) might result from contamination with apoE-rich and apoA-I HDL since these fractions contained small amounts of these apolipoproteins.

CETP deficiency in the proband is caused by the presence of a novel C → T substitution at position 836 in exon 9 as demonstrated by DNA sequencing (Figure 3.7). This single base pair substitution resulted in the conversion of Arg-268 to a nonsense codon. The proband is homozygous for this mutation as evidenced by bidirectional direct sequencing and digestion with the restriction enzyme *Mae*III (Figure 3.8). No other mutation was found within the coding sequence or at any intron/exon splice junction.

The Arg-268 → STOP mutation would result in a prematurely truncated protein and hence, if secreted would exhibit no lipid transfer activity as the functional domains are located at the C-terminus of the protein (Figure 3.3). The monoclonal antibodies used were directed to the C-terminal and thus would not be expected to react with the CETP truncated at residue 268. Thus, it is not known if this truncated CETP is expressed and secreted into the plasma. The amplification of exon 9 with the 26 bp-mismatched primer resulting in a restriction site for *Mae*III (Figure 3.8) in the presence of this mutation would allow for rapid screening of subjects with hypercholesterolemia. This novel mutation is the first to be characterized in a Caucasian North American.

CETP and atherosclerosis

Since CETP deficiency results in hyperalphalipoproteinemia, it was previously proposed that CETP deficiency might represent a longevity syndrome. Moreover, animal species with very low levels of CETP activity are resistant to atherosclerosis. Earlier studies showed that the over-expression of simian CETP in transgenic mice (Marrotti et al., 1993) resulted in the early lesion formation after an atherogenic diet. In addition, a positive correlation with LDL cholesterol levels and plasma CETP concentrations was found in cynomolgous monkeys fed a high fat, high cholesterol diet (Quinet et al., 1991). These studies favour the concept that the presence of CETP is pro-atherogenic as it decreases protective HDL and increases atherogenic LDL levels.

However, CETP plays a central role in reverse cholesterol transport, a system that is thought to prevent atherosclerosis via the HDL-mediated transport of cholesterol from peripheral tissues including the arterial wall, to the liver for excretion in bile. Some studies have shown that hyperalphalipoproteinemia in CETP deficiency can be associated

with coronary heart disease (CHD) (Hirano et al., 1995a) especially in the presence of other genetic defects such as decreased hepatic lipase activity (Hirano et al., 1995b). Although CETP deficiency results in a lipoprotein profile that appears to be anti-atherogenic (i.e. increase HDL and decreased LDL), the HDL_2 that accumulate are highly enriched in cholesteryl esters, may be atherogenic, and are not efficiently removed. In addition, the large, cholesteryl ester rich HDL_2 particles have a reduced ability to mediate cholesterol efflux and could not prevent macrophages from accumulating cholesterol from acetylated LDL or remove cholesterol from lipid-laden macrophages (Ishigami et al., 1994). Furthermore, LDL levels are not always decreased in CETP deficiency and may still acquire a high content of cholesteryl ester as a result of LCAT activity as evident in this proband (Teh et al., 1998). The LDL in CETP deficiency are also small and polydispersed (Yamashita et al., 1988; Sakai et al., 1991), and have a lower affinity for the LDL receptor (Sakai et al., 1995). Since CETP also acts in synergism with LCAT and hepatic lipase to promote cholesterol efflux and uptake by the SR-BI receptor (Collet et al., 1999), in the absence of CETP these mechanisms may become ineffective.

Numerous studies in human populations have provided some support for the anti-atherogenic role of CETP. A study by Zhong et al. (1996) on Japanese-Americans living in Hawaii showed an increased incidence of CHD in the presence of CETP deficiency (either the intron 14 or Asp-442 → Gly mutations). Population studies of Omagari City where CETP deficiency is prevalent showed that the risk for coronary heart disease in CETP deficiency was no different to that of men without CETP mutations when HDL levels were between 1.55 and 1.81 mmol/l (Hirano et al., 1997). The "U" shaped relationship between the HDL cholesterol concentration and the incidence of ischemic

ECG revealed that CETP-deficient subjects with lower HDL concentrations at 1.03 mmol/l (in heterozygous CETP deficiency) or with HDL concentrations above 2.32 mmol/l had an increased risk for CHD. Homozygotes for CETP-deficiency were also decreased in the subjects greater than 80 years of age suggesting that CETP deficiency is not a longevity syndrome. In humans, CETP activity appears to be driven by the extent of hypertriglyceridemia (Tall et al., 1986; Mann et al., 1991). CETP expression inhibited the development of early atherosclerotic lesions in hypertriglyceridemic apoC-III transgenic mice despite the reduced HDL levels (Hayek et al., 1995). Hence, it appears that high levels of HDL do not always necessarily reflect an efficient RCT pathway and other genetic and environmental factors that influence the risk for CHD have to be considered.

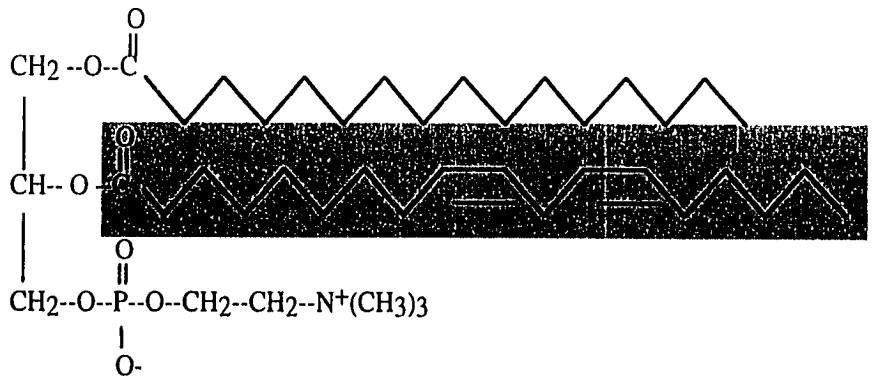
In summary, a Nova Scotian patient exhibiting most of the phenotypic characteristics of CETP deficiency has been identified (Teh et al., 1998). Molecular analysis of her CETP gene revealed the presence of a novel mutation resulting in the conversion of Arg-268 → STOP which, if expressed would result in a truncated protein lacking neutral lipid transfer activity. The proband is homozygous for this mutation. This novel mutation can be rapidly identified by PCR using a mismatched primer engineered to generate a *Mae*III site in the presence of the mutation. The identification of CETP deficiency in North American Caucasian offers the opportunity to study the absence of CETP in a different (non-Japanese) genetic background.

4. Lecithin:cholesterol Acyltransferase

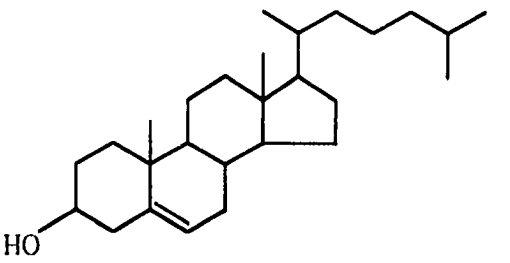
A) Perspectives

The nature and concentrations of plasma lipoproteins are one of the many factors that determine the genesis, progression, and regression of atherosclerosis. Consequently, structure/function studies of plasma proteins that modulate lipoprotein metabolism are important in refining our understanding of this complex pathway. Lecithin:cholesterol acyltransferase (LCAT; EC 2.3.1.43) has received significant attention because of its role in trapping cell-derived cholesterol in plasma HDL and thereby mediating an initial step in the RCT pathway. The formation of water-insoluble cholesteryl ester maintains the concentration gradient for net cholesterol efflux (Glomset, 1968; Fielding, 1984). This pathway is thought to account for the protective role of HDL against cardiovascular disease. In conjunction with this, LCAT is responsible for the maturation of HDL particles from their nascent discoidal form to spherical plasma HDL. Finally, LCAT can also utilize cholesterol on circulating lipoproteins (Fielding et al., 1991) and on the membranes of erythrocytes following its acquisition by lipoproteins. Hence, LCAT can modulate plasma triglyceride-rich lipoprotein composition either by direct esterification of cholesterol on these particles or following CETP-mediated transfer of LCAT-derived cholesteryl esters from HDL (Barter, 1983; Barter et al., 1984).

LCAT is a serine esterase that has both a phospholipase A_2–like activity and an acyltransferase activity. It catalyses the transacylation of the sn-2 fatty acyl chain of phosphatidylcholine to the 3β-hydroxyl group of cholesterol forming cholesteryl esters and lysophosphatidylcholine (Glomset, 1968; Assmann et al., 1978) (Figure 4.1).

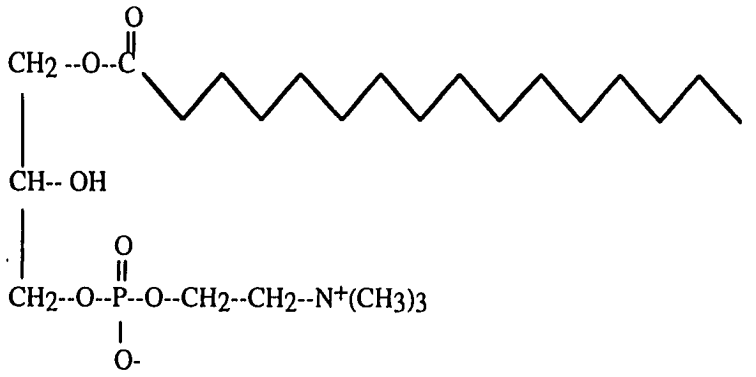


PHOSPHATIDYLCHOLINE

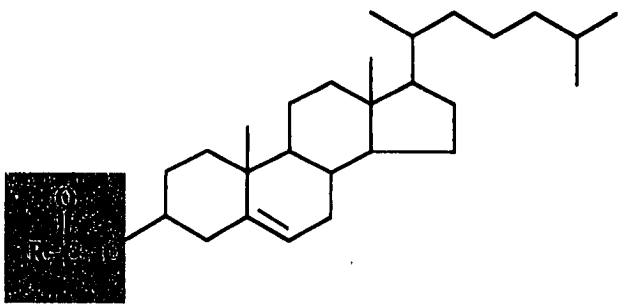


CHOLESTEROL

LCAT



LYSOPHOSPHATIDYLCHOLINE



CHOLESTERYL ESTER

R =

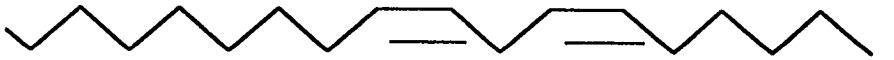


Figure 4.1: The main reaction catalyzed by lecithin:cholesterol acyltrasnferase (LCAT).

Consequently, LCAT is responsible for the formation of the majority of the plasma cholesteryl esters and indirectly controls the levels of cholesterol and cholesteryl esters in various cells and tissues (Glomset, 1968).

As the LCAT substrates are water-insoluble, the LCAT reaction takes place at the interface of lipoprotein particles. In the plasma, LCAT associates preferentially with HDL that contain its main activator, apoA-I (Fielding et al., 1972; Francone et al., 1989). The activation of LCAT by the apolipoprotein cofactor is poorly understood. It is thought that the cofactor may facilitate the interaction of the enzyme with the lipid interface and/or LCAT may interact directly with the apolipoprotein cofactor. The mechanism is undoubtedly similar to that of other interfacially active lipases (i.e., lipoprotein lipase and pancreatic lipase) that require co-lipase activator proteins (Dolphin, 1992). LCAT is also present at very low concentrations in lipid-free form or in association with LDL (Glomset, 1972). The affinity of LCAT with LDL is about 5 fold lower than for HDL and less than 20% of lipoprotein-associated LCAT can be expected to be on LDL (Jonas, 1998).

In addition to cholesterol esterification, LCAT also functions as a phospholipase A_2 in the absence of cholesterol or a suitable sterol acceptor, generating free fatty acids (Piran and Nishida, 1976; Aron et al., 1978). LCAT can also transfer the acyl group back to lysophosphatidylcholine when lysophosphatidylcholine concentrations are elevated in a reaction termed the lysolecithin acyltransferase (LAT) (Subbaiah et al., 1980). This reaction may be responsible for the regeneration of phosphatidylcholines from lysophosphatidylcholines that are formed from oxidatively damaged lipids (Goyal et al., 1997). Furthermore, the LCAT-catalyzed cholesteryl ester hydrolysis followed by re-

esterification of a second cholesterol molecule has also been reported (Sorci-Thomas et al., 1990) and this activity was termed the cholesterol acyltransferase (CAT) (Jauhiainen and Dolphin (1990). All these reactions require an apolipoprotein cofactor. However, in the absence of apoA-I, LCAT can hydrolyze water-soluble esters (Bonelli and Jonas, 1989) as well as platelet-activating factor (PAF) (Liu and Subbaiah, 1994).

B) The LCAT gene and protein

The human LCAT gene is located in the region of chromosome 16q22.1 and is present as a single copy (Azoulay et al., 1987). The entire gene spans about 4.5 kb and consist of 6 exons separated by 5 introns (McLean et al., 1986; Tata et al., 1987). Exon 1 of the human LCAT gene codes for the 24 residues of the hydrophobic leader sequence and 28 residues of the mature peptide. Exons 2-4 code for a small portion of the mature protein including the region of the LCAT containing the putative "lid" involved in substrate recognition and binding (Peelman et al., 1999a). Exon 5 codes for amino acid residues 151-226 that contains the active Ser-181 residue. Exon 6, the largest of the exons codes for the remaining 50% of the mature peptide (res. 227 – 416) including the two residues (His-377 and Asp-345) (Peelman et al., 1998) that form the catalytic triad with Ser-181 (Jauhiainen and Dolphin, 1986; Francone and Fielding, 1991a). The LCAT gene has a relatively small promoter that spans 71 bases and includes the classical TATA box and two Sp1 binding sites that are essential for expression, together with an LFA1 motif (Meroni et al., 1991) (Figure 4.2).

The LCAT mRNA length is about 1.5 kb (McLean et el., 1986) and consists of a 5'-untranslated region of 22 bases, 1250 bases of transcribed sequence, a short 3'-

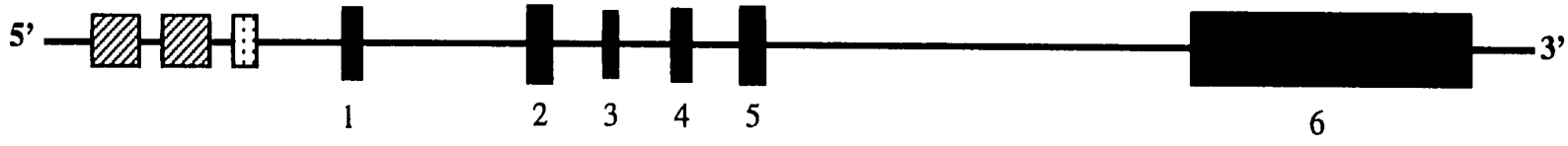


Figure 4.2: Schematic representation of the human lecithin:cholesterol acyltrasnferase (LCAT) gene. The LCAT gene contains 6 exons separated by 5 introns. The entire gene spans about 4.5 kb. The exons are represented by the black boxes. The 5' regulatory site contains the TATA box (denoted by the dotted box) and two Sp1 binding sites.

untranslated region of 23 bases, and a poly(A) tail of 200 bases (Fielding, 1990). Alternative splicing of the human LCAT mRNA also occurs as an insertion of a 95 base Alu cassette between exon 5 and 6 (Miller and Zeller, 1997). Alternative transcripts are present at low levels but there is currently no evidence that protein synthesis and secretion occurs from these transcripts. In humans, the liver is the major source of LCAT mRNA (McLean et al., 1986). The human hepatoma HepG2 and rat hepatocytes also synthesize LCAT (Chen et al., 1986; Erickson and Fielding, 1986). In rats, LCAT mRNA is also expressed at low but significant levels in the brain and testes (Warden et al., 1989). The expression of LCAT in humans is relatively constant in adults but changes during embryonic, post-natal development, and in liver disease (Warden et al., 1989). Overall, the human LCAT gene expression appears to be unaffected by diet and drug treatments (Warden et al., 1989). Avian LCAT, on the other hand, is expressed at very high levels in the liver during early development but when they begin laying eggs, the brain expresses the LCAT mRNA up to five-fold higher than that in the liver. This developmental pattern is only seen in hens and not roosters (Hengstschlager-Ottnad et al., 1995).

The amino acid sequence of human LCAT was deduced by cDNA and genomic sequencing experiments (McLean et al., 1986) (Figure 4.3). The calculated polypeptide mass of LCAT is approximately 47 kDa and has 416 residues in the mature, secreted protein. The primary protein sequence reveals a high content of hydrophobic residues and is heavily N-glycosylated at positions Asn-20, Asn-84, Asn-272, and Asn-384 (Yang et al., 1987). It also contains two O-linked carbohydrate chains at positions Thr-407 and Ser-409 (Schindler et al., 1995). The total carbohydrate content is

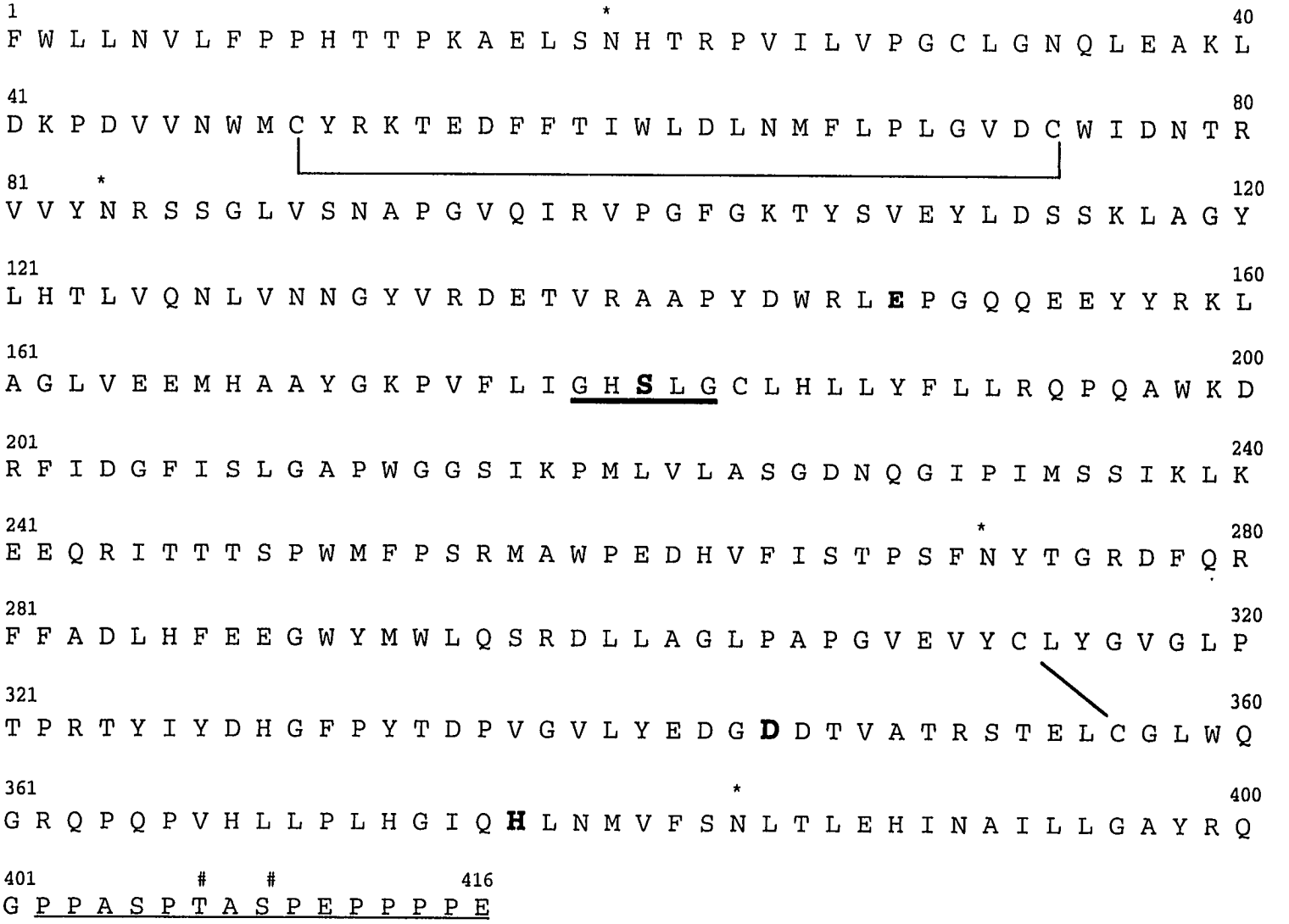


Figure 4.3: Primary sequence of human LCAT.

The mature LCAT protein consists of 416 amino acids. The (★) represents the N-linked glycosylation sites and (#) the two O-linked glycosylation sites. The two free Cys residues are at position 31 and 184. The lines between the other Cys residues represent disulphide bonds. The disulphide bond between Cys-50 and Cys-74 is the putative "lid" region. The heavy line under the residues GX**S**XG represents the conserved sequence observed among esterases and lipases. The catalytic triad includes the active site Ser-181, Asp-345, and His-377 that are in bold. The Glu-149 in bold type determines the fatty-acyl chain specificity at the sn-2 position. The underlined proline-rich sequence at the C-terminus region is not required for interfacial activation. Reproduced from Jonas, 1998.

approximately 25% (Chung et al., 1979; Chong et al., 1983) of the enzyme mass which has an apparent molecular mass varying from 65 to 69 kDa by SDS-gel electrophoresis (Marcel, 1982). Earlier studies by O et al. (1993) showed that all 4 N-linked carbohydrate chains were utilized but were not necessary for secretion and that the pattern of glycosylation had a profound effect on the catalytic activity of the enzyme (Collet and Fielding, 1991). For example, culture studies in COS cells involving the removal of Asn-20 drastically reduced enzymatic activity while the removal of Asn-384 increased activity by two-fold compared to wild-type (O et al., 1993). Similar observations were also made in BHK cells (O et al., 1995). Both these studies suggest that Asn-384 may have inhibitory effects in its natural conformation. In contrast, Francone et al. (1993) found Asn-272 functionally important for acyltransferase activity in LCAT secreted by CHO cells. Although these studies are not in agreement with each other, the carbohydrate chains are clearly important for the full function of LCAT. Removal of the carbohydrate chains also reduced the thermostability of the LCAT protein (O et al., 1995). Altogether, these studies suggest that the carbohydrate moieties may have an important role in the maintenance of the active conformation of LCAT as well as their ability to interact with substrates (Miller et al., 1996), and contribute to the conformational stability in an aqueous environment (Lacko et al. 1998). In contrast, the 2 O-linked residues are not essential for function (Schindler et al., 1995) as their loss in the C-terminal truncations of the enzyme do not affect LCAT activity (Lee et al., 1997).

LCAT also has six cysteine residues organized as two disulphide bridges (Cys-50 and Cys-74; Cys-313 and Cys-356) and two free cysteines (Cys-31 and Cys-184) (Yang et al., 1987). Both the disulphide bridges are important in maintaining the structure and

function of the enzyme (Yang et al., 1987; Qu et al., 1994). However, the role of the two free cysteines has not been completely elucidated. It is known that the free cysteines are vicinal within 3.5-3.6 Å and are close to the catalytic site (Jauhiainen et al. 1988). Mutagenesis studies revealed that these residues are not required for catalysis (Francone and Fielding, 1991b) and when disulphide bridged may serve to sterically hinder access to the active site region (Qu et al., 1993). Comparison of the human LCAT sequence with that of the baboon (Hixson et al., 1993), rabbit (Murata et al., 1996), rat (Meroni et al., 1990) and mouse (Warden et al., 1989) LCAT shows a high sequence conservation (85%) while the chicken (Hengstschlager-Ottnad et al., 1995) LCAT is less conserved (75%) (Figure 4.4).

The precise tertiary structure of the LCAT protein has yet to be defined. Recently, a 3-dimensional model for the folding of LCAT was proposed by Peelman et al. (1998) (Figure 4.5). This model was created based on known X-ray structures of pancreatic lipase and *Candida antartica* lipase and on the sequence homology of LCAT with a variety of other lipases. Although this model excludes the putative "lid" region and only accounts for about a third of the secondary structure of LCAT, it does provide for some important insights for further study.

C) Substrate and fatty acyl specificity

The active site of human LCAT recognizes the phosphate group of phospholipids but has no strict specificity for the head groups. For example, di- and triacylglycerols are not substrates for LCAT, and although phosphatidylcholine is the major acyl donor for LCAT, phosphatidylethanolamine is actually a better substrate (Pownall et al., 1985).

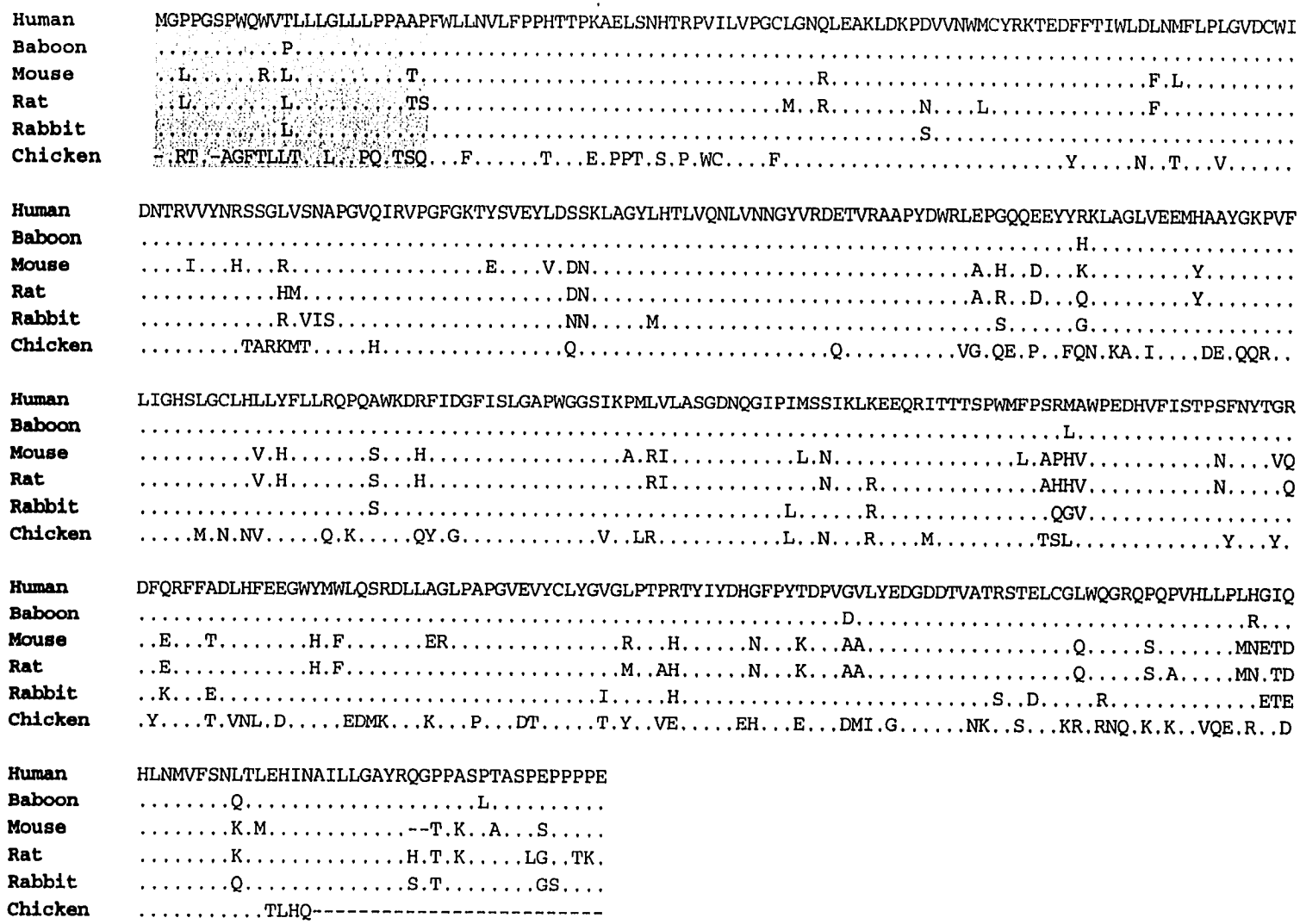


Figure 4.4: Amino acid sequence alignment of LCAT from six species. Identical sequences are represented by (.) and missing amino acids are represented by (-). Sequence identity between the human and baboon is 98%, human and mouse (85%) and human and chicken (75%). The highlighted sequences represents the signal peptide.

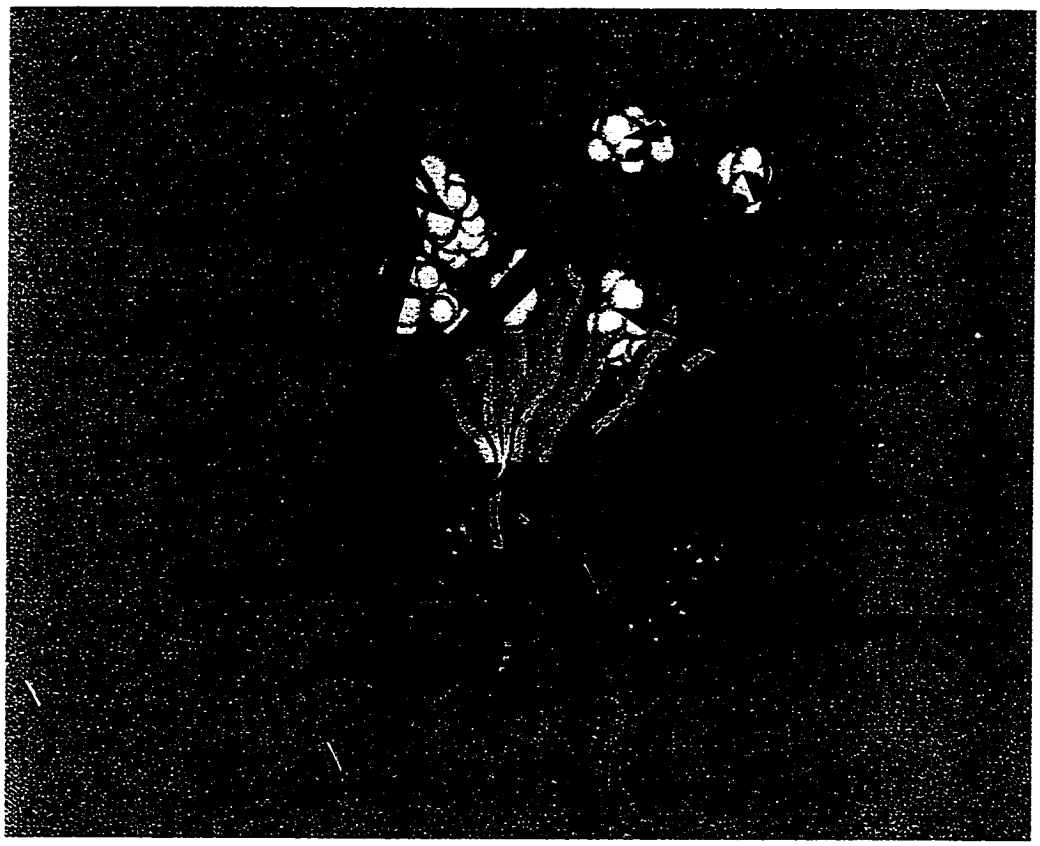


Figure 4.5: The proposed model of lecithin:cholesterol acyltransferase (LCAT). LCAT belongs to the å/ß hydrolase fold family. The catalytic triad consist of Ser-181, Asp-345, and His-377. From Peelman et al., 1998.

Furthermore, the sn-2 position is the preferential fatty acyl chain transferred to cholesterol (Subbaiah et al., 1992; Subbaiah et al., 1994a). This selectivity is influenced by the fatty acyl composition of the phosphatidylcholine (Pownall et al., 1985). Subbaiah et al. (1992) reported that when the sn-1, 16:0; sn-2, 20:4 phosphatidylcholine was used as a substrate, human LCAT would preferentially transacylate the sn-1 fatty acyl chain to produce 16:0 cholesteryl ester. Studies on the phosphatidylcholine selectivity from various animal species showed that human LCAT prefers shorter, more saturated fatty acyl chains such as 16:0, 18:1 and 18:2, while rat and mouse LCAT can bind and react with phosphatidylcholines containing longer, more unsaturated fatty acyl chains such as 20:4 (Portman and Sugano, 1964; Liu et al., 1995). To further elucidate which region of the LCAT was responsible for the phosphatidylcholine binding selectivity, chimeric proteins were constructed. The human-mouse chimeras containing the sequence of one of the LCATs between residues 130-306 were replaced with the corresponding sequence in the other enzyme. The chimera with the human LCAT sequence at residues 130-306 inserted into the mouse LCAT exhibited specificity of that of human LCAT and vice versa (Subbaiah et al., 1994b). Hence, the region between residues 130-306 may contain the phosphatidylcholine binding site. Recently, Wang et al., (1997) showed that a single residue substitution in human LCAT to that of rat LCAT (Glu-149 → Ala) changed the selectivity of the human LCAT from shorter, more saturated acyl chains (18:1) to that of rat LCAT that prefers longer, more unsaturated fatty acyl chains (20:4). The residue Glu-149 may not be directly involved with phosphatidylcholine binding but its substitution may cause a conformational change in the enzyme that affects the active site pocket (Wang et al., 1997). Alternatively, Glu-149 could be located near the site where the end

of the sn-2 chain contacts LCAT (Jonas, 1998). Primary sequence alignments of known LCAT sequences from various species showed that Glu-149 is present in species that show a preference similar to that of human LCAT.

The major acyl chain acceptor of the phospholipase A_2 reaction of LCAT is cholesterol. However, other sterols, water, and lysophosphatidylcholine can act as acceptors and *in vitro*, long chain alcohols, diacylglycerols and monoacylglycerols can also accept the acyl chain (Piran and Nishida, 1979; Jonas, 1998).

D) LCAT Deficiencies

Natural mutations in the human LCAT gene are associated with lipid abnormalities such as hypoalphalipoproteinemia and an increase in the unesterified to esterified cholesterol ratio. These mutations are expressed as either familial LCAT deficiency (FLD) or fish-eye disease (FED). Both are rare and are inherited in an autosomal recessive manner (Norum et al., 1989).

FLD was first reported in a Norwegian family by Norum and Gjone (1967). The biochemical features that characterize FLD are low levels or absence of plasma LCAT activity due to reduced LCAT mass, resulting in severely reduced levels of cholesteryl ester, and decreased levels of HDL and apoA-I caused by increased catabolism (Glomset et al., 1989; Rader et al., 1994). The molar ratio of cholesteryl ester to cholesterol is markedly decreased. Furthermore, individuals with LCAT deficiency often present with high levels of LDL, and sometimes larger cholesterol and phospholipid-rich particles termed lipoprotein-X (Lp-X) (Forte et al., 1974; Guérin et al., 1993). The major clinical

findings in these patients include corneal opacity, anemia, proteinuria, and progressive renal failure (Glomset et al., 1989; Pritchard and Hill, 1993).

FED or partial LCAT deficiency was first described by Carlson and Philipson in 1979 in two Swedish families (Carlson, 1982). Biochemically, FED can be distinguished from FLD by the selective inability of LCAT to esterify HDL cholesterol (α-LCAT activity) but not VLDL and LDL cholesterol (β-LCAT activity). Due to the β-LCAT activity, plasma cholesteryl ester levels and cholesteryl esterification rates are usually normal although there is a partial reduction in LCAT mass and activity (Carlson and Philipson, 1979; Carlson, 1982). Consequently, cholesteryl ester levels in VLDL and LDL are normal (Carlson, 1982) but cholesteryl ester levels in HDL are extremely low (Carlson and Holmquist, 1983). However, HDL deficiency and reduced levels of apoA-I are still apparent. Clinically, FED patients suffer only from an age-dependent corneal opacification. Recently, Klein et al. (1993) reported an FED phenotype with both the α- and β-LCAT activity. In view of this, it was suggested that the residual levels of total LCAT activity in a patient's plasma dictates the different clinical manifestations of FLD and FED and is not necessarily caused by the selective deficiency of α-LCAT activity (Klein et al., 1993). Rather, FED could occur because of impaired binding or reactivity towards all or specific subclasses of HDL and LDL which would result in low LCAT specific activity toward one or more lipoprotein classes (Blanco-Vaca et al., 1997). Furthermore, the identification of a selective deficiency in β-LCAT activity and not in α-LCAT activity would be difficult to characterize since cholesterol esterification in HDL followed by the subsequent transfer by CETP to LDL would compensate for this defect.

Recently, a compilation of the molecular defects causing LCAT deficiency was reported by Kuivenhoven et al. (1997). The known LCAT mutations were classified into five distinct classes (Table 4.1). Class 1 mutations are considered null mutations of the LCAT gene leading to FLD and can be caused by premature stop codons, defective mRNA splicing, or frameshifts that result in the synthesis of truncated/nonsense LCAT. Deletions and insertions were also considered Class 1 mutations. Class 2 mutations are missense mutations that cause FLD with a complete or near complete loss of LCAT activity. Missense mutations or minor deletions that may cause partial or total loss of activity against HDL and LDL or partial loss against LDL substrates are considered Class 3 mutations while Class 4 represents the typical FED phenotype where there is a specific loss of α-LCAT activity. Finally, Class 5 represents mutations that do not fall into any of the above categories. The mutations in this category include Tyr-144 → Cys, Met-293 → Ile, and Arg-158 → Cys and are described in detail by Kuivenhoven et al. (1997).

Since this publication, four novel mutations have been reported. These include three FLD mutations: Thr-13 → Met, Pro-307 → Ser (Argyropoulos et al., 1998), and a nonsense mutation at Thr-171 (Guérin et al., 1997); and one FED mutant, Arg-99 → Cys (Blanco-Vaca et al., 1997). Based on the classification scheme by Kuivenhoven et al. (1997), the Thr-13 → Met and Pro-307 → Ser mutants can be classified under Class 2, whereas Thr-171 → Stop can be classified as a Class 1 mutation. The Arg-99 → Cys can be classified as a Class 4 mutation.

Table 4.1: Classes of LCAT gene defects

Class	Biochemical Phenotype	Defect
1. Null mutations causing FLD	Total loss of catalytic activity. LCAT mass: (virtually) absent	30 bp insertion (codon 4) C-insertion (codon 9, 10) Tyr-83 → stop Thr-171 → stop * Gly-141-insertion Intron 4 defect A–T substitution C-deletion (codon 120) C-deletion (codon 168) G-deletion (codon 264) A-insertion (codon 376)
2. Missense mutations causing FLD	Total loss of catalytic activity LCAT mass: normal/ reduced/ absent	Thr-13 → Met ** Gly-30 → Ser Leu-32 → Pro Gly-33 → Arg Ala-93 → Thr Arg-135 →Trp Arg-135 → Gln Arg-140 → His Arg-147 → Trp Tyr-156 → Asn Gly-183 → Ser Leu-209 → Pro Asn-228 → Lys Arg-244 → Gly Met-252 → Lys Pro-307 → Ser ** Thr-321 → Met Gly-344 → Ser Thr-347 → Met Arg-399 → Cys

Class	Biochemical Phenotype	Defect

3. Missense mutations and minor deletions causing FED	Partial loss of activity against a) LDL, or b) both HDL and LDL. LCAT mass: reduced	Asn-131 → Asp Leu-300 deletion Asn-391 → Ser
4. Missense mutations causing FED	Partial loss of activity against HDL only,. LCAT mass: reduced.	Pro-10 → Leu Pro-10 → Gln Arg-99 → Cys † Thr-123 → Ile
5. Unclassified mutations		Tyr-144 → Cys Met-293 → Ile Arg-158 → Cys

The different phenotypes of LCAT deficiency categorized into five classes by Kuivenhoven et al. (1997). Class 1 and 2 represents FLD phenotypes, while Class 3 and 4 are FEDs. The Class 5 mutations represent obscure mutations. * (Guerin et al., 1997), ** (Argyropoulos et al., 1998), and † (Blanco-Vaca et al., 1997) are newly published mutations since this publication.

E) Transgenic and knockout animals

The overexpression of human LCAT has been studied in mice (Vaisman et al., 1995; Mehlum et al., 1995; Francone et al., 1995; Francone et al., 1997) and rabbits (Hoeg et al., 1996a; Brousseau et al., 1996; Hoeg et al., 1996b; Brousseau et al., 1997). The expression of LCAT activity in these animals occurs in a gene dose-dependent manner and can result in over a 100-fold increase in the plasma LCAT activity of the transgenic animals (Vaisman et al., 1995; Mehlum et al., 1995). In both animal models, a significant increase was observed for total cholesterol, HDL cholesterol, apoA-I, apoA-II and apoE with concomitant decrease in the apoB-containing particles. In rabbits, while apoA-I clearance was delayed (Brousseau et al., 1996), the clearance of the apoB-containing particles was enhanced (Brousseau et al., 1997). The lipoprotein patterns generated by the LCAT overexpression with increased HDL cholesterol and decreased LDL cholesterol appears to be anti-atherogenic. However, transgenic mice fed an atherogenic diet are more susceptible to atherosclerotic lesions than control mice (Berard et al., 1997) whereas rabbits become less susceptible than their control counterparts (Hoeg et al., 1996).

LCAT knock-out mice have also been generated and studied (Sakai et al., 1997; Ng et al., 1997). The lipoprotein patterns in these animals resemble those of human LCAT deficiency with reduced total plasma cholesterol, HDL and apoA-I concentrations. The triglycerides levels in these animals were also elevated and an increase in discoidal pre-β HDL were observed.

F) Structure and Functional Domains of LCAT

The predicted 3-dimensional model placed LCAT into the α/β-hydrolase fold family similar to other lipases (Peelman et al., 1998) (Figure 4.5). Like the members of the α/β-hydrolase fold family, the central domain of LCAT consists of one anti-parallel and six conserved parallel β-strands connected by four α-helices and separated by functionally important loops (Peelman et al., 1998). The functional domains of LCAT were predicted based on the conserved features in the α/β-hydrolase fold family.

i.) The Catalytic Site

The susceptibility of LCAT activity to chemical modifications of the active Ser, His and Cys residues (Chong et al., 1983) led to the proposal that the catalytic site of LCAT involved an active Ser residue in a catalytic Asp-His-Ser triad similar to that of serine proteases and lipases (Jauhiainen and Dolphin, 1986). The Asp-His-Ser residues serve to form a H-bonded network wherein, upon addition of substrate, a proton is transferred from the Ser to His resulting in Ser-O^- and a positively charged imidazole ring. The charged imidazole-ring is subsequently stabilized by the electrostatic interactions with the negatively charged Asp (or Glu) residue in a proton relay charge system (Lohse et al., 1997). Indeed, it was shown by Jauhiainen and Dolphin (1986; Dolphin and Jauhiainen, 1987) through chemical modifications that both the Ser and His residues are involved in the phospholipase A_2 step, and carboxyl group modification by carbodiimides can inactivate LCAT (Jauhiainen and Dolphin, 1991). Furthermore, Jauhiainen et al. (1987, 1989) demonstrated the formation of a fatty acyl-enzyme intermediate. Hence, the LCAT catalytic mechanism involves cleavage of the sn-2 ester

bond following nucleophilic attack of the Ser oxygen leading to the formation of a tetrahedral adduct which decays to form a Ser-O-fatty acyl intermediate. The fatty acid is then transferred to cholesterol (Jauhiainen and Dolphin, 1986). Francone and Fielding (1991a) subsequently provided evidence for Ser-181 as the active Ser residue through site-directed mutagenesis studies that were later confirmed by Qu et al. (1994). This active Ser-181 is also located within the highly conserved region of GXSXG shared by serine proteases and lipases (McLean et al, 1986).

Due to the high sequence conservation of Asp and His residues between the different species, it was difficult to assign which of the Asp and His would form part of the catalytic triad. Both the His and Asp residues were assigned based on results observed through site-directed mutagenesis following the application of threading techniques to produce the molecular model (Peelman et al., 1998). There were three fully conserved His residues (His-263, His-268 and His 377) in the sequenced human, baboon, rat, mouse, and chicken LCAT downstream of the Ser-181 that had the potential to be the catalytic site His. These residues were chosen based on the observation that the triad residues are in the same orientation in the primary sequence of a large number of lipases (Lohse et al., 1997; Peelman et al., 1998). In two separate studies both the phospholipase and acyltransferase activity were lost only when His-377 was mutated to Ala (Peelman et al., 1998), Gly or Ser (Adimoolam et al, 1998). Based on these results, His-377 was identified as the catalytic site His residue of LCAT in all six species (Peelman et al., 1998). Using the predicted secondary structure of LCAT, the Asp residue candidates (six in total) were separately mutated to Asn and only Asp-345 → Asn resulted in the total

lost of activity strongly suggesting its role in catalysis. Thus, the catalytic triad of LCAT is thought to consist of Ser-181, Asp-345, and His-377.

One of the essential structural features required for the catalytic action of serine residues is an oxyanion hole that is in a pocket of the enzyme (Branden and Tooze, 1991; Ollis et al., 1992). In addition to the His and Asp residue, the oxyanion hole residues are responsible for forming hydrogen bonds with the negatively charged oxygen atom of Ser to confer a tight binding and stabilize the tetrahedral transition state intermediate. The residues in LCAT identified as those forming the oxyanion hole are Phe-103 and Leu-182. Leu-182 was assigned based on the fact that enzymes with the α/β-hydrolase fold family have one of the oxyanion hole residues at position +1 of the active Ser (Peelman et al., 1998). Phe-103 was identified through sequence alignment and secondary structure prediction of all LCAT species and confirmed by site-directed mutagenesis (Peelman et al., 1998).

ii.) Interfacial binding sites

Another important feature that is shared by all members of the α/β-hydrolase fold family is the "lid" or "flap" domain (Ollis et al., 1992). This region consists of a highly mobile segment that covers the hydrophobic active site of the enzyme and is closed by a disulphide bridge (Ollis et al., 1992; Winkler et al, 1990). The lids usually have 15-45 residues and are characterized by the presence of an amphipathic α-helix (Peelman et al., 1999a). In the closed conformation, the helix covers the catalytic site of the enzyme with its hydrophobic side orientated towards the core of the enzyme. Upon interfacial activation, the helix flips open and the core is exposed to create a large hydrophobic

surface that contributes to the interaction with the lipid interface and to substrate binding (Derewenda et al., 1992; Grochulski et al, 1994; Egloff et al., 1995). Such a structure has been identified in human pancreatic lipase (Winkler et al., 1990; van Tilbeurgh et al., 1993), and has been inferred by homology for both lipoprotein lipase and hepatic lipase (Hide et al., 1992; van Tilbeurgh et al., 1993). By analogy to the other lipases, this lid domain may be involved in the primary interaction between LCAT and its lipoprotein substrates. The putative "lid" domain was proposed to be between Cys residues 50-74 of human LCAT and represents the interfacial recognition site (Adimoolam and Jonas, 1997). Removal of this region resulted in the secretion of LCAT with no catalytic activity. This was later confirmed by Peelman et al. (1999a) who further investigated the role of the residues at position 56-68. This region highly resembles that of the amphipathic helix in pancreatic and *C.rugosa* lipase lids (Peelman et al., 1999a) and was hypothesized to have the ability to destabilize the organized lipid substrates (Brasseur et al., 1997). Deletion of these residues (56-68) resulted in the complete inactivation of the enzyme, which is consistent with their proposed role in substrate recognition (Peelman et al., 1999a). Furthermore, Trp-61 aligns with Trp-255 in the amphipathic helix of pancreatic lipase which, in the closed conformation of the enzyme fills the hydrophobic cavity impairing access of substrates into the catalytic site (Winkler al., 1990). The substitution of Trp-61 for Leu or Gly resulted in total inactivity of the LCAT enzyme (Peelman et al., 1998) but substitution of Trp-61 for either Phe or Tyr produced an enzyme with a three fold increase in enzymatic activity towards water-soluble monomeric phosphatidylcholine (Peelman et al., 1999a). Furthermore, the activity of Trp-61 → Phe and Trp-61 → Tyr was retained on HDL but decreased on LDL. Thus, it

was proposed that an aromatic residues at position 61 is required for efficient interaction of LCAT with its lipid substrate and the smaller aromatic residues decreases the steric hindrance to the catalytic site (Peelman et al., 1999a).

Two other conserved basic residues Arg-52 and Lys-53 are also important for the structural integrity and function of the LCAT lid. Substitution of these residues by Ala resulted in decreased activity towards HDL and LDL but retained full activity on water-soluble monomeric substrates (Peelman et al., 1999a).

Fielding and Fielding (1995) have proposed a region of LCAT between residues 151-175 to have the potential for interfacial and sterol binding. The hypothesis that residues 151-175 may have potential sterol binding is based on the fact that this region in LCAT shows sequence similarity with the C-terminal amphipathic segment of apoE (268-288) which associates with phospholipids. Support for this hypothesis is weak. Results from studies by Peelman et al. (1997) suggest that the decreased activity observed in the natural LCAT mutant of Tyr-156 → Arg might be caused by the decreased affinity for lipid binding. In contrast, site-directed mutagenesis studies by Wang et al. (1998) of the three glutamic acids in this region (154, 155 and 165) concluded that these residues are not necessary for LCAT activity.

iii.) Other regions of LCAT

Beside the aforementioned, the far N-terminus is speculated to be unimportant for function or structural integrity. This comes from the observation that removal of up to 6 residues of LCAT results in a functional enzyme (Jin et al., 1997). However, mutations in this region cause FED and FLD. The N-terminal domain is also very highly conserved

and this is often indicative of important structural and/or functional elements within the primary sequence.

A region of the LCAT sequence that has been assigned unimportant for either function or structural integrity is the 18 residues from the C-terminus that contains the proline-rich region (Francone et al., 1996; Lee et al, 1997). Deletion of this region did not affect the phospholipase and transacylase activity of LCAT (Francone et al, 1996; Lee et al., 1997) nor the stability of the enzyme at 37°C (Francone et al., 1996). Interestingly, the phospholipase A_2 activity increased 8-fold for the water-soluble ester, p-nitrophenylbutyrate (PNPB) (Lee et al, 1997). In accordance with this, the chicken LCAT sequence lacks the proline-rich C-terminus common to mammalian sequences described (Figure 4.4).

iv.) Activation of LCAT by apolipoproteins

The LCAT reaction on lipoproteins and lipid vesicles is stimulated by apoA-I (Fielding et al., 1972), the major protein on HDL and to a lesser extent by apoE (Soutar et al., 1975; Zorich et al., 1985), apoA-IV (Chen and Albers, 1985), and apoC-I (Steyrer and Kostner, 1988). It is important to note that the activation of LCAT by apolipoproteins also depends on the nature of the phospholipids and type of substrate particles (Jonas, 1998). The second most abundant apolipoprotein, apoA-II seemed to inhibit the activation of LCAT but this inhibitory effect is attributed to the fact that apoA-II can displace both the apoA-I and apoC-I from lipoprotein surfaces (Scanu et al., 1980).

Human apoA-I is synthesized by the liver and intestine as a prepropeptide. It is cotranslationally cleaved to pro-apoA-I, and following secretion, processed to the mature

form of 243 amino acids (Brewer et al., 1978). ApoA-I circulates in the plasma as the major protein component of HDL (Assmann and Brewer, 1974). The secondary structure of apoA-I contains a series of 22-amino acid amphipathic α-helical repeats (Karathanasis et al., 1983; Li et al., 1988), punctuated by Pro residues (Brewer et al., 1978). The Pro residues act as helix-breakers providing apoA-I with the flexibility to conform to the surface of the HDL as the diameter of the lipoprotein increases due to LCAT action (Cheung et al., 1987). These helices are able to form stable complexes with phospholipids (Kanellis et al., 1980; Segrest et al., 1983).

The activation of LCAT by apoA-I requires that apoA-I have at least 2 functional domains. These domains would be 1) regions in apoA-I which mediate the interaction with and binding to the lipoproteins, and 2) regions that would stimulate the catalytic activity of LCAT (Dolphin, 1992). Mutagenic studies have indicted that residues 143-187 are critical for LCAT activation (Sorci-Thomas et al., 1993) and the C-terminus of apoA-I is important for the initial binding of the protein to the lipid (Minnich et al., 1992; Holvoet et al., 1995; Laccotripe et al., 1997). The conformation of the apoA-I can also regulate the activation of LCAT. Natural mutations that affect the structure of the apoA-I helices between residues 143-187 inhibit LCAT activation (Jonas, 1998). In the absence of apoA-I, LCAT can esterify cholesterol on HDL particles containing other apolipoproteins and on LDL.

The process by which LCAT is activated by apoA-I is still only speculative. It has been proposed that apoA-I might be involved in several different steps of the LCAT reaction (Jonas, 1998). As summarized by Jonas (1998), apoA-I could either act at the initial steps of LCAT activation through mediating the binding of LCAT to lipid surfaces

or stabilizing the activated conformation of LCAT after binding to the lipid surfaces. Alternatively, after the lipid-protein–protein complex formation, apoA-I might facilitate substrate availability by concentrating the lipid substrates to the vicinity of LCAT or orientating the substrates in an optimal conformation, and subsequently facilitate product removal. Finally, apoA-I may facilitate the dissociation of the enzyme from the lipid complex.

It is unlikely that apoA-I mediates the binding of LCAT to the lipid substrates because LCAT can bind to lipid surfaces in the absence of apolipoproteins (Furakawa and Nishida, 1979) and there is no evidence for LCAT association with lipid-free apoA-I (Jonas, 1998). The possibility that both the apoA-I and LCAT interact while bound to the lipid surface is more likely and has some supporting evidence. Through fluorescence energy transfer experiments, Knipping et al. (1993) have shown that apoA-I and LCAT are close to each other on the surface of lipid vesicles. By analogy with the interfacial activation of pancreatic lipase by co-lipase, apoA-I might stabilize the activated conformation of LCAT as they both undergo structural changes (Jonas, 1998). The region in LCAT mediating apoA-I activation is still undefined but may be localized to the N-terminus of LCAT since mutations in this region (Pro-10 and Thr-123) cause FED. FED mutants are defective in their reaction with HDL but not with lipoproteins lacking apoA-I which further suggest that LCAT and apoA-I interact with each other on the surface of HDL.

G) Objectives of the Study

The objectives of this research include 1) DNA sequence analysis of the LCAT gene from an individual exhibiting the FLD phenotype and 2) to evaluate the structure and functional significance of the N-terminal 80 residues of the mature LCAT protein.

Natural mutations can sometimes provide useful insights in defining the structure and function of a protein. LCAT mutations are rare and its phenotypic expression is highly heterogenous. Furthermore, the mutations identified to date have not provided much information on how structure might relate to function, as they are widespread throughout the gene and can cause either FED or FLD. This study was undertaken to identify the underlying mutation causing the FLD phenotype in a French patient.

Although LCAT shares some structural similarity with other lipases in the α/β-hydrolase fold family, it lacks homology to any known proteins. Consequently, the proposed structure of LCAT only accounts for one-third of the protein (Peelman et al., 1998) and only regions that share some structural similarities with known proteins were modelled. Due to the lack of a suitable template, the N-terminal residues 1-73 were not modelled. Figure 4.4 shows the primary sequence alignment of the human LCAT with that of the baboon, mouse, rat, rabbit, and chicken. With the exception of the chicken LCAT, a high degree of sequence identity exists between the various species. In fact, the N-terminal region of LCAT is the most highly conserved region. Furthermore, the differences in amino acids with respect to human LCAT are mostly conservative substitutions.

The N-terminal region also contains the putative "lid" (residues 50-74) which, in concert with other lipases, is thought to open and expose the catalytic residues upon

substrate binding and interfacial activation. This "lid" region has been shown to be functionally important since deletion of this region results in a catalytically inactive protein (Adimoolam et al., 1997; Peelman et al., 1998). Certain key residues within this region include the Trp-61, Arg-52, and Lys-53, which are conserved in all six species (Figure 4.4). Trp-61 is required for full enzymatic activity and Arg-52 and Lys-53 are thought to contribute to the folding of the lid (Peelman et al., 1998). This region is clearly important for regulating the interaction of the enzyme with lipoprotein substrates.

Lastly, natural mutations that occur within the first 80 residues cause both phenotypes of LCAT deficiency. These include missense mutations at residue Pro-10 and Thr-13 (Carlson and Philipson, 1979; Skretting and Prydz, 1992; Klein et al., 1995; Kuivenhoven et al., 1996; Argyropoulos et al., 1998). Based on the high sequence similarity of the N-terminus, it is hypothesized that LCAT chimeras containing residues 1-80 from either the rat, mouse or chicken LCAT with residues 81-416 of human LCAT will be catalytically active. The results of this study may provide information on the relative flexibility of the LCAT protein to conservative changes and potentially, the role of residues 15-23 in the chicken LCAT which does not appear to be conserved. Additionally, it has been established that LCAT is usually more reactive with its homologous apoA-I (Chen and Albers, 1983; Sparrow et al., 1995). Therefore, if the apoA-I activation site is within the first 80 residues of LCAT, the chimeric protein may have a selective reactivity with rHDL containing its homologous apoA-I.

It was previously reported that the first six residues of the mature LCAT protein are not required for secretion and function of the protein (Jin et al. 1997). However, mutations at Pro-10 cause FED (Carlson and Philipson, 1979; Skretting and Prydz, 1992;

Klein et al., 1995; Kuivenhoven et al., 1996) and a mutation at Thr-13 (Argyropoulos et al., 1998) results in FLD. These natural mutations suggest that although the first six residues are not required, residue 10 is important for LCAT function. The goal of this study is to investigate how many residues at the N-terminal are required for secretion and function. It is hypothesized that the far N-terminus residues are important for functionality and are required for the proper alignment of the lid region. The far N-terminus may provide the first anchor point for the lid through hydrophobic interactions.

H) Results

i.) Familial LCAT deficiency in a French patient

Lipoprotein and apolipoprotein data

The proband in this study did not suffer from proteinuria or show signs of either renal failure or premature atherosclerosis although she was mildly anemic (Hemoglobin: 11.6 $\pm$ 0.03 g/l; Normal: 14 $\pm$ 2 g/l) and had corneal opacity requiring transplant. Total plasma lipids and apolipoprotein values of the proband and a control female individual are summarized in Table 4.2. The proband's total plasma cholesterol was low (2.34 mmol/l; Normal: 3.93 mmol/l) with only 14.8% of the total cholesterol present in the esterified form giving a marked increase in the cholesterol to cholesterol ester molar ratio (10.9:1). This abnormal lipid profile is characteristic of the FLD phenotype. However, her plasma triglycerides were low at 0.47 mmol/l (Normal: 0.99 $\pm$ 0.28 mmol/l) with very low amounts of apo B (40.5 mg/dl; Normal: 90-110 mg/dl). Plasma apo A-I, A-II, and E levels were also low. Her plasma apo A-1 (14.7 mg/dl, Normal: 100-200 mg/dl) and apo A-II (6.8 mg/dl; Normal: 30-40 mg/dl) levels were less than 25% of normal values. The

Table 4.2: Plasma lipids and apolipoproteins of a patient with FLD

Component	* LCAT deficient	Control
	mmol/l	
Total cholesterol	2.34	3.93
Unesterified cholesterol	2.13	1.00
Cholesteryl esters	0.21	2.91
Triglycerides	0.47	0.55
Phospholipids	1.85	2.24
	mg/dl	
apoA-I	14.7	120.0
apoA-II	6.8	31.0
apoB	40.5	80.0
apoC-II	0.9	3.5
apoC-III	2.0	12.1
apoE	2.1	5.3

Plasma lipids and apolipoproteins of the LCAT-deficient patient and a control female subject. Plasma lipids were determined by gas chromatographic total lipid profiling (Kuksis et al., 1978) and plasma apolipoproteins were quantified by electroimmunoassay (Dolphin et al., 1984). * From Teh et al., 1999.

low level of apo B could be attributed to the decrease in VLDL. When whole serum was subjected to agarose gel electrophoresis (Figure. 4.6), there was an increased presence of heterogeneous β-migrating lipoproteins with very faint bands in the α-migrating region, indicative of a lack of normal HDL particles. The lipoprotein distribution and concentration of each component in individual density gradient fractions from the proband's plasma is represented in Figure 4.7. The proband exhibited a lipoprotein profile that showed a pronounced shift of the HDL fractions (*d:* 1.063 - 1.179 g/ml) into the lower density range (*d:* 1.019-1.063 g/ml), as typically observed in LCAT deficient individuals. The resultant broad peak in the LDL density range is enriched in cholesterol and phospholipids. Esterified cholesterol was also markedly decreased in the VLDL and LDL fractions (1-12), all indicative of FLD. HDL deficiency was quite apparent from both the agarose gel electrophoresis and lipoprotein distribution (Figure 4.6 and 4.7). Fractions 13-22 represent the HDL population (*d:* 1.063-1.179 g/ml). FLD was verified by the absence of LCAT activity against both endogenous and exogenous substrates (data not shown).

The molar ratio of cholesteryl ester to apoB for the apoB-rich lipoprotein fractions isolated by density ultracentrifugation of a normal individual, the LCAT-deficient proband, and a previously reported CETP-deficient patient (Teh et al., 1998) are shown in Table 4.3. In both the LCAT and CETP deficient individuals, the ratio for VLDL and IDL were much lower than that observed in the normal subject. In the LCAT-deficient patient, the low ratio values are observed throughout the LDL fractions but are increased in the CETP-deficient individual. This suggests that LCAT deficiency has a marked effect on the molar ratios of cholesteryl ester/apoB throughout the lipoprotein spectrum.

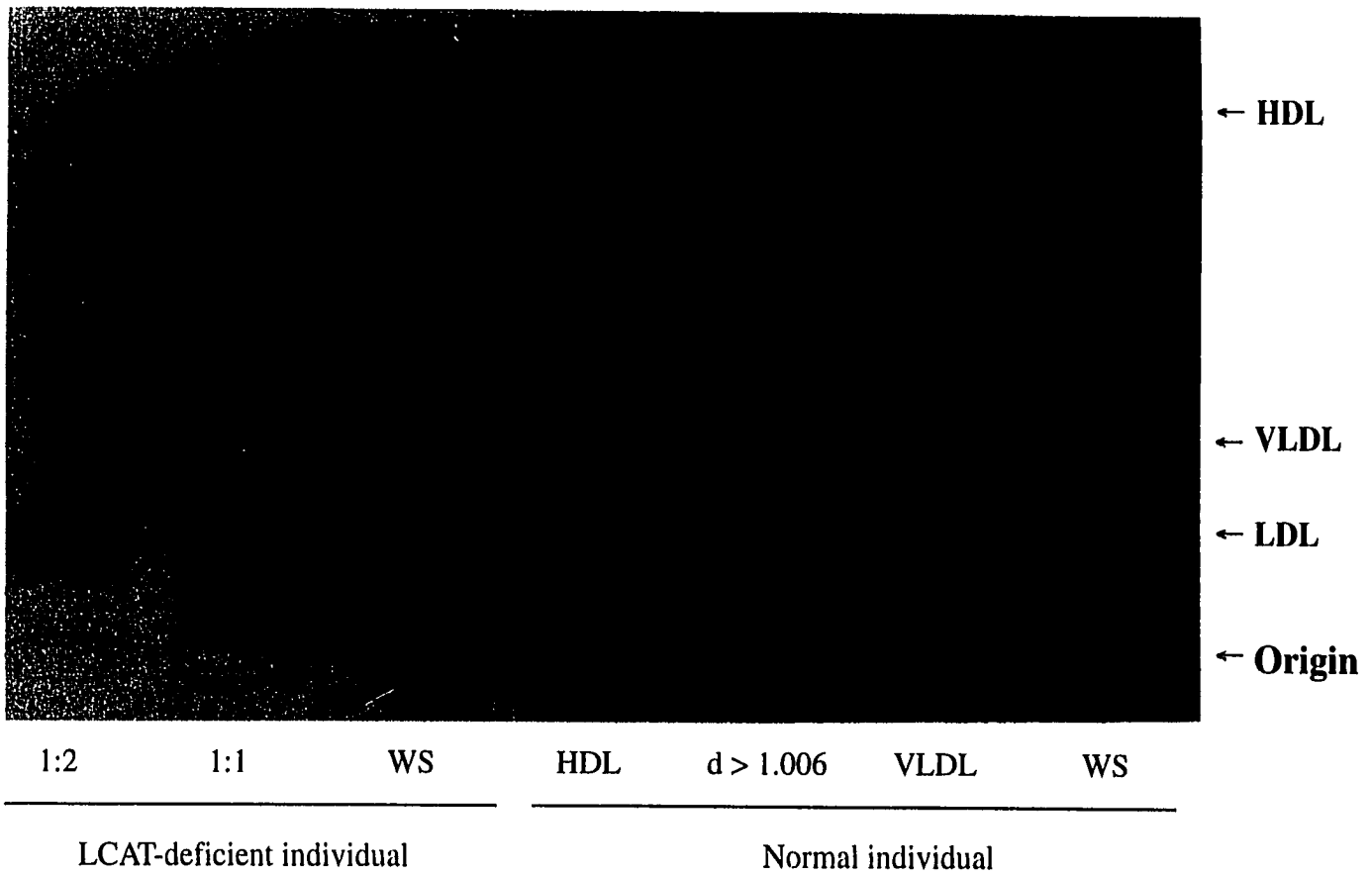


Figure 4.6: Agarose gel electropohoresis. A 0.7% agarose gel electrophoresis of isolated liporpotein fraction and whole serum (WS) from a normal individual and the LCAT-deficient proband showing an increase in ß-migrating lipoproteins. From Teh et al., 1999.

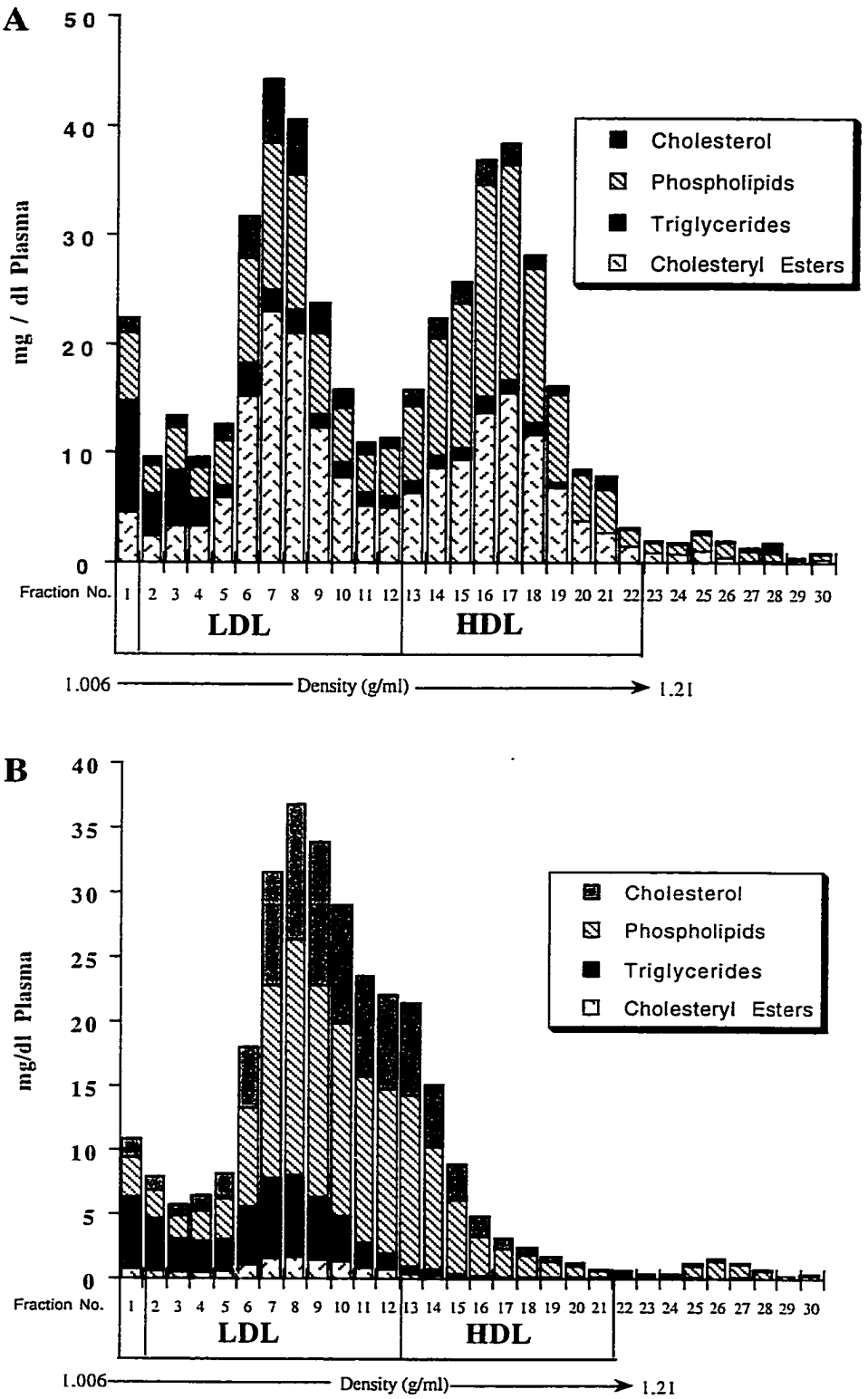


Figure 4.7: Plasma lipid distribution of a normolipidemic individual (A) and the LCAT deficient proband (B). The fractions were isolated by density gradient ultracentrifugation by the method of Chapman et al., 1981. From Teh et al., 1999.

Table 4.3: Molar Proportions of Cholesteryl Ester and ApoB in ApoB-rich Fractions from Density Gradient Ultracentrifugation

Density gradient Fraction		CE / apoB molar ratio		
		CETP-deficient subject	Control female subject	LCAT -deficient subject
1	VLDL	330	1825	433
2	VLDL	307	1668	342
3		739	1920	549
4		1015	2479	481
5		1362	2353	302
6		1345	1636	910
7		1427	1489	884
8		1707	1535	437
9		1813	1448	319

Molar proportions of cholesteryl ester to apoB from the previously described CETP-deficient subject (Teh et al., 1998), a control subject, and the LCAT-deficient patient. The ratios were calculated from the lipid and apolipoprotein analyses of the density gradient ultracentrifugation fractions. Fractions 1 and 2 contain VLDL, fractions 3 and 4 contain IDL and fractions 5-9 are LDL. From Teh et al., 1999.

Sequence analysis of genomic DNA

Since mutations in the LCAT gene are causative for the disorder, the gene was studied as a candidate for the molecular basis of the clinical phenotype. Sequence analysis was carried out on the entire coding region including the consensus splice sites. All exons and exon/intron boundaries were sequenced bidirectionally and found to be normal except for exon 4. A two base pair (TG) deletion involving codons 138-139 of the LCAT protein was found (Figure 4.8) (Teh et al., 1999). This deletion resulted in a frameshift that generated a premature stop codon further downstream in exon 4 at amino acid residue 144. Two clones from three different PCR products of exon 4 from the patient were sequenced on both strands and all showed the missing TG base pairs (results not shown). Direct sequencing of the PCR product (Figure 4.8) was also performed on both strands to evaluate the genotype and confirm the mutation. From these data, it was concluded that the proband is homozygous for the deleted TG nucleotides. Although this mutation did not result in the loss or gain of a restriction enzyme site, it was possible to create a diagnostic restriction site using a mismatched primer that allowed for convenient and rapid detection. The results shown in Figure 4.9 represent the restriction analysis of the PCR-amplified exon 4 with the reverse 53 bp mismatched primer described in the Materials and Methods (Figure 2.2). In the case of the deleted di (TG)-nucleotides, the *Fsp* I restriction site is lost, and only one band representing the PCR product of exon 4 is observed. This differed from two normal individuals that did have the TG nucloetides which were recognized by *Fsp* I and resulted in 103 bp and 52 bp fragments. The absence of any smaller fragments resulting from the digest of the proband's DNA further

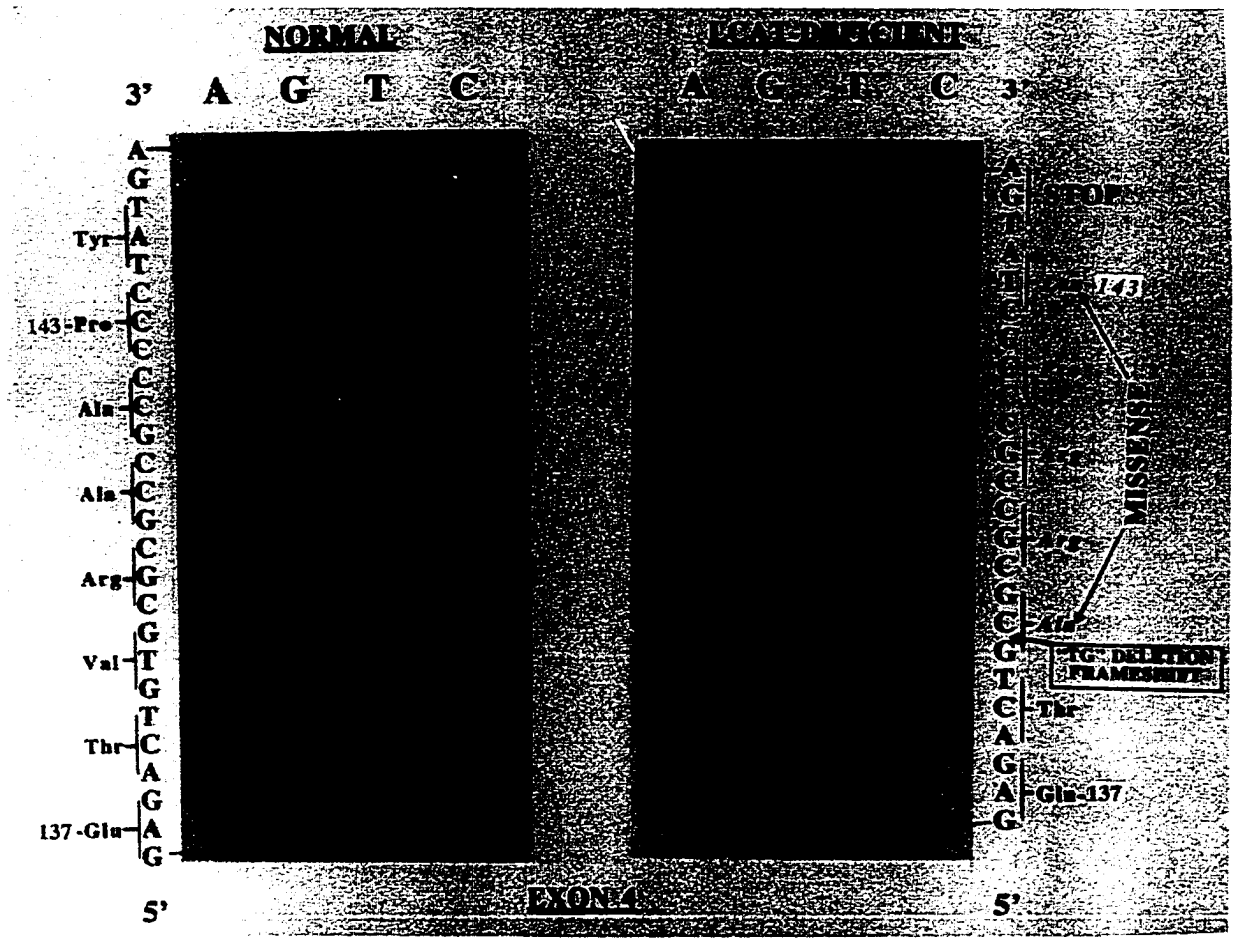


Figure 4.8: DNA sequencing of the PCR products of exon 4. A di(TG)-nucleotide deletion was identified by dideoxy sequencing of the PCR product. This deletion resulted in a frameshift which would cause the protein to be terminated prematurely at residue 144. From Teh et al., 1999.

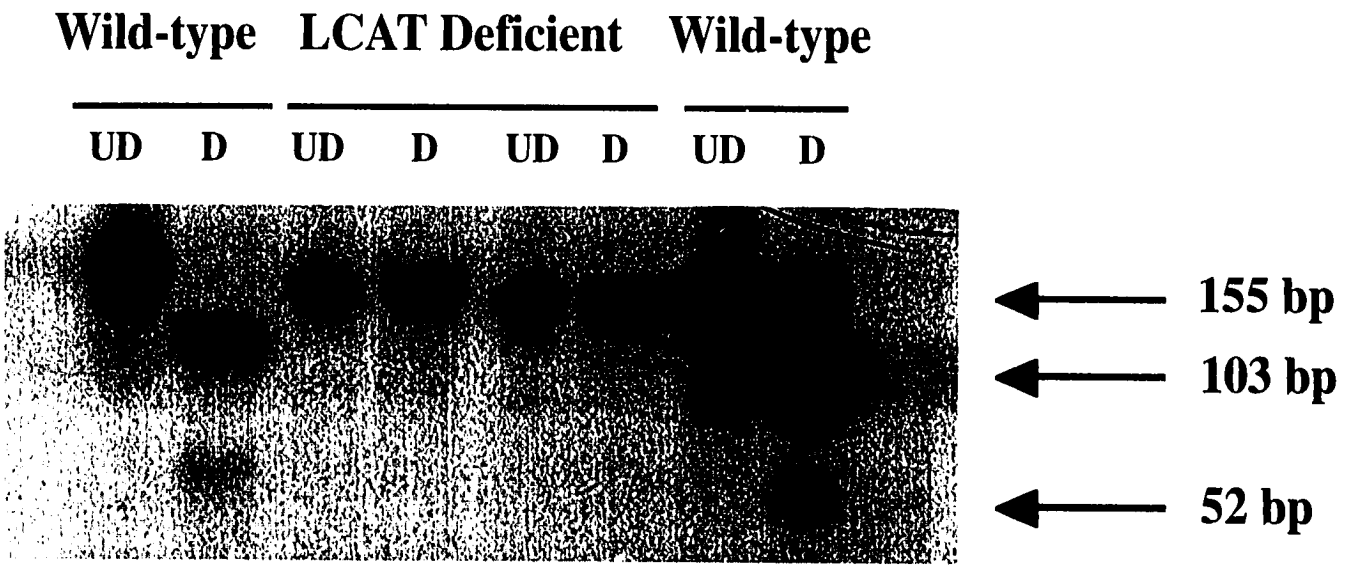


Figure 4.9: Rapid diagnosis for the exon 4 mutation in the LCAT gene by *Fsp*I digestion. The *Fsp*I restriction site was generated in the wild-type sequence by a mismatched primer used in the PCR amplification of exon 4. Upon digestion with *Fsp*I the 155 bp product was cleaved to produce 2 fragments at at 103 bp and 52 bp in the wild-type. The undigested PCR product from the LCAT deficient individual confirmed the presence of the di(TG)-nucleotide deletion (see Materials and Methods, Figure 2.2). The lack of any digestion products in the mutant sequence confirms the homozygous state. (UD: undigested; D: digested). From Teh et al., 1999.

substantiates that she is homozygous for this mutation. No family members were available for this study.

ii.) Investigating the structure and function of the N-terminal domain of human LCAT

Although the human and rat LCAT shares 87% identity, the N-terminal region (first 80 residues) has the highest sequence identity (98.8%) (Figure 4.10). There are only five different amino acids within the first 80 residues of this region. The rat80-human (R80H) chimera was constructed using the PCR overlap technique as described in Materials and Methods (Figure 2.3) using the human and rat cDNA as templates. The successful construction of the R80H chimera was verified by the complete bidirectional sequencing of the cDNA. The chimeric R80H construct was transiently expressed in COS-7 cells along with the recombinant human and rat LCAT sequences.

Expression of recombinant rat-human chimera LCAT protein in COS-7 cells

The expression of the recombinant human LCAT and R80H chimeric LCAT was confirmed by Western blot analysis of the cell lysates (Figure 4.11A) and culture medium (Figure 4.11B). The expression vector, pCMV5 was used as a negative control and a purified recombinant His-tagged LCAT (kindly provided by Dr. John Parks, Dept. of Comparative Medicine, Wake Forest University, Winston-Salem, NC.) was used as a positive control. It appeared that the R80H chimera is expressed and secreted at slightly lower levels than the human LCAT, however the LCAT mass was not determined. The Western blot analysis further showed that R80H chimeric LCAT had the same molecular weight as that of the human LCAT and appeared to be fully glycosylated although this

```
Human   MGPPGSPWQWVTLLLGLLLPPAAPFWLLNVLFPPHTTPKAELSNHTRPVILVPGCLGNQLEAKLDKPDVVNWMCYRKTEDFFTIWLDLNMFLPLGVDCWI
Rat     ..L.....L.....TS.....M..R.....N...L.....F.....

Human   DNTRVVYNRSSGLVSNAPGVQIRVPGFGKTYSVEYLDSSKLAGYLHTLVQNLVNNGYVRDETVRAAPYDWRLEPGQQEEYYRKLAGLVEEMHAAYGKPVF
Rat     .....HM.....DN.....A.R..D...Q.....Y.....

Human   LIGHSLGCLHLLYFLLRQPQAWKDRFIDGFISLGAPWGGSIKPMLVLASGDNQGIPIMSSIKLKEEQRITTTSPWMFPSRMAWPEDHVFISTPSFNYTGR
Rat     .....V.H.....S...H.....RI.....N...R.....AHHV.....N.....Q

Human   DFQRFFADLHFEEGWYMWLQSRDLLAGLPAPGVEVYCLYGVGLPTPRTYIYDHGFPYTDPVGVLYEDGDDTVATRSTELCGLWQGRQPQPVHLLPLHGIQ
Rat     ..E.....H.F.....M..AH.....N...K...AA.....Q.....S.A.....MN.TD

Human   HLNMVFSNLTLEHINAILLGAYRQGPPASPTASPEPPPPE
Rat     .....K.....H.T.K.....LG..TK.
```

Figure 4.10: Primary sequence alignment of the human and rat LCAT. The highlighted sequence represents the first 80 residues of the mature protein. The human and rat LCAT share 87.5% sequence identity. The N-terminal region is the most highly conserved region. A single amino acid change at position 149 changes the fatty-acyl specificity. There are only 5 amino acid differences within the first 80 residues between the two species. Identical residues are represented by (.).

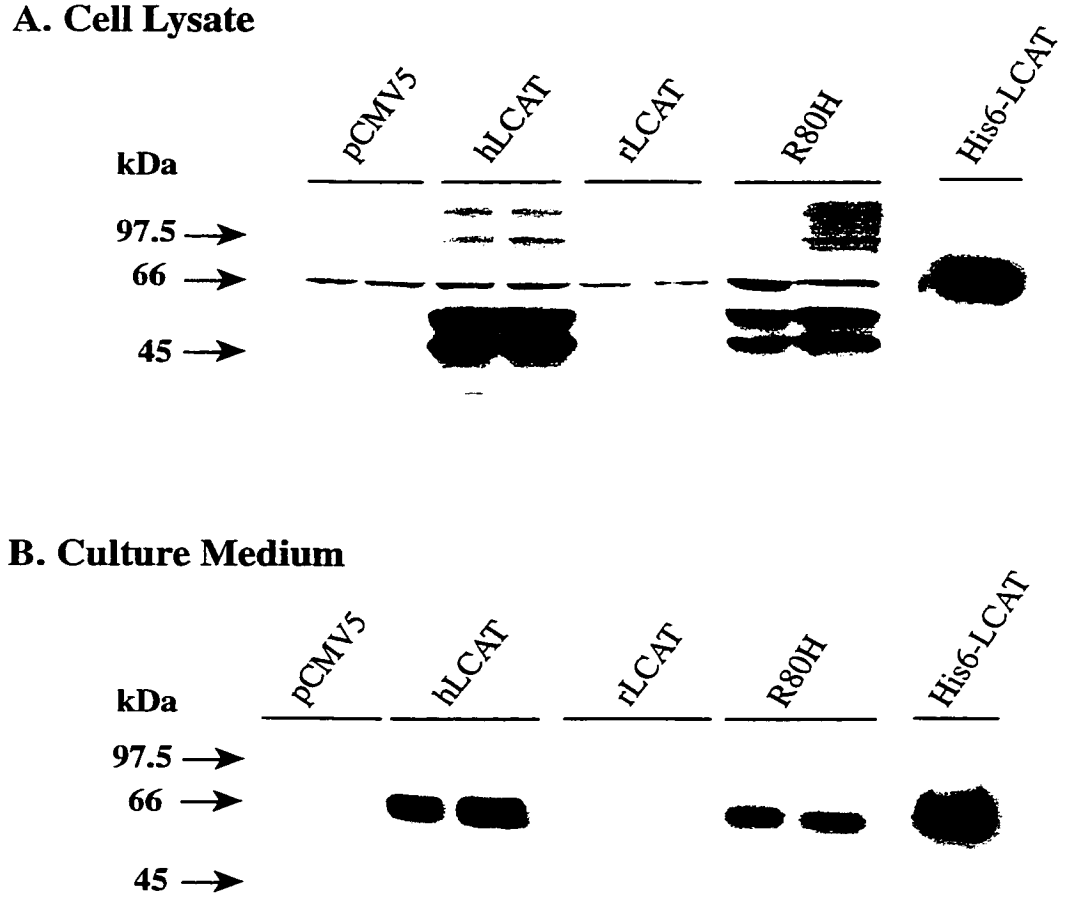


Figure 4.11: Rat-human (R80H) chimeric protein detected by Western blot analysis. Duplicate lanes represent separate transfections from the same experiment. pCMV5 was used as a negative control and the purified His-tagged LCAT was used as a positive control. **A. Cell lysates of COS cells transfected with recombinant LCAT. B. Culture medium harvested from COS cells after 48h.** The samples were loaded on a 10% SDS-PAGE gel and reacted with mouse anti-human LCAT (5D4) and an HRP-conjugated secondary antibody. The bands were visualized by ECL detection and exposure to X-ray film.

was not experimentally determined or confirmed. The 66 kDa bands in the cell lysate are due to non-specific recognition by the antibody. The doublets detected in both the recombinant human LCAT and chimeric R80H LCAT represent the unglycosylated LCAT (at approximately 45 kDa) and possibly the partially glycosylated species. It is of interest to note that the antibody 5D4 did not detect the recombinant rat LCAT protein. However, the expression of the rat LCAT protein was confirmed by activity assays (Table 4.4) and Northern Blot analysis (Figure 4.13). The mRNA levels for both the rat LCAT and R80H chimera were low (20% and 40% respectively) compared to human LCAT.

LCAT activity assays with rHDL and labelled human LDL

The medium was assayed for LCAT activity using rHDL as substrates containing POPC, cholesterol, and either human apoA-I or rat apoA-I. These results are shown in Table 4.4. The LCAT activity is represented as nmol cholesteryl ester formed / h / ml. As the LCAT mass was not measured, the specific activity could not be determined. The human apoA-I / rat apoA-I activity ratio for the recombinant human LCAT was 0.69 and there was no significant difference in activity with rHDL containing human or rat apoA-I. (Figure 4.12). The recombinant rat LCAT, although not detected by Western analysis, displayed activity and was consistently more reactive with rHDL containing rat apoA-I. The rat LCAT had an activity ratio of 0.48. The R80H chimeric LCAT consistently showed a higher preference for rHDL containing human apoA-I over rHDL containing rat apoA-I, and had an activity ratio of 1.62 ($P = 0.02$ with human LCAT and $P = 0.003$ with rat LCAT). The rat LCAT had a higher preference for rat apoA-I, consistent with a previous finding that the homologous apoA-I is a better activator for the LCAT enzyme

Table 4.4: Cholesterol esterification activity in media of COS 7 cells expressing human, rat and R80H chimeric-recombinant LCAT

LCAT source	CE formation		Activity ratio
	h-apoA-I	r-apoA-I	(h-apoA-1/r-apo-AI)
	nmol CE / h / ml		
Human LCAT	5.24 ± 1.67	7.42 ± 1.74	0.69 ± 0.13
Rat LCAT	1.53 ± 0.33	3.64 ± 0.77	0.48 ± 0.11
R80H chimera	4.76 ± 1.48	2.94 ± 0.75	1.62 ± 0.21*

Media was harvested after 48h from transiently transfected COS 7 cells and assayed for LCAT activity using rHDL containing POPC, [^{3}H]-cholesterol, and human or rat apoA-I (72.7:10:1 and 94.7:14.2:1 molar ratio, respectively). Values represent mean ± S.E.M. of three separate transfections. The R80H chimeric cDNA encoded an LCAT protein that contained the first 80 residues from the rat sequence and 81-416 of the human sequence. *Student's t-test were performed on the activity ratio and the R80H chimera is significantly different from both the human ($P = 0.020$) and rat LCAT ($P = 0.003$).

Table 4.5: Cholesterol esterification rate (CER) for chimeric R80H LCAT

LCAT source	CER
	nmol / h / ml
Human LCAT	40.55 ± 13.84
R80H LCAT	52.23 ± 8.00

CERs were measured using human LDL radiolabelled with [^{3}H]-cholesterol as described in Materials and Methods. Activity assays were performed for 6h at 37°C and at 4°C. Values represent mean ± S.E.M. from three separate experiments and their duplicates. Media collected from cells transfected with pCMV5 showed no activity. The CER for human and R80H were not significantly different as shown by Student's t-test.

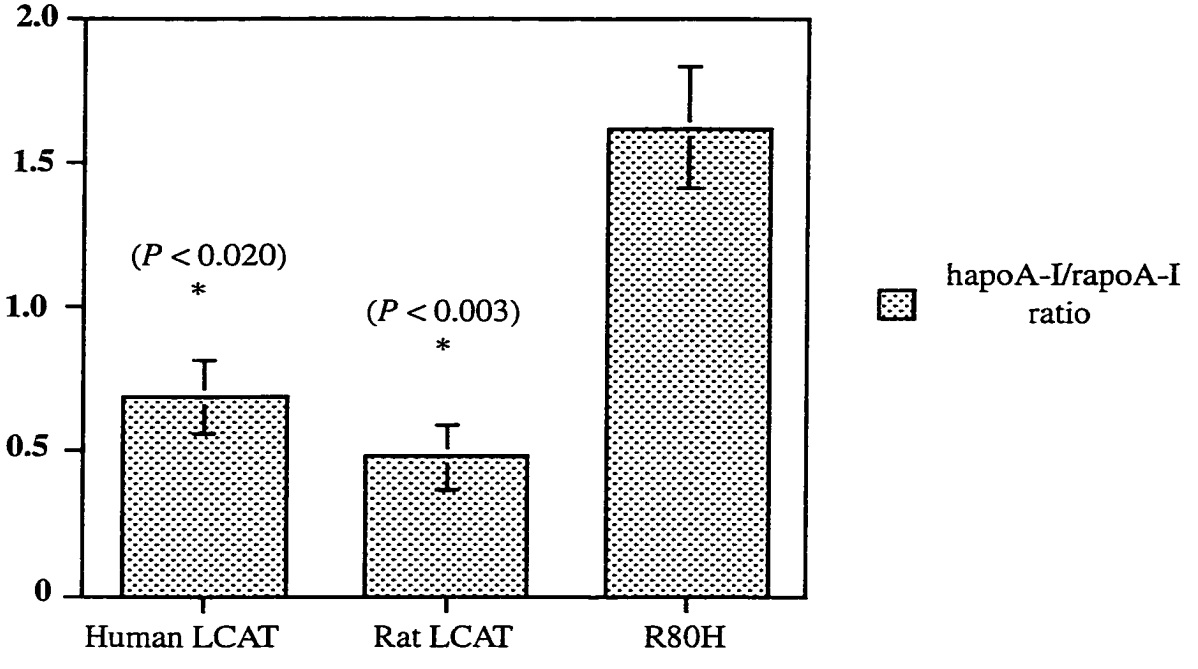


Figure 4.12: Human apoA-I / rat apoA-I ratio of human, rat and chimeric R80H LCAT. The values are from Table 4.4 and represent mean ± S.E.M.. *P* values were calculated by Student's t-tests and (*) are significantly different from R80H.

(Chen and Albers, 1983; Sparrow et al., 1995). Although there was a decrease in LCAT mass in the R80H chimera, the activity against rHDL containing human apoA-I was enhanced.

To further characterize the enzymatic activity of the chimeric R80H LCAT, LCAT activity was measured against human LDL. Table 4.5 represents the cholesterol esterification rate in nmol / h / ml of the recombinant human LCAT and the R80H chimera. Both the human and R80H LCAT exhibited similar rates of cholesterol esterification with 40.55 $\pm$ 13.84 and 52.23 $\pm$ 8.00 nmol /h / ml, respectively. This is inconsistent with the lower amount of LCAT mass secreted by the R80H chimera.

Expression of the LCAT deletion mutants and mRNA analysis

N-terminal deletion mutants of the mature human LCAT were also constructed to investigate their functional significance in the tertiary structure. The N-terminal deletion mutants include the deletions of the first 8 residues (Δ8), the first 10 residues (Δ10) and the first 12 residues (Δ12) of the mature LCAT protein. The successful constructions of all three mutants were confirmed by bidirectional sequencing as seen in Figure 4.14. After transfection into COS-7 cells, the expressions of the deletion mutants (Δ8, Δ10, and Δ12) were evaluated by Western blot analysis. Of the three mutants, only Δ10 was expressed and secreted as shown in Figure 4.15A and 4.15B. Furthermore, the slight decrease in the molecular weight of Δ10 corresponded to the 10 amino acid deletion. The protein appeared to be fully glycosylated and was secreted at comparable levels to wild-type LCAT. The fact that this mutant was secreted suggests that the first 10 residues are not required for secretion. Therefore, it was surprising that the Δ8 mutant was not secreted or expressed. In order to investigate this further, Northern blot hybridization

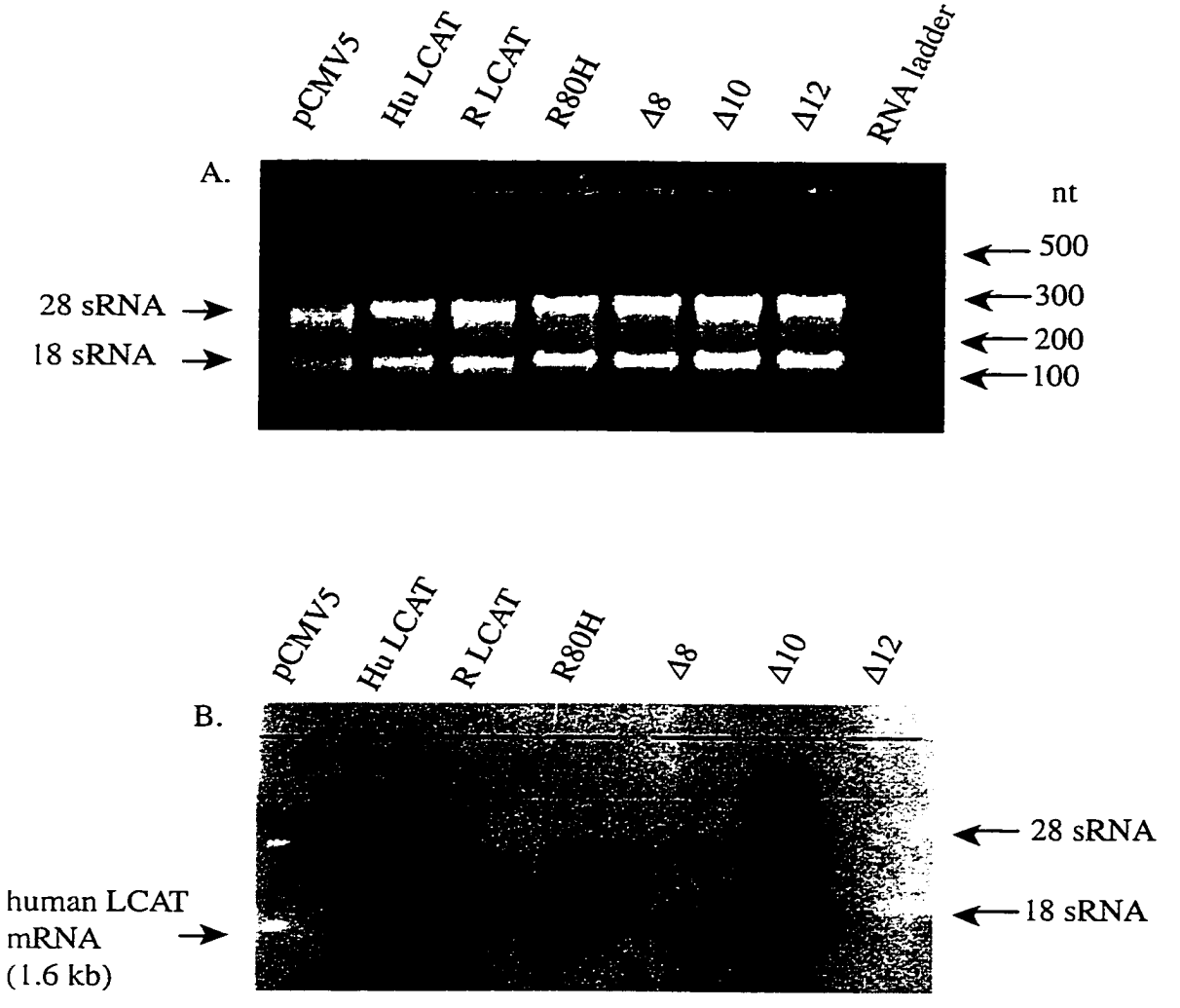


Figure 4.12: A. Northern gel analysis. A total of 20 μg of total RNA isolated from COS-7 cells were loaded into each well. **B. Northern blot hybridization with the full-length human cDNA.** the pCMV5 was used as a negative control. The rat LCAT, R80H, and Δ8 all had very low levels of LCAT mRNA (20%, 40% and 30%, respectively). The Δ10 expression was 88% of that of human LCAT. The Δ12 mutant did not appear to be expressed.

Figure 4.14: N-terminal deletion mutants of the human LCAT cDNA

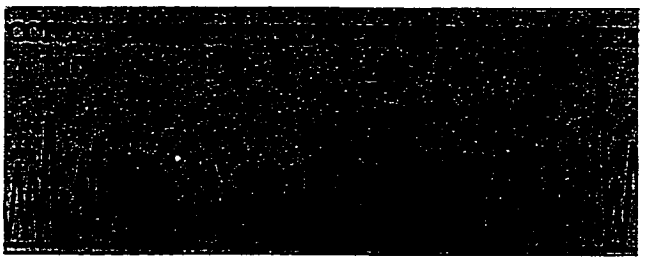
signal peptide (SP) Mature peptide

P P A A P F W L L N V L F P P H T T P K A

Human LCAT

SP

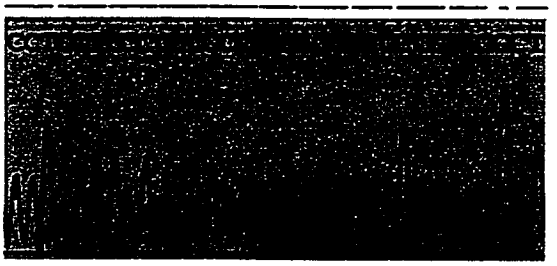
P P A A P P P H T T P K A



Δ 8 mutant of human LCAT

SP

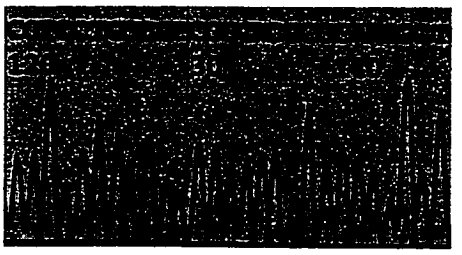
P P A A P H T T P K A



Δ 10 mutant of human LCAT

SP

P P A A P T P K A



Δ 12 mutant of human LCAT

The deletion mutants were constructed from wild-type cDNA using the PCR overlap technique of Ho et al. (1989). Sequencing was first performed manually using the T7 ™ Sequencing Kit to confirm the deletion. The bidirectional sequencing of the entire cDNA was performed using the ABI Prism automated sequencer.

using the full-length human LCAT cDNA was performed to evaluate the Δ8 and Δ12 mutants at the RNA level (Figure 4.13). LCAT mRNA was expressed at the normal size of 1.6 kb. A larger mRNA transcript was also detected but does not appear to be non-specific as it was not observed in the negative control (RNA from cells transfected with pCMV5) nor in the Δ12 mutant which had no detectable LCAT mRNA. Both the rat and chimeric R80H LCAT mRNA were detectable but at much lower levels (20% and 40% with respect to human LCAT) as mentioned previously. The Δ10 mRNA was expressed at comparable levels (88%) when compared to human LCAT mRNA while the Δ8 was expressed at much lower levels (30%). The absence of mRNA in the Δ12 mutant could result from mRNA instability or unsuccessful transfection. To further identify the problem, PCR was used to detect DNA in the cell lysates of transiently transfected COS-7 cells to determine if the Δ12 construct was present. PCR amplification was performed with specific primers targetted to the C-terminus of human LCAT that would result in a single band of 800 bp in the presence of recombinant LCAT. The results showed the Δ8, but not the Δ12 to have detectable DNA (Figure 4.16), again confirming the presence of the Δ8 construct in the cells. These results collectively suggest that the Δ8 was successfully transfected and possibly expressed at very low levels rendering it undetectable by Western analysis. The medium from the Δ8 mutant was subsequently concentrated at least 10X before Western blot analysis but no protein was detectable. In contrast, the Δ12 mutant did not appear to have been successfully transfected since no DNA was detectable.

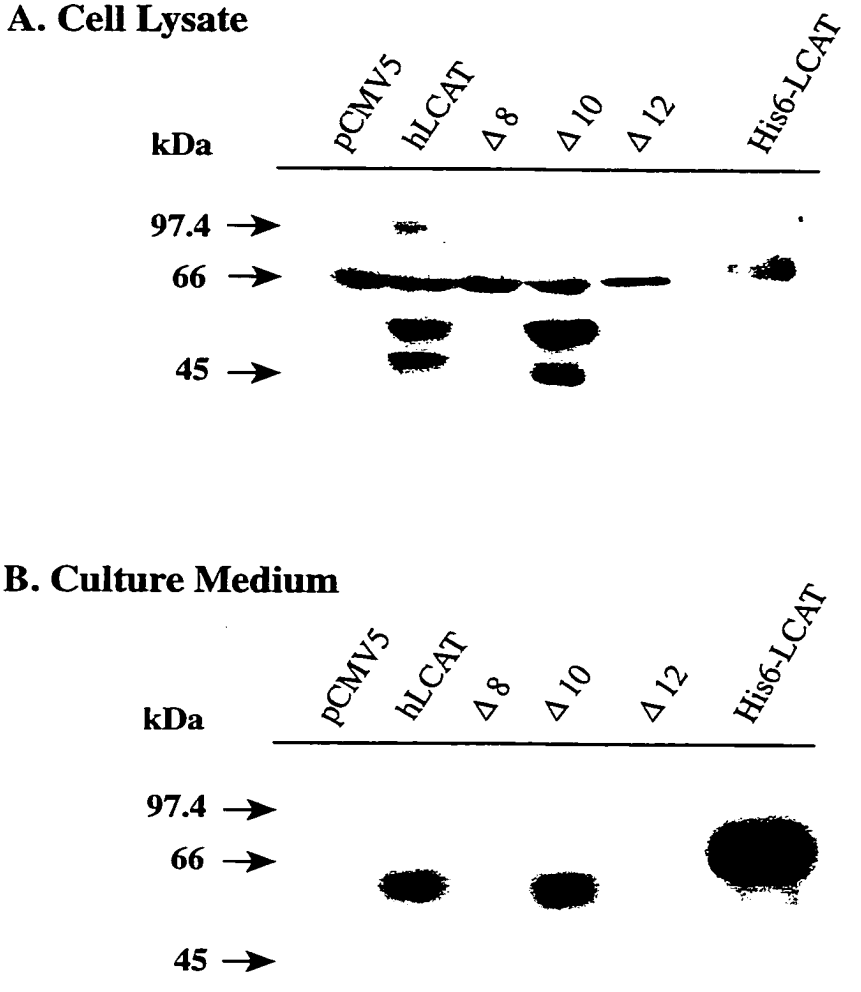


Figure 4.15: Western blot analysis of the N-terminal deletion mutants. A 15 µl aliquot of the cell lysate from each sample and 20 µl of cell medium were loaded on a 10 % SDS-PAGE gel. Purified His6-LCAT was loaded as a control. After electrophoresis, proteins were transferred to PVDF membranes, blocked with Blotto and reacted with monoclonal antibody 5D4, and subsequently with HRP-conjugated secondary antibody. The bands were visualized by ECL detection and exposure to X-ray film.

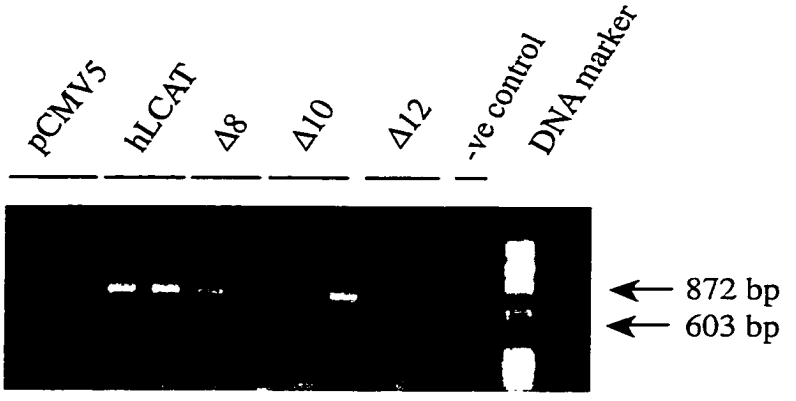


Figure 4.16: PCR amplification of the cell lysates from COS-7 cells transfected with recombinant DNA. Duplicate lanes represent separate transfections of the same experiment. The PCR products were separated on a 0.8% agarose gel and stained with ethidim bromide. The negative control represents the PCR amplification with no template.

Table 4.6: LCAT activities in the cell media of Δ10

LCAT source	CE formation	
	Activity with rHDL	Activity with LDL
	nmol CE / h / ml	*% CER (nmol / h / ml)*
Human LCAT	4.95 ± 0.37	84.60 ± 21.96
Δ- 10 mutant	0.0	*0.0

Values are mean ± S.E.M. of three separate experiments. LCAT activity was assayed using rHDL containing POPC, [^{3}H]-cholesterol, and human apoA-I (72.7:10:1 molar ratio). LDL was isolated as described in Materials and Methods. The COS-7 cells were transiently transfected with human and N-terminally deleted mutant LCAT cDNA. The media was collected after 48h. *Values obtained were negative. Media collected from cells transfected with pCMV5 showed no activity.

LCAT activity assays of the Δ10 mutant

In order to establish the functionality of the Δ10 mutant, LCAT activity assays were performed against rHDL (α-LCAT activity) and human plasma LDL (β-LCAT activity). These results are summarized in Table 4.6. The Δ10 mutant was inactive with both the rHDL and LDL.

I) Discussion

i.) Familial LCAT deficiency in a French patient

There are currently 42 unique LCAT mutations identified and the majority of these result from single base changes, deletions or insertions which may or may not cause frameshifts (Kuivenhoven et al., 1997; Blanco-Vaca et al., 1997; Guerin et al., 1997; Argyropoulos et al., 1998). However, mutations throughout the LCAT gene have not provided much information on the variability of the phenotypic and genotypic expressions.

We have identified a novel di-nucleotide (TG) deletion at residues 138-139 in exon 4 from a French patient who is homozygous for familial LCAT deficiency (Teh et al, 1999). The 2-bp deletion caused a frameshift resulting in the premature truncation of the LCAT protein at residue 144. The mutation was detected by sequence analysis of the PCR products containing the exon/intron boundaries and coding regions of the LCAT gene, and confirmed with an engineered restriction fragment length polymorphism (RFLP) (Figure 2.2). The 53-bp mismatched primer used in the amplification of the PCR product creates an *Fsp*1 RFLP site in the absence of a mutation (wild-type sequence). The lack of LCAT enzyme activity can be attributed to the missing 262 amino acids at

the carboxyl terminal of the protein, including the catalytic triad Ser-181 (Francone and Fielding, 1991a; Qu et al., 1994), and the proposed Asp-345 and His-377 (Peelman et al., 1998). It is not known whether the inactive truncated protein is rapidly degraded upon secretion, or not secreted at all. *In vitro* truncations of the C-terminal showed that deletion past residue 391 results in instability of the protein (Francone et al., 1996; Lee et al., 1997). Based on the classification scheme of Kuivenhoven et al. (1997), this mutation can likely be categorized as Class 1. This class represents FLD deficiency caused by null mutations such as deletions, insertions, or substitutions that cause premature truncation, and defective mRNA splicing resulting in the total loss of catalytic activity and the virtual absence of LCAT mass.

FLD is characterized by severe hypoalphalipoproteinemia, an increased in total plasma cholesterol resulting in an increased molar ratio of cholesterol to cholesteryl esters, corneal opacities, and varying degrees of renal disease (Norum et al., 1989). It is distinguished from FED by the lack of LCAT activity against both the α- (HDL) and β-lipoproteins (LDL). Consistent with the FLD phenotype, our proband lacked both the α- and β-LCAT activities and was severely hypoalphalipoproteinemic. Furthermore, her cholesterol to cholesteryl ester molar ratio was very high (10.9:1). The decreased levels of apoA-I and apoA-II are most likely explained by the increased fractional catabolic rate (Brinton et al., 1991; Emmerich et al., 1993; Horowitz et al., 1993) usually observed in FLD patients.

She developed corneal opacities at age 40 and although the clinical and pathological features of her cornea were very similar to those of FED, there was a lesser degree of lipid deposition (data not shown). Corneal opacities have also been reported in

several other types of HDL deficiencies including FED and Tangier Disease (Winder et al., 1985; Bron, 1989; Assmann, 1990). It has been proposed that reverse cholesterol transport may be essential for the removal of lipids in the cornea and maintenance of lipid homeostasis in the eye (Gjone et al., 1974), as well as other extrahepatic tissues. However, the exact nature of this process remains unclear. A recent study of the corneal lipids in an FED individual showed that the lipids that are deposited in the cornea are mainly cholesterol and phospholipids which are substrates of the LCAT reaction (Blanco-Vaca et al., 1997). The lack of cholesteryl ester accumulation suggest that the flux of cholesteryl esters between the cornea and the plasma compartment may be normal and that LCAT is required for the maintenance of phospholipid and cholesterol homeostasis (Blanco-Vaca et al., 1997). Since the corneal stroma has many structural components similar to that of the arterial wall, it may behave like the extracellular matrix of the arterial wall and favour lipoprotein deposition.

The proband was also slightly anemic, but did not suffer from proteinuria or renal disease as observed in most FLD patients. Although interesting, this is not unusual since there have been reported cases of FLD patients in the 5th decade of life (Gjone et al., 1974; Borysiewicz et al., 1982; Murayama et al., 1984a; Guerin et al., 1993) with no signs of proteinuria or renal failure. It is also noteworthy that male patients are more likely to demonstrate both renal disease and CHD than their female counterparts (Gjone et al., 1974; Borysiewicz et al., 1982; Murayama et al., 1984a). Also, in LCAT transgenic mice studies, over-expression of LCAT seems to have a more significant impact on HDL cholesterol in transgenic males than in transgenic female mice (Vaisman et al., 1995). Moreover, patients who are vegetarians are also asymptomatic for renal

disease (Murayama et al., 1984b). Thus, it is likely that diet and sex hormones are factors that may affect not only the development of CHD but also in the development of renal disease (Pritchard and Hill, 1993). The proband's unusually low total plasma cholesterol levels could potentially account for the lack of some clinical manifestations such as anemia, proteinuria and renal failure. For example, reduced plasma cholesterol may result in a lowered tendency to enrich red cell membranes with cholesterol which would render her erythrocytes less fragile and hence less likely to lyse than in other LCAT-deficient individuals presenting a much higher plasma cholesterol level. Renal failure has been linked to elevated levels of Lp-X particles (Guerin et al., 1993) which was not apparent in our proband.

It was previously demonstrated that in the absence of CETP, the cholesteryl ester/apoB molar ratio appears to be very low in the VLDL fraction (Teh et al., 1998). This can be attributed to the fact that the amount of cholesteryl ester secreted as VLDL is very low and requires cholesteryl ester derived form LCAT to be transferred by CETP from HDL particles to assume the cholesteryl ester/apoB ratio observed in the control individual. Based on this, it can be assumed that in the absence of LCAT the cholesteryl ester/apoB ratio should also be low as LCAT is responsible for forming the majority of plasma cholesteryl ester. The LCAT deficient individual did indeed exhibit a very low cholesteryl ester/apoB molar ratio similar to that of the CETP deficient individual and in contrast, this trend is observed throughout the IDL-LDL range. Although CETP is present, there are very low amounts of cholesteryl ester available for transfer. However, if high amounts of cholesteryl ester were secreted in the VLDL particle, the cholesteryl ester/apoB ratio in the LCAT deficient individual should be similar to that of the normal

individual. These low ratios are thus a reflection of the amount of cholesteryl ester secreted by the liver in the VLDL particle.

LCAT deficiency and atherosclerosis

Decreased levels of HDL-cholesterol has been used as a marker for an increased risk for coronary heart disease (CHD) since epidemiological studies have shown a strong positive correlation between the absence of HDL-cholesterol and premature CHD. Furthermore, the role of HDL as a transport vehicle for excess cholesterol from the periphery to the liver in reverse cholesterol transport is thought to account for one of the many protective effects of HDL against atherosclerotic vascular disease. Some controversy still surrounds the suggestion that low levels of HDL-cholesterol are in fact, a risk indicator for the development of premature CHD. Despite the low levels of HDL in FLD, FED, and Tangier disease, CHD is present in only half the reported cases. Our patient appeared lesion free at age 45. In contrast, in cases where apoA-I is not synthesized and is completely absent from the plasma, fulminant atherosclerosis occurs at an early age (Norum et al., 1982; Calabresi and Franceschini, 1997). Thus, apoA-I turnover rates may be a more significant factor in risk determinations than absolute plasma levels. Recently, more reports of FED are showing an increased risk in CHD possibly due to the retained β-LCAT activity that could potentially cause an increase in the levels of atherogenic LDL cholesteryl ester. Another plausible explanation may reside within the fact that cholesterol present in plasma lipoprotein can freely exchange with cell membranes. These membranes provide a large "sink" within the body for cholesterol. LDL cholesteryl esters, in contrast, do not readily exchange between LDL and cell membranes. Rather, their uptake is targeted to those cells such as fibroblasts,

smooth muscle cells, and macrophages capable of recognizing and taking up either native or oxidized LDL species and thereby, potentially contributing to the atherogenic process. Since LDL cholesteryl ester levels are very low in FLD, the targeted uptake of this lipid, which could contribute to plaque formation, would be reduced when compared to normal individuals. LDL cholesteryl ester uptake in the FED phenotype, however, could approach normal values since the plasma cholesterol esterification rate is near normal as LDL cholesterol is esterified by the β-LCAT activity of the mutated yet functional enzyme. Other factors, that are secondary causes of CHD, may also contribute to the risk of developing premature CHD in individuals with LCAT deficiency.

The question remains as to what role HDL plays in the absence of LCAT that may be beneficial in some way in preventing vascular disease. As proposed by Fielding et al. (1982), there may be another mechanism which allows for cholesterol egress out of the cell without cholesterol esterification by LCAT. Small HDL particles enriched in apoE, quite common in FLD, are thought to facilitate the efflux and influx of cholesterol to maintain homeostasis (Fielding et al., 1982). Other important factors such as those of a physical and genetic nature would also contribute to the multifactorial development of premature atherosclerosis.

More studies concerning the structure and function of LCAT are required. Once the 3-dimensional structure of LCAT is firmly established, it will allow us to study LCAT function more effectively and discover why different phenotypes are expressed with mutations in certain areas of the gene and the different catalytic activities that are displayed by this unique enzyme. The role of LCAT in the development of atherosclerosis remains a central topic that has yet to be fully understood.

ii.) Structure and functional significance of the far N-terminal region of LCAT

The structure and functional role of the N-terminal region of LCAT is unknown. Although this region is the most highly conserved between species, it does not share any structural homology with any known proteins (Peelman et al., 1998). As a result, the predicted 3-D model of LCAT does not include the N-terminal region. The first 80 residues of LCAT contains the putative "lid" region that has been shown to be required for LCAT activity but not for structural integrity (Adimoolam et al., 1997; Peelman et al., 1998). Furthermore, this region might be involved in LCAT activation by apoA-I as mutations present at Pro-10 result in FED (Skretting and Prydz, 1992; Qu et al., 1995; Klein et al., 1995).

Chimeric R80H LCAT

The first 80 residues of the different LCAT species are very highly conserved. Although the chicken LCAT shares an overall 75% sequence identity with that of human LCAT, the sequence identity of the first 80 residues is 96.5%. The first 80 residues of rat and human LCAT on the other hand, shares a 98.8% sequence identity. Sequence conservation between species is usually significant because it indicates a structural and/or functional role. Based on this observation, it was hypothesized that the chimeric R80H LCAT will be secreted and will be functionally active. Indeed, the chimeric R80H LCAT was secreted with the correct molecular weight and appeared to be similarly glycosylated when compared to the human LCAT. This is not surprising as the glycosylation site at Asn-20 in the human LCAT is conserved in the rat LCAT sequence. Furthermore, the chimeric R80H was functionally active against rHDL (α-LCAT activity) and LDL (β-LCAT activity). The relative activity against the rHDL from the chimeric R80H LCAT

was about 90% of the human LCAT against rHDL containing human apoA-I and slightly less than 50% with rHDL containing rat apoA-I suggesting that the R80H chimera had an enhanced activity in the presence of human apoA-I. This was not consistent with the results observed for the protein and mRNA expression. The mRNA expression of the R80H chimera was significantly lower (40%) than human LCAT. The LCAT protein is quite sensitive to changes as seen in FED where LCAT mass is decreased even when the protein is functional. Additionally, the β-LCAT activity was also similar to that of the human LCAT.

It has been established that between species, LCAT is always more reactive with its homologous apoA-I (Chen and Albers, 1983; Sparrow et al., 1995). Although the rat LCAT consistently showed a higher reactivity with rHDL containing rat apoA-I, the human LCAT seemed more reactive with rHDL containing rat apoA-I. Interestingly, the chimeric R80H LCAT showed a preference for rHDL containing human apoA-I (activity ratio of 1.62 ± 0.21). This suggest that the five amino acid differences in the first 80 residues between the human and rat LCAT may cause some subtle changes in the folding of the LCAT protein. These different residues in the N-terminal region may confer conformational changes on the LCAT such that the interaction with or activation by apoA-I is altered. As discussed previously, the mechanism of activation of LCAT by apoA-I remains unresolved. It was suggested that apoA-I and LCAT interact on the surface of the HDL and that apoA-I may stabilize the activated conformation of LCAT. Alternatively, these observations may be due entirely to conformational changes unrelated to apoA-I activation or interaction with LCAT and hence it is premature to suggest that the first 80 residues might involve apoA-I.

The five differences in amino acids between the human and rat LCAT at the first 80 residues are at positions 32, 35, 44, 49, and 64. These residues, from human to rat represent changes from polar to non-polar residue or vice-versa (Leu-32 → Met, Met-49 → Leu), polar to charged residues or vice versa (Gln-35 → Arg, Asp-44 → Asn), and non-polar to an aromatic residue (Leu-64 → Phe). The introduction of charged residues could potentially disrupt the conformation of the protein especially when the corresponding residue from the human LCAT is unchanged. A natural mutation at Leu-32 → Pro (McLean, 1992) of the human LCAT causes FLD. Missense mutations at residue 30 (Gly-30 → Ser) (Bron et al., 1975; Owen et al., 1996) and residue 33 (Gly-33 → Arg) (Wiebusch et al., 1995) also cause FLD. This region of the LCAT enzyme, which is in the vicinity of Cys-31and Cys-184, has been shown to be in close spatial proximity to the catalytic Ser-181 (Jauhiainen et al., 1988). Although the Cys residues are highly conserved in the mammalian LCAT species, the Leu-32 in rat LCAT is substituted with a non-polar Met residue, and in both the mouse and the rat Gln-35 is substituted by a positively charged Arg. These residues in the R80H chimera collectively could confer conformational changes such that both the α- and β- LCAT activity are retained, whereas if these substitutions were separately introduced into the wild-type human LCAT, the α- or β- LCAT activity may be disrupted. The decrease in the R80H LCAT mass may also be caused by structural changes in residues 1-80 since *in vitro* expression of the natural mutations occurring at residues 30, 32 and 33 are secreted by COS cells at 66-90% of that of wild-type LCAT (Qu et al., 1995; Yang et al., 1997; Peelman et al., 1999b).

Furthermore, the Leu-64 → Phe is within the putative "lid" region and residues 56-68 have been shown to have the characteristics of an oblique lipid destabilizing peptide involved in substrate interaction. The oblique peptide has a hydrophobicity gradient along its helical axis (Perez-Mendez et al., 1998) and is involved in interfacial binding of substrates. The more hydrophobic end of the helix inserts into the membrane at an angle and perturbs the regular lipid organization (Peelman et al., 1999a). Studies by Peelman et al. (1999a) showed that both the Met-65 and Asn-66 are involved in maintaining the peptide obliquity as mutagenesis of Met-65 →Asn and Asn-66 → Met decreased activity of LCAT on all substrates, particularly LDL. A more hydrophobic residue at position 64 (Leu → Phe) might increase the ability of the LCAT "lid" to interact with LDL that has a more condensed phospholipid monolayer than HDL due to its increased saturated phospholipids, cholesterol and sphingomyelin content.

In order to evaluate whether the first 80 residues actually changes the α- and β-activity, the specific activities of the recombinant LCAT are required. The sequential mutagenesis of the five residues of the rat LCAT sequence (res. 1-80) in the chimeric R80H LCAT to that of the human sequence (res.81-416) could potentially provide information on the functional roles of these residues.

N-terminal Deletion Mutant

The N-terminal deletion mutants include the Δ8, Δ10, and Δ12. These mutants were constructed to investigate their role in aligning the putative "lid" region. The proteins for the Δ8 and Δ12 were undetectable by Western blot analysis even after concentration of the culture medium. DNA and RNA analysis suggest that the Δ8 mutant was successfully transfected and expressed but at very low levels. The low levels of

expression are likely the cause of the inability to detect protein in the media by Western Blot analysis. In view of the fact that the Δ10 protein has a much larger deletion and is secreted at higher levels than that of the wild-type human LCAT, it is unlikely that the Δ8 is not secreted because of structural defects. However, there is the possibility that the residues between 6 and 10 might exert some structural role in the presence of the partial sequence but does not affect the protein when completely removed (i.e. Δ10). Alternatively, the Δ8 mutant might cause translational defects such as the proper cleavage of the pre-peptide due to the presence of the proline-rich region (Figure 4.14) adjacent to the signal sequence. This could cause the rapid degradation of the protein resulting in a lack of secretion. In order to address these questions, proteosome inhibitors such as N-acetyl-leucly-leucyl-norleucyl (aLLN) or carbobenzoxyl-leucinyl-leucinyl-luecinal (MG132) could be used to prevent the degradation of the protein through the ubiquitin-proteasome pathway. Previous preliminary experiments with wild-type human LCAT showed that in the presence of such inhibitors (10 μM of aLLN or 1 μM of MG132) there was an accumulation of the protein in the cell lysates. Hence, if similar results are observed with the Δ8 mutant, it can be concluded that the absence of the protein in the cell lysate and medium is a result of rapid degradation of the protein.

The translational efficiency of the Δ8, on the other hand, can be assessed using the in vitro transcription/translation cell-free system with and without microsomes. This experiment would provide information on the translational processing of the Δ8 mutant and whether the N-terminal Pro-1 and Pro-2 residues of the Δ8 mutant affects its translation. In contrast, transfection of the Δ12 mutant construct was unsuccessful.

The Δ10 mutant was expressed and secreted at comparable levels to that of the wild-type human LCAT. This suggests that the first 10 residues are not essential for protein secretion. This region is therefore most likely located on the surface of the enzyme such that its deletion does not drastically affect the overall structure of LCAT. However, LCAT activity was abolished towards rHDL and LDL substrates. The role of the far N-terminal hydrophobic residues is unknown but natural mutations at Pro-10 and Thr-13 causes FED and FLD respectively. These results suggest that the residues at the far N-terminus are important for function. Jin et al. (1997) in contrast, were able to produce a fully functional enzyme lacking the first six residues of the mature LCAT protein. In the absence of any other data from the Δ8 mutation, it can be suggested that residues between 6 and 10 are important for full enzymatic activity. The hydrophobic residues at these positions may serve to anchor the lid to the enzyme through hydrophobic interactions. A fully functional lid not only requires that it has the ability to interact with lipoprotein substrates but also interact with the surrounding residues required for the conformational changes upon interfacial activation. The residues at the N-terminal region may have a similar function as that of residues Arg-52 and Lys-53 that were proposed to be involved in the folding of the lid structure. In addition, the hydrophobic residues at the far N-terminus may function to keep the lid in place upon interfacial activation or keep the lid "closed" prior to interfacial activation. Clearly more experiments are required to fully delineate the role of this region. Sequential deletion of the N-terminus would provide information on which residues are required for secretion and which are required for function.

In summary, human apoA-I is a better activator of the chimeric R80H LCAT than wild-type LCAT. The β-LCAT activity of the R80H chimera also appears to be enhanced. The sequential substitution of the five amino acids in the rat LCAT sequence of the R80H chimera would provide information on the significance of these residues in the function of LCAT, specifically in the possible interaction with or activation by apoA-I. The far N-terminal residues are also important for enzyme function. Deletion of the first 10 residues resulted in the total loss of LCAT activity against both rHDL and LDL. The hydrophobic amino acids between residues 6 and 10 are clearly important and may be involved in anchoring the lid of LCAT. The sequential deletion of each residue from the N-terminal would pinpoint the residues important for functional and/or structural integrity.

5. Final Conclusions and potential future directions

Familial CETP deficiency has been reported in the Japanese and European populations (Table 3.1). In Japan, the Int14(A) and Asp-442 → Gly mutations are the most prevalent and the heterozygote frequencies for these mutations are 2% and 7%, respectively (Hirano et al., 1993; Inazu et al., 1990; Inazu et al., 1994; Sakai et al., 1995). The partial expressions of these mutations also appear to be genetically dominant. Clinically, patients with CETP deficiency have elevated total cholesterol, which is a result of increased HDL-cholesterol and therefore is usually unresponsive to lipid-lowering drugs such as the statins. Owing to increased HDL levels that have been shown in epidemiological studies to be a negative risk factor for CHD, CETP deficiency was originally proposed to be a longevity syndrome. However, current evidence from human population and transgenic animal studies have questioned this view and have suggested that CETP deficiency may in fact be a cause of premature CHD.

The CETP mutation identified in this study is a novel mutation and the first to be characterized in a Caucasian North American (Teh et al., 1998). The C → T transition at the first position of codon 268 in exon 9 of the CETP gene results in the substitution of Arg-268 to a nonsense codon. The premature truncation of the CETP protein would result in a lack of lipid transfer activity. A diagnostic approach to rapidly screen for this mutation was developed and involves the amplification of a 168 bp fragment in the region of the mutation. In the presence of the mutation, a *Mae*III site is generated.

As no family members were available for the study, pedigree analysis could not be performed. It is important to recognize and properly diagnose CETP deficiency since

in dyslipidemias such as hypertriglyceridemia, the lowering of CETP activity may be beneficial to reduce the transfer of cholesteryl esters to atherogenic lipoproteins such as the VLDL, IDL and LDL. Furthermore, the "U" shaped relationship between HDL cholesterol concentrations and CHD (Hirano et al., 1997) suggests that it may be beneficial to increase HDL cholesterol levels in heterozygous CETP-deficient patients to the range between 1.55 -1.81 mmol/l. On the other hand, in homozygotes that have markedly elevated HDL cholesterol levels, it would be preferable to decrease HDL levels to the above range. The molecular analysis provided here for the rapid identification of this novel mutation should be used as a tool for screening patients who are hypercholesterolemic and drug resistant especially in the South shore region of Nova Scotia.

A new mutation was also identified in the LCAT gene of a French patient with familial LCAT deficiency (Teh et al., 1999). The consecutive deletion of two nucleotides (TG) at the third position of codon 138 and the first position of codon 139 causes a frameshift that results in the premature termination of the LCAT protein at residue 144. This mutation can also be rapidly detected using a mismatched primer that creates a site for the restriction enzyme *Fsp*I in the absence of a mutation.

The phenotypic hallmark of FLD is severe hypoalphalipoproteinemia, increased total cholesterol resulting in an increased molar ratio of cholesterol to cholesteryl esters, corneal opacities and varying degrees of renal disease. The LCAT-deficient proband had corneal opacity and received a bilateral corneal transplant. Although her total cholesterol levels were low, the cholesterol / cholesteryl ester molar ratio was high. The inconsistencies observed with HDL cholesterol and atherosclerosis in LCAT and CETP

deficiency suggest that HDL cholesterol levels per se, are not totally indicative of an efficient RCT pathway. Genetic LCAT or CETP deficiency that perturbs that RCT pathway would offer some insights into lipoprotein metabolism and atherosclerosis. Recently, the discovery of the scavenger receptor B1 as a putative HDL receptor has provided new insights into HDL metabolism.

The functionally important regions of LCAT remain to be identified. In particular, focus on the N-terminal region of LCAT is lacking. A rat-human (R80H) LCAT chimera and N-terminal deletion mutants of human LCAT were used to determine the structural and functional significance of the far N-terminus. The results suggest that human apoA-I is a better activator of the chimeric R80H LCAT. The sequential substitution of the five non-identical residues in the rat LCAT sequence of the R80H chimera would provide further information on the significance of these residues in LCAT function specifically, in the interaction of LCAT with its substrates, and perhaps in the activation by or interaction with apoA-I which remains to be identified.

It was proposed that the hydrophobic residues at the far N-terminus anchor the putative lid to the enzyme through hydrophobic interactions. The deletion of LCAT from the far N-terminus showed that residue 10 is essential for full enzymatic function but not for structural stability as determined by protein synthesis and secretion. However, no solid conclusion can be drawn from the Δ8 mutant. More experiments are obviously required to fully elucidate the role of the first 10 residues. There is the possibility that residues between 6 and 10 are required for structural stability. Sequential deletion from the N-terminus is therefore a logical follow up to confirm these findings.

The increasing number of protein and enzyme defects that are currently being identified allow for a more accurate evaluation of the impact of mutations in different genes on CHD risk. As all steps in the RCT are modulated by proteins and enzymes it is necessary to further define the mechanism(s) by which they function and modulate lipoprotein metabolism. The molecular approach used for identifying the functional residues at the N-terminal domain of LCAT may provide a better understanding of protein function and hopefully, contribute to the incomplete 3-D model for LCAT. A better insight into the complex RCT pathway may permit pharmacological manipulations of protein action to treat or manage CHD.

6. Appendices

I. Primers used in CETP gene amplification for the identification of the gene mutation.

Primer name	Sequence (5' → 3')
Exon 1	CGGACATACATATACGGGCTC CTGGCATAGTGGGTGTCCATG
Exon 2	CATCTCAGAGAGGCTGAGTC CCAGCCAGGTCACACCCGAG
Exon 3	CTATCTTCCACCCTCGCCTAG GCATCAGCTGACAGGAATGC
Exon 4	CCTCTCTGGGTGGAGGGCTGAATG GAACCCTCTGCCTGCACAAC
Exon 5	GCATGTGGATACCATCTGATAG GTGGACACACTAACAGGATG
Exon 6	GTACACACTAGGCGCTCCATG GAAGGTGGGAGTGGCACCTTC
Exon 7	GTGAGTGAGGCTGGCTGACT CAGCAGACAGAAACGCACTCA
Exon 8	GACGGTGACTCAGGGCAATTC CAGGACTCTTGGCTGCCATAAG
Exon 9	GACACACAGGGTCCAGCCAG CATACCACATGCCATCTGGATC
Exon 10	CAGTCCTTGAAACTGCCCTTG CTCTTGAAGCCAGAGCAGCATTG
Exon 11	CATGGACTGCTGCTCCGTGATG CTGCGGCCCATATGTGAGTC
Exon 12	GTCATCCTTGCCTCTCCAGTC GAGAGGTTTGCGGAGTTGAC
Exon 13	AGGCCTGAGCTATGAGACA TGGACCACCTGGTCACATTCT
Exon 14	CCGTGCAGCATCTGCCTTGT

	AAAAGGTGAAATGGGAAGCT*
Exon 15	GTCTCAGCATCTCCCACTAC
	CAGGAATTCTGTCTGGGCCTTCTCTC†
Exon 16	CTCTACCAGCTTGGCTCCCTC
	CCAGGGTTCCAGCTGTGAGC

* From Brown et al., 1989.
† From Takahashi et al., 1993.

II. Primers used for the construction of the Rat-80 human chimeric protein.

Primer name	Sequence (5' → 3')
HuRI.EcoRI	CGTCAGAATTCTCGAGCTCGTCG
RatHu.2r	CACATTGAGGAGCCAGAAGGGGGTCGGC
HuRat.3f	CTGCTCCCTCCTGCCGCCCCCTTCTGGCTCCTCAATGTGCTCTTC
HuRat.4r	GCTCCGGTTGTAGACAACCCTGGTGTTATCAATCCAGCA
RatHu.5f	CTCGGGGTGGACTGCTGGATTGATAACACCAGGGTTGTCTACAAC
HuR2.BamHI	CACCCGGGGATCCTTATTCAGGAGGCGGGGGCTCTG

II. Primers used for the construction of the deletion mutants.

Primer name	Sequence (5' → 3')
LCAT-Δ8	CTGCTCCCTCCTGCCGCCCCCCCCCCGCACACCACGACCAAGGCTG and CTCAGCCTTGGGCGTGGTGTGCGGGGGGGGGGCGGCAGGAGGGAG
LCAT-Δ10	CTGCTCCCTCCTGCCGCCCCCCACACCACGCCCAAGGCTGAG and GAGCTCAGCCTTGGGCGTGGTGTGGGGGGCGGCAGGAGGGAG
LCAT-Δ12	CTGCTCCCTCCTGCCGCCCCCACGCCCAAGGCTGAGCTC and ACTGAGCTCAGCCTTCCCCGTGGGGGCGGCAGGAGGGAG

7. References

Acton, S., A Rigotti, K. T. Landschulz, S. Xu, H. H. Hobbs, and M. Krieger. (1996). Identification of a scavenger receptor SR-BI as a high density lipoprotein receptor. *Science*. **271**: 518-520.

Adimoolam, S., and A. Jonas. (1997) Identification of a domain of lecithin-cholesterol acyltransferase that is involved in interfacial recognition. *Biochem. Biophys. Res. Comm.* **232**: 783-787.

Adimoolam, S., Y.-P. Lee, and A. Jonas. (1998). Mutagenesis of highly conserved histidines in lecithin:cholesterol acyltransferase: Identification of an essentil histidine (His 377). *Biochem. Biophys. Res. Comm.* **243**: 337-341.

Agellon, L. B., E. M. Quinet, T. G. Gillette, D. T. Drayna, M. L. Brown, and A. R. Tall. (1990). Organization of the human cholesteryl ester transfer protein gene. *Biochemisty.* **29**: 1372-1376.

Agellon, L. B., A. Walsh, T. Hayek, P. Moulin, X.-C. Jiang, S. A. Shelanski, J. L. Breslow, and A. R. Tall. (1991) Reduced high density lipoprotein cholesterol in human cholesteryl ester transfer protein transgenic mice. *J Biol. Chem.* **266:** 10796-10801.

Agellon, L. B., P. Zhang, B. Christie, L. Mendelsohn, and A. R. Tall. (1992). The CCAAT/Enhancer binding protein *trans* activates the human cholesteryl ester transfer protein gene promoter. *J. Biol. Chem.* **267**: 223336-223339.

Alaupovic, P. (1985). The physiochemical and immunological heterogeneity of human plasma high-density lipoproteins *In* Clinical and Metabolic aspects of high density liporpoteins. Miller, N. E., Miller G. J. (eds). Amsteram, Elsevier Science Publishers BV. 1-43.

Albers, J. J., J. H. Tollefson, C. H. Chen, and A. Steinmetz. (1984). Isolation and characterization of human plasma lipid transfer proteins. *Arteriosclerosis*. **4:** 49-58.

Arai, T., S. Yamashita, N. Sakai, K. Hirano, S. Okada, M. Ishigami, T. Maruyama, M. Yamane, H. Kobayashi, S. Nozaki, T. Funahashi, K. Kameda-Takemura, N. Nakajima, and Y. Matsuzawa. (1996). A novel nonsense mutation (G181X) in the human cholesteryl ester transfer protein gene in Japanese hyperalphalipoproteinemia subjects. *J. Lipid Res.* **37**: 2145-2154.

Argyropoulos, G. A. Jenkins, R. L. Klien, T. Lyons, B. Wagenhorst, J. St. Armand, S. M. Marcovina, J. J. Albers, P. H. Hadyn. (1998). Transmission of two novel mutations in a pedigree with familial lecithin:cholesterol acyltransferase deficiency: structure-

function relationships and studies in a compound heterozygous. *J. Lipid Res.* **39:** 1870-1876.

Aron, L., S. Jones, and C. Fielding. (1978). Human plasma lecithin-cholesterol acyltransferase. Characterization of cofactor-dependent phospholipase activity.*J. Biol. Chem.* **253:** 7220-7226.

Assmann, G., and H. B. Brewer Jr. (1974). Lipid-protein interactions in high density lipoproteins. *Proc. Natl. Acad. Sci. USA.* **71:** 989-993.

Assmann, G., G. Schmitz, N. Donath, and D. Lekim. (1978). Phosphoatidylcholine substrate specificity of lecithin:cholesterol acyltransferase. *Scand. J. Clin. Lab. Invest.* **38 (Suppl. 150)**: 16-20.

Assmann, G. (1990). Genes and dyslipoproteinaemias. *Eur. Heart J.* **11(Suppl H):** 4-8.

Assmann, G., A. von Eckardstein, and H. Funke. (1992). Mutations in apolipoprotein gene and HDL metabolism. *In* Structure and function of apolipoproteins. M. Rosseneu. (ed). CRC Press, Boca Raton, Fl. 85-122.

Au-Young, J., and C. J. Fielding. (1992). Synthesis and secretion of wild-type and mutant human plasma cholesteryl ester transfer protein in baculovirus-transfected insect cells; the carboxy-terminal region is required for both lipoprotein binding and catalysis of transfer. *Proc. Natl. Acad. Sci. USA.* **89:** 4049-4098.

Azoulay, M., I. Henry, F. Tata, D. Weil, K. H. Grzeschik, M. E. Chaves, N. McIntyre, R. Williamson, S. E. Humpries, and C. Junien. (1987). The structural gene for lecithin:cholesterol acyl transferase (LCAT) maps to 16q22. *Ann. Hum. Genet.* **51**: 129-136.

Babbitt, J., B. Trigatti, A., Rigotti, E. J. Smart, R. G. W. Anderson, S. Xu, and M. Krieger. (1997). Murine SR-B1, a high density lipoprotein receptor that mediates selective uptake, is N-glycosylated and fatty acylated and colocalized with plasma membrane caveolae. *J.Biol. Chem.* **272**: 13242-13249.

Banka, C. L. (1996). High density lipoprotein and lipoprotein oxidation. *Curr. Opion. Lipidol.* **7:** 139-142.

Barbaras, R., P. Puchois, J. C. Fruchart, and G. Ailhaud. (1987). Cholesterol efflux from cultured adipose cells is mediated by LpA-I particles but not by LpA-I:A-II particles. *Biochem. Biophys. Res. Commun.* **142**: 63-69.

Barter, P. J. (1983). Evidence that lecithin:cholesterol acyltransferase acts on both high density and low density lipoproteins. *Biochim. Biophys. Acta.* **751**: 261-270.

Barter, P. J., G. J. Hopkins, and L. Gorjatschko. (1984). Comparison of human plasma low- and high-density lipoproteins as substrates for lecithin:cholesterol acyltransferase. *Biochim. Biophys. Acta.* **792**: 1-5.

Barrans, A., B. Jaspard, R. Barbaras, H. Chap, B. Perret, X., Collet. (1996). Pre-β HDL: structure and metabolism. *Biochim. Biophys. Acta.* **1300:** 73-85.

Beisiegel, U., W. Weber, and G. Bengtsson-Olivecrona. (1991). Lipoprotein lipase enhances the inding of chylomicrons to low density lipoprotein receptor-related protein. *Proc. Natl. Acad, Sci, USA.* **88**: 8342-8346.

Beisiegel, U. (1998). Lipoprotein metabolism. *Eur. Heart J.* **19 (Suppl. A):** 20-23.

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., S. Santamarina-Fojo. (1997). High plasma HDL concentrations associated with enhanced atherosclerosis in transgenic mice overexpressing lecithin-cholesteryl acyltransferase. *Nature Medicine.* **3:** 744-749.

Bisgaier, C. L., M.V. Siebenkas, M. L. Brown, A. Inazu, J. Koizumi, H. Mabuchi, and A. R. Tall. (1991). Familial cholesteryl ester transfer protein deficiency is associated with triglyceride-rich low density lipoproteins containing cholesteryl esters of probable intracellular origin. *J. Lipid Res.* **32**: 21-33.

Blanche, P. J., E. L.Gong, T. M. Forte, and A. V. Nichols. (1981). Characterization of high density lipoproteins by gradient gel electrophoresis. *Biochim. Biophys. Acta.* **665**: 408-419.

Blanco-Vaca, F., S.-J. Qu, C. Fiol, H.-Z. Fan, Q. Pao, A. Marzal-Casacuberta, J. J. Albers, I. Hurtado, V.Gracia, X. Pinto, T. Marti, and H. J. Pownall. (1997). Molecular basis of fish-eye disease in a patient from Spain. Characterization of a novel mutation in the LCAT gene and lipid analysis of the cornea. *Arterioscl. Thromb. Vasc. Biol.* **17:** 1382-1391.

Bonelli, F. S., and A. Jonas. (1989). Reaction of lecithin cholesterol acyltransferase with water-soluble substrates. *J. Biol. Chem.* **264:** 14723-14728.

Borysiewicz, L. K., A. K. Soutar, D. J. Evans, G. R. Thompson, A. J. Rees. (1982). Renal failure in familial lecithin:cholesterol acyltransferase deficiency. *Quarterly J. of Med.* **51**:411-426.

Branden, C., and J. Tooze. (eds) (1991). An exmaple of enzyme catalysis: serine proteinases. *In* Introduction to protein structure. Garland Publishing Inc., New York. 231-245.

Brasseur, R., T. Pillot, L. Lins, J. Vandekerckhove, and M. Rosseneu. (1997). Peptides in membranes: tipping the balance of membrane stability. *Trends Biochem. Sci.* **22:** 167-171.

Breckenridge, W. C., P. J. Dolphin, and M. H. Tan. (1984). Distribution of the molecular species of phsopholipids in human umbilical cord blood. *Lipids.* **19:** 337-340.

Brewer, H. B. Jr., T. Fairwell, A. LaRue, R. Ronan, A. Houser, and T. J. Bronzert. (1978). The amino acid sequence of human APOA-I, an apolipoprotein isolated from high density lipoproteins. *Biochem. Biophys. Res. Commun.* **80:** 623-630.

Brewer, H. B. Jr., and D. J. Rader. (1990). HDL: structure, function and metabolism. *Prog. Lipid Res.* **30:** 139-144.

Brinton, E. A., S Eisenberg, and J. L. Breslow. (1991). Increased apo A-I and apo A-II fractional catabolic rate in patients with low high density lipoprotein cholesterol levels with or without hypertriglyceridemia. *J. Clin. Invest.* **87**: 536-544.

Bron, A. J., J. K. Llyod, A. S. Fosbrooke, A. F. Winder, and R. C. Tripathi. (1975). Primary L.C.A.T. –deficiency disease. *Lancet.* **1:** 928-929.

Bron, A. J. (1989). Corneal changes in dyslipoproteinaemias. *Cornea.* **8**: 135-140.

Brousseau, M. E., S. Santamarina-Fojo, L. A. Zech, A. M. Berard, B. L. Vaisman, S. M. Meyn, D. Powell, H. N. Brewer Jr., and J. M. Hoeg. (1996). Hyperalphalipoproteinemia in human lecithin cholesterol acyltransferase transgenic rabbits. In vivo apolipoprotein A-I catabolism is delayed in a gene-dose dependent manner. *J. Clin. Invest.* **97:** 1844-1851.

Brousseau, M. E., S. Santamarina-Fojo, B. L. Vaisman, D. Applebaum-Bowden, A. M. Berard, G. D. Talley, H. B. Brewer Jr., and J. M. Hoeg. (1997). Overexpression of human lecithin:cholesterol acyltransferase in cholesterol-fed rabbits: LDL metabolism and HDL metabolism are affected in a gene dose-dependent manner. *J. Lipid Res.* **38:** 2537-2547.

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

Brown, M. L., A. Inazu, C. B. Hesler, L. B. Agellon, C. Mann, M. E. Whitlock, Y. L. Marcel, R. W. Milne, J. Koizumi, H. Mabuchi, R. Takeda, and A. R. Tall. (1989). Molecular basis of lipid transfer protein deficiency in a family with increased high density lipoproteins. *Nature (London).* **342**: 448-451.

Bruce, C., D. S. Sharp, and A. R. Tall. (1998a). Relationship of HDL and coronary heart disease to a common amino acid polymorphism in the cholesteryl ester transfer protein in men with and without hypertriglyceridemia. *J. Lipid Res.* **39:** 1071-8.

Bruce, C., L. J. Beamer, and A. R. Tall. (1998b). The implications of the structure of the bactericidal/permeability-increasing protein on the lipid-transfer function of the cholesteryl ester transfer protein. *Curr. Opin. Struc. Biol.* **8:** 426-434.

Calabresi, L., and G. Franceschini. (1997). High density lipoproteins and coronary heart disease: insights from mutations leading to low HDL. *Curr. Opin. Lipidol.* **8:** 219-224.

Carlson, L. A., and B. Philipson. (1979). Fish-eye Disease: a new familial condition with massive corneal opacities and dyslipoproteinaemia. *Lancet.* **2**: 921-924.

Carlson, L. A. (1982). FED: a new familial condition with massive corneal opacities and dyslipoproteinemia. *Eur. J. Clin. Invest.* **12:** 41-53.

Carlson, L. A., and L. Holmquist. (1983). Studies on high density lipoproteins in FED. *Acta Med. Scand.* **213:** 177-182.

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

Chan, L. (1992). Apolipoprotein B, the major protein component of triglyceride-rich and low density lipoproteins. *J. Biol. Chem.* **276**: 25621-25624.

Chapman, M. J., S. Goldstein, S. Lagrange, and P. M. Lapland. (1981). A density gradient ultracentrifugatal procedure for the isolation of the major classes from human sera. *J. Lipid Res.* **22**: 339-356.

Chapman, M. J., Guerin, M., and E. Bruckert. (1998). Atherogenic, dense low-density lipoproteins. Pathophysiology and new therapeutic approaches. *Eur. Heart J.* **19 (Suppl. A):** 24-30.

Chen, C. H., and J. J. Albers. (1983). Interspecies activation of lecithin-cholesterol acyltrasnferase by apolipoprotein A-I isolated from the plasma of humans, horses, sheep, goats, and rabbits. *Biochim. Biophys. Acta.* **753:** 40-46.

Chen, C. H., and J. J. Albers. (1985). A rapid large-scale procedure for purification of lecithin:cholesterol acyltransferase from human and animal plasma. *Biochim. Biocphys. Acta.* **826:** 279-286.

Chen, C. H., T. H. Forte, B. E. Cahoon, R. N. Thrift, and J. J. Albers. (1986). Synthesis and secretion of lecithin-cholesterol acyltransferase by the human hepatoma cell line HepG2. *Biochim. Biophys. Acta* **877**: 433-439.

Cheung, M. C., J. P. Segrest, J. J. Albers, J. T. Cone, C. G. Brouilette, B. H. Chung, M. Kashyap, M. A. Glasscock, and G. M. Ananthatanaiah. (1987). *J. Lipid Res.* **28:** 913-929.

Cheung, M. C. and A. C. Wolf. (1989). *In vitro* trnasformation of apoA-I-containing lipoprotein subpopulations: role of lecithin:cholesterol acyltransferase. *J. Lipid Res.* **30**: 499-509.

Cho, K.-H., J.-Y. Lee, M.-S. Choi, J. M. Cho, J.-S. Lim, and Y. B. Park. (1998). A peptide from hog plasma that inhibit human cholesteryl ester transfer protein. *Biochim. Biophys. Acta.* **1391:** 133-144.

Chong, K. S., M. Jahani, S. Hara, and A. G. Lacko. (1983). Characterization of lecithin-cholesterol acyltransferase from human plasma. 3. Chemical properties of the enzyme. *Can J. Biochem. Cell Biol.* **61:** 875-881.

Chung, J., D. A. Abano, G. M. Fless, and A. M. Scanu. (1979). Isolation, properties, and mechanism of in vitro action of lecithin cholesterol acyltransferase from human plasma. *J. Biol. Chem.* **254:** 7456-7464.

Clark, R. W., J. B. Moberly, and M. J. Bamberger. (1995). Low level quantification of cholesteryl ester transfer protein in plasma subfractions and cell culture media by monoclonal antibody-based immunoassay. *J. Lipid Res.* **261**: 876-889.

Clay, M. A., H. H. Newnham, and P. J. Barter. (1990). Hepatic lipase promotes a loss of apolipoprotein A-I from triglyceride-enriched human high density lipoproteins during incubations in vitro. *Arterioscler. Thromb.* **11:** 415-422.

Collet, X., and C. J. Fielding. (1991). Effects of inhibitors of N-linked oligosaccharide processing on the secretion, stability, and activity of lecithin:cholesterol acyltransferase. *Biochemistry.* **30:** 3228-3234.

Collet, X., A. R. Tall, H. Serajuddin, K. Guendouzi, L. Royer, H. Oliveira, R. Barbaras, X.-C., Jiang, and O. L. Francone. (1999). Remodelling 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:** 1185-1193.

Connolly, D. T., D. Heuvelman, and K. Glenn. (1996a). Inactivation of cholesteryl ester transfer protein by cysteine modification. *Biochem. Biophys. Res. Comm.* **223:** 42-47.

Connolly, D. T., J. McIntyre, D. Heuvelman, E. E. Remsen, R. E. McKinnie, L. Vu, M. Melton, R. Monsell, E. S. Krul, and K. Glenn. (1996b). Physical and kinetic characterization of recombinant human cholesteryl ester transfer protein. *Biochem. J.* **320:** 39-47.

Day, J.R., J. J. Albers, C. E. Lofton-Day, T. L. Gilbert, A. F. T. Ching, F. J. Grant, P.J. O'Hara. (1994). Complete cDNA encoding human phospholipid transfer protein from human endothelial cells. *J. Biol. Chem.* **269**: 9388-9391.

Davis, R. A., and J. E. Vance. (1996). Structure, assembly and secretion of lipoproteins. *In* Biochemistry of lipids, lipoproteins and membranes. D. E. Vance, and J. Vance, (eds.) Elsevier Press, Amsterdam. 473-493.

Derewenda, U., A. M. Brzozowski, D. M. Lawson, and Z. S. Derewenda. (1992). Catalysis at the interface: the anatomy of a conformational change in triglyceride lipase. *Biochemisrty.* **31:** 1532-1541.

Dessi, M., C. Motti, C. Cortese, E. Leonardis, C. Giovannini, G. Federici, and F. Piemonte. (1997). Alternative splicing of human plasma cholesteryl ester transfer protein mRNA in Caca-2 cells and its modulation by oleic acid. *Mol. and Cell. Biochem.* **177:** 107-112.

Dinchuk, J., J. Hart, G. Gonzalez, G. Karmann, D. Schmidt, and D. O. Wirak. (1995). Remodelling of lipoproteins in transgenic mice expressing human cholesteryl ester transfer protein. *Biochim. Biophys. Acta.* **1255:** 301-310.

Dobiásová, M., and J. J. Frohlich. (1994). Structural and functional assessment of high density lipoprotein heterogeneity. *Clin. Chem.* **40**: 1554-1558.

Dolphin, P. J. (1980). Organic extraction and preparative isoelectric focussing of rat serum apoliporpoteins. *FEBS Lett.* **117:** 252-258.

Dolphin, P. J., W. C. Breckenridge, M. A. Dolphin, and M. H. Tan. (1984). The lipoproteins of human umbilical cord blood. Apolipoprotein and lipid levels. *Atherosclerosis.* **51**: 109-122.

Dolphin, P. J. (1985). Lipoprotein metabolism and the role of apolipoproteins as metabolic programmers. *Can J. Biochem. Cell Biol.* **63:** 850-869.

Dolphin, P. J., and M. Jauhiainen. (1987). Human plasma lecithin-cholesterol acyltransferase-the catalytic mechanism of action. *Atheroma.* **13:** 16- 28.

Dolphin, P. J. (1992). Lipolytic enzymes and the role of apolipoproteins in the regulation of their activity. *In* Structure and function of apolipoproteins. M. Rosseneu. (ed). CRC Press, Boca Raton, Fl. 295-362.

Dousset, N., A. M. Julia, H. Chap, and L. Douste-Blazy. (1988). Enhanced cholesteryl ester transfer activity in cyclophoaphamide-treated rabbits: relationship with lipoplytic enzymes. *Adv. Exp. Med. Biol.* **243:** 255-261.

Drayna, D., A. S. Jarnagin, J. McLean, W. Henzel, W. Kohr, C. Fielding, and R. Lawn. (1987). Cloning and sequencing of the human cholesteryl ester transfer protein cDNA. *Nature (London).* **327**: 632-634.

Egloff, M.-P., F. Marguet, G. Buono, R. Verger, C. Cambillau, and van Tilbeurgh. (1995). The 2.46 A resolution structure of the pancreatic lipase-colipase complex inhibited by a C11 alkyl phosphonate. *Biochemistry.* **34:** 2751-2762.

Emmerich, J., B. Verges, I. Taureron, D. Rader, S. Santamarina-Fojo, J. Schaefer, M. Ayrault-Jarrier, P. Thieblot, H. B. Brewer Jr.. (1993). Familial HDL deficiency due to marked hypercatabolism of normal apo A-I. *Arterioscler. Thromb.* **13**: 1299-1306.

Erickson, S. K., and C. J. Fielding. (1986). Parameters of cholesterol metabolism in the human hepatoma cell line, Hep-G2. *J. Lipid Res.* **27**: 875-883.

Fielding, C. J., V. G. Shore, and P. E. Fielding. (1972). A protein cofactor of lecithin:cholesterol acyltransferase. *Biochem. Biophys. Res. Commum.* **46**: 1493-1498.

Fielding, C. J., J. Frohlich, K. Moser, and P. E. Fielding. (1982). Promotion of sterol efflux and net transport by apolipoprotein E in lecithin:cholesterol acyltransferase deficiency. *Metabolism.* **31**: 1023-1028.

Fielding, C. J. (1984). The origin and properties of free cholesterol potential gradients in plasma and their relation to atherogenesis. *J. Lipid Res.* **25:** 1624-1628.

Fielding, C. J. (1990). Lecithin:cholesterol acyltransferase *In* Advances in Cholesterol Research. M. Esfahani and J. B. Swaney. (eds). The Telford Press, New Jersey. 271-314.

Fielding, P. E., T. Miida, and C. J. Fielding. (1991). Metabolism of low-density lipoprotein free cholesterol by human plasma lecithin-cholesterol acyltransferase. *Biochemistry.* **30:** 8551-8557.

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

Fielding, P. E., and C. J. Fielding. (1980). A cholesteryl ester transfer complex in human plasma. *Proc. Natl. Acad. Sci. USA.* **77:** 3327-3330.

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

Fielding, P. E., and C. J. Fielding. (1996). Dyanamics of lipoprotein transport in the circulatory system. *In* Biochemistry of lipids, lipoproteins and membranes. D. E. Vance, and J. Vance, (eds.) Elsevier Press, Amsterdam. 495-516.

Folch, J., M. Lees, and G. H. Sloane-Stanley. (1957). A simple method for the isolation and purification of total lipids from animal tissue. *J. Biol. Chem.* **226**: 497-509.

Forte, T. M., J. K. Bielicki, R. Goth-Goldstein, J. Selmek, and M. R. McCall. (1995). Recruitment of cell phospholipid and cholesterol by apolipoprotein 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**: 148-157.

Forte, T., A. V. Nichols, J. A. Glomset, K. R. Norum. (1974). The ultrastructure of plasma lipoproteins in lecithin:cholesterol acyltransferase deficiency. *Scand. J. Clin. Lab Invest.* **137**: 121-134.

Francone, O. L., L. Royer, and M. Haghpassand. (1996). Increased preβ-HDL levels, cholesterol efflux, and LCAT-mediated esterification in mice expressing the human cholesteryl ester transfer protein (CETP) and human apolipoprotein A-I transgenes. *J. Lipid Res.* **37:** 1268-1277.

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 functional unit containing these activities together with apolipoprotein A-I and D that catalyzes the esterification and transfer of cell-derived choelsterol. *J. Biol. Chem.* **264:** 7066-7072.

Francone,O. L., A. Gurakar, and C. J. 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 apo A-I and D that catalyzes the esterification and transfer of cell derived cholesterol.*J. Biol. Chem.* **264:** 7066-7072.

Francone, O. L., and C. J. Fielding. (1991a). Effects of site-directed mutagenesis at residues cysteine-31 and cysteines-184 on lecithin-cholesterol acyltransferase activity. *Proc. Natl. Acad. Sci. USA.* **88**: 1716-1720.

Francone, O. L., and C. J. Fielding. (1991b). Structure-function relationships in human lecithin:cholesterol acyltransferase. Site directed mutagenesis at serine residues 181 and 216. *Biochemistry.* **30**: 10074-10077.

Francone, O. L., L. Evangelista, and C. J. Fielding. (1993). Lecithin-cholesterol acyltransferase: effects of mutagenesis at N-linked oligosaccharide attachment sites on acyl aceptor specificity. *Biochim. Biophys. Acta.* **1166**: 301-304.

Francone, O. L., E. L. Gong, D. S. Ng, C. J. Fielding, and E. M. Rubin. (1995). Expression of human lecithin-cholesterol acyltransferase in transgenic mice. Effect of human apolipoprotein AI and human apoliporpotein AII on plasma lipoprotein choelsterol metabolism. *J. Clin. Invest.* **96:** 1440-1448.

Francone, O. L., L. Evangelista, and C. J. Fielding. (1996). Effects of carboxy-terminal truncation on human lecithin;cholesterol acyltransferase activity. *J. Lipid Res.* **37**: 1609-1615.

Francone, O. L., M. Haghpassand, J. A. Bennett, L. Royer, J. McNeish. (1997). Expression of human lecithin:cholesterol acyltransferase in transgenic mice: effects on cholesterol efflux, esterification, and transport. *J. Lipid Res.* **38:** 813-822.

Fruchart, J. C., G. Ailhaud, J. M. Bard. (1993). Heterogeneity of high density lipoprotein particles. *Circulation.* **87(suppl 3):** 22-27.

Funke, H., H. Wiebusch, L. Fuer, S. Muntoni, H. Schulte, and G. Assmann. (1994). Identification of mutations in the cholesteryl ester transfer protein in Europeans with elevated high density lipoprotein cholesterol. *Circulation.* **90 (pt 2)**: I-241. (Abstr.).

Fukasawa, M. H. Arai, and K. Inoue. (1992). Establishment of anti-human cholesteryl ester transfer protein monoclonal antobodies and radioimmunoassaying of the level of cholesteryl ester transfer protein in human plasma. *J. Biochem.* **111:** 696-698.

Furakawa, Y., and T. Nishida. (1979). Stability and properties of lecithin-cholesterol acyltransferase. *J. Biol. Chem.* **254:** 7213-7219.

Gaudet, F., and G. S. Ginsburg. (1995). Transcriptional regulation of the cholesteryl ester transfer protein gene by the orphan nuclear hormone receptor apolipoprotein A-I regulatory protein-1. *J. Biol. Chem.* **270:** 29916-29922.

Gauthier, B., M. Robb, F. Gaudet, G. S. Ginsburg, and R. McPherson. (1999). Characterization of a cholesterol response element (CRE) in the promoter of the cholesteryl ester transfer protein gene: functional role in transcription factors SREBP-1a, -2, and YY1. *J. Lipid Res.* **40:** 1284-1293.

Ginsberg, H., and M. –R. Taskinen. (1997). New insights into the regulation of lipoprotein metabolism: studies in procaryotes, eukaryotes, rodents, pigs, and people. *Curr. Opin. Lipidol.* **8**: 127-130.

Gjone, E., J. P. Blomhoff, A. J. Skarbovik. (1974). Possible association between an abnormal low density lipoprotein and nephropathy in lecithin:cholesterol acyltransferase deficiency. *Clinica. Chim. Acta.* **54**: 11-18.

Glass, C., R. C. Pittman, D. B. Weinstein, and D. Steinberg. (1983). Dissociation of tissue uptake of cholesteryl ester from that of apoprotein A-I of rat plasma high density lipoprotein: selective delivery of cholesteryl ester to liver, adrenal, and gonad. *Proc. Natl. Acad. Sci. USA.* **80**: 5435-5439.

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

Glomset, J. A. (1972). *In* Blood lipids and lipoproteins. G. J. Nelson. (ed.). Wiley Interscience, New York. 745-787.

Glomset, J. A., G. Assmann, E. Gjone, and K. R. Norum. (1989). Lecithin:cholesterol acyltransferase deficiency and fish-eye disease. *In* The metabolic and molecular basis of inherited disease. C. R. Scriver, A. L. Beaudet, W. S. Sly, J. B. Standbury, J. B. Wyngaarden, and D. S. Fredrickson. (eds.). McGraw-Hill, Inc., New York. 1933-1951.

Gordon, D. and B. M. Rifkind. (1989). Current concepts: High density lipoproteins – the clinical implications of recent studies. *N. Engl. J. Med.* **321**: 1311-1315.

Gotoda, T., M. Kinoshita, H. Shimano, K. Harada, M. Shimada, J. Ohsuga, T. Teramoto, Y. Yazaki, and N. Yamada. (1993). Cholesteryl ester transfer protein deficiency caused by a nonsense mutation detected in the patient's macrophage mRNA. *Biochem. Biophys. Res. Commun.* **194**: 519-524.

Goyal, J., K. Wang, M. Liu, and P. V. Subbaiah. (1997). Novel function of lecithin:cholesterol aclytransferase. Hydrolysis of oxidized polar phospholipids generated during lipoprotein oxidation. *J. Biol. Chem.* **272**: 16231-16239.

Grochulski, P., Y. Li, J. D. Schrag, and M. Cygler. (1994). Two conformational states of Candida rugosa lipase. *Protein Sci.* **3:** 82-91.

Guérin, M., P. J. Dolphin, and M. J. Chapman. (1993). Familial lecithin:cholesterol acyltansferase deficiency: further resolution of lipoprotein particle heterogeneity in the low density interval. *Atherosclerosis.* **104**: 195-212.

Guérin, M., P. J. Dolphin, and M. J. Chapman. (1994). A new in vitro method for the simultaneous evaluation of cholesteryl ester exchange and mass transfer between HDL and apoB-containing lipoprotein subspecies. *Arterioscler. Thromb.* **14**: 199-206.

Guérin, M., C. Dachet, S. Goulinet, D. Chevet, P. J. Dolphin, M. J. Chapman, and M. Rouis. (1997). Familial lecithin:cholesterol acyltransferase deficiency: molecular analysis of a compound heterozygote: LCAT (Arg^{147} -> Trp) and LCAT (Tyr^{171} -> stop). *Atherosclerosis.* **131**: 85-95.

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

Ha, Y. C., L. B. F. Chang, and P. J. Barter. (1985). Effects of injecting exogenous lipid transfer protein into rats. *Biochim. Biophys. Acta.* **833:** 203-210.

Hayek, T. T. Chajek-Shaul, A. Walsh, L. B. Agellon, P. Moulin, A. R. Tall, J. L. Breslow. (1992). An interaction between the human cholesteryl ester transfer protein (CETP) and apolipoprotein A-I genes in transgenic mice results in a profound CETP-mediated depression of HDL cholesterol levels. *J. Clin. Invest.* **90:** 505-510.

Hayek, T., N. Azrolan, R. B. Verdery, A. Walsh, T. Chajek-Shaul, L. B. Agellon, A. R. Tall, and J. L. Breslow. (1993). Hypertriglyceridemia and cholesteryl ester transfer protein interact dramatically alter high density lipoprotein levels, particle sizes, and metabolism; studies in transgenic mice. *J. Clin Invest.* **92:** 1143-1152.

Hayek, T., L. Masucci-Magoulas, X. Jiang, A. Walsh, E. Rubin, J. L. Breslow, and A. R. Tall. (1995). Decreased early atherosclerotic lediaons in hypertriglyceridemic mice expressing cholesteryl ester transfer protein transgene. *J. Clin Invest.* **96:** 2071-2074.

Hengstschlager-Ottnad, E., K. Kuchler, and W. J. Schneider. (1995). Chicken lecithin-cholesterol acyltransferase. *J. Biol. Chem.* **270**: 26139-26145.

Hesler, C. B., T. L. Swenson, and A. R. Tall. (1987). Purification and characterization of human plasma cholesteryl ester transfer protein. *J. Biol. Chem.* **262**: 2275-2282.

Hesler, C. B., A. R. Tall, T. L. Swenson, P. K. Weech, Y. L. Marcel, and R. W. Milne. (1988). Monoclonal antibodies to the M_r 74,000 cholesteryl ester transfer protein neutralize all of the cholesteryl ester and triglyceride transfer activities in human plasma. *J. Biol. Chem.* **263**: 5020-5023.

Hesler, C. B., M. L. Brown, D. S. Feuer, Y. L. Marcel, R. W. Milne, and A. R. Tall. (1989). Structure-function analysis of plasma cholesteryl ester transfer protein by protease digestion and expression of cDNA fragments in *Escherichia coli. J. Biol. Chem.* **264**: 11317-11325.

Hide, W. A., L. Chan, and W.-H. Li. (1992). Structure and evolution of the lipase superfamily. *J. Lipid Res.* **33:** 167-178.

Hirano, K., S. Yamashita, T. Funahashi, N. Sakai, M. Menju, M. Ishigami, H. Hirakoa, K. Kameda-Takemura, K. Tokunaga, T. Hoshino, K. Kumasaka, K. Kawano, and Y. Matsuzawa. (1993). Frequency of intron 14 splicing defect of cholesteryl ester transfer protein gene in the Japanese general population – relation between the mutation and hyperalphalipoproteinemia. *Atherosclerosis.* **100:** 85-90.

Hirano, K., S. Yamashita, N. Sakai, T. Arai, Y. Yoshida, S. Nozaki, K. Kameda-Takemura, and Y. Matsuzawa. (1995a). Molecular defect and atherogenecity in cholesteryl ester transfer protein deficiency. *Ann. N. Y. Acad. Sci.* **748:** 606-608.

Hirano, K., S. Yamashita, Y. Kuga, N. Sakai, S. Nozaki, s. Kihara, T. Arai, K. Yanagi, S. Takami, M. Menju, M. Ishigami, Y. Yoshida, K. Kameda-Takemura, K. Hayashi, and Y. Matsuzawa. (1995b). Atherosclerotic disease in marked hyperalphalipoproteinemia.

Combined reduction of cholesteryl ester transfer protein and hepatic lipase. *Arterioscler. Thromb. Vasc. Biol.* **15:** 1849-1856.

Hirano, K., S. Yamashita, N. Nakajima, T. Arai, T. Murayama, Y. Toshida, M. Ishigami, N. Sakai, K. Kameda-Takemura, and Y. Matsuzawa. (1997). Genetic cholestryl ester transfer protein seficiency is extremely frequent in the Omagari area of Japn. Marked hyperalphalipoproteinemia caused by CETP gene mutaion is not assiciated with longevity. *Arterioscler. Thromb. Vasc. Biol.* **17:** 1053-1059.

Hixson, J. E., D. M. Driscoll, S. Birnbaum, and M. L. Britten. (1993). Baboon lecithin cholesterol acyltransferase (LCAT): cDNA sequences of two alleles, evolution, and gene expression. *Gene.* **128:** 295-299.

Ho, S. N., H. D. Hunt, R. M. Horton, J. K. Pullen, and L. R. Pease. (1989). Site-directed mutagenesis by overlap extension using the polymerase chain reaction. *Gene.* **77**: 51-59.

Hoeg, J. M., B. L. Vaisman, S. J. Demosky, Jr., S. M. meyn, G. D. Talley, R. F. Hoyt, Jr., S. Feldman, A. M. Bérard, N. Sakai, D. Wood, M. E. Brousseau, S. Marcovina, H. B. Brewer, Jr., and S. Santamarina-Fojo. (1996a). Lecithin:cholesterol acyltransferase overexpression generates hyperalpha-lipoproteinemia and a nonatherogenic lipoprotein pattern in transgenic rabbits. *J. Biol. Chem.* **271**: 4396-4402.

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. Demonsky 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. USA.* **93:** 11448-11453.

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

Horwitz, A. H., S. D. Leigh, S. Abrahamson, H. Gazzano-Santoro, P. S. Liu, R. E. William, S. F. Carroll, and G. Theofan. (1996). Expression and characterization of cysteine-modified variants of an amino-terminal fragment of bactericidal/permeability-increasing protein. *Protein Expr. Purif.* **8:** 28-40.

Howell, K. E. and G. E. Palade. (1982). Heterogeneity of lipoprotein particles in hepatic Golgi fractions. *J. Cell. Biol.* **92:** 833-845.

Huang, Y., A. von Eckardstein, S. Wu, N. Maeda, and G. Assmann. (1994). A plasma lipoprotein containing only apoE and with γ-mobility on electrophoresis releases cholesterol from cells. *Proc. Natl. Acad. Sci. USA.* **91**: 1834-1838.

Hubacek, J. A., C. Buchler, C Aslanidis, and G. Schmitz. (1997). The genomic organization of the genes for human lipopolysaccharide binding protein (LBP) and bactericidal permeability increasing protein (BPI) is highly conserved. *Biochem. Biophys. Res. Commun.* **236:** 427-30.

Huukonenen, J.,

Ikewaki, K., M. Nishiwaki, T. Sakamoto, T. Ishikawa, T. Fairwell, L. A. Zec, M. Nagano, H. Nakamura, H. B. Brewer, Jr., and D. J. Rader. (1995). Increased catabolic rate of low density lipoproteins in humans with cholesteryl ester transfer protein deficiency. *J. Clin. Invest.* **96**: 1573-1581.

Inazu, A., M. L. Brown, C. B. Hesler, L. B. Agellon, J. Koizumi, K. Takata, Y. Maruhama, H. Mabuchi, and A. R. Tall. (1990). Increased high-density lipoprotein levels caused by a common cholesteryl ester transfer protein gene mutation. *N. Engl. J. Med.* **323**: 1234-1238.

Inazu, A., E. M. Quinet, S. Wang, M. L. Brown, S. Stevenson, M. L. Barr, P. Moulin, and A. R. Tall. (1992). Alternative splicing of the mRNA encoding the human cholesteryl ester transfer protein. *Biochemistry* **31**: 2352-2358.

Inazu, A., X.-C. Jiang, T. Haraki, K. Yagi, N. Kamon, J. Koizumi, H. Mabuchi, R. Takeda, K. Takata, Y. Moriyama, M. Doi, and A. R. Tall. (1994). Genetic cholesteryl ester transfer protein deficiency caused by two prevalent mutations as a major determinant of increased levels of high density lipoprotein cholesterol. *J. Clin. Invest.* **94**: 1872-1882.

Ishigami, M., S. Yamashita, N. Sakai, T. Arai, K. Hirano, H. Hiraoka, K. Takemura, and Y. Matsuzawa. (1994). Large and cholesteryl ester-rich high density lipoprotein deficiency can not protect macrophages from choelsterol accumulation induced by acetylated low density lipoproteins. *J. Biochem.* **116:** 257-262.

Jauhiainen, M., N. D. Ridgway, and P. J. Dolphin. (1987). Aromatic boronic as probes of the catalytic site of human plasma lecithin-cholesterol acyltransferase. *Biochim. Biophys. Acta.* **918**: 175-188.

Jauhiainen, M., and P. J. Dolphin. (1986). Human plasma lecithin:cholesterol acyltransferase. An elucidation of the catalytic mechanism. *J. Biol. Chem.* **261**: 7032-7043.

Jauhiainen, M., K. J. Stevenson, and P. J. Dolphin. (1988). Human plasma lecithin:cholesterol acyltransferase. The vicinal nature of cysteine 31 and cysteine 184 in the catalytic site. *J. Biol. Chem.* **263**: 6525-6533.

Jauhiainen, M., W. Yuan, M. H. Gelb, and P. J. Dolphin. (1989). Human plasma lecithin;cholesterol acyltransferase. Inhibition of the phospholipase A_2-like activity by *sn*-2-difluoroketone phosphatidylcholine analogues. *J. Biol. Chem.* **264**: 1963-1967.

Jauhiainen, M., and P. J. Dolphin. (1990). Human plasma lecithin:cholesterol acyltransferase (LCAT). On the role of essential carboxyl groups in catalysis. *In* Hypercholesterolemia, hypocholesterolemia, hypertriglyceridemia. C. L. Malmendier. (ed.) Plenum Press, New York. 71.

Jauhiainen, M., and P. J. Dolphin. (1991). Human plasma lecithin:cholesterol acyltransferase (LCAT). On the role of essential carboxyl groups in catalysis. *Adv. Exp. Med. Biol.* **285**: 71-75.

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**: 4032-4036.

Jeoung, N. H., W. G. Jang, J. I. Nam, Y. K. Pak, and Y. B. Park. (1999). Identification of retinoic acid receptor element in human cholesteryl ester transfer protein gene. *Biochem. Biophys. Res. Comm.* **258:** 411-415.

Jiang, X., P. Moulin, E. Quinet, I. J. Golberg, Y. K., Yacoub, L. B. Agellon. D. Compton, R. Schnitzer-Polokoff, and A. R. Tall . (1991) Mammalian adipose tissue and muscle are major sources of lipid transfer protein mRNA. *J. Biol. Chem* **266**: 4631-4639.

Jiang, X.-C., L. B. Agellon., A. Walsh, J. L. Breslow, and A. R. Tall. (1992). Dietary cholesterol increases transcription of the human cholesteryl ester transfer protein gene in transgenic mice: dependence on natural flanking sequences. *J. Clin Invest.* **90**:1290-1295.

Jiang, X. –C., L. Masucci-Magoulas, J. Mar, M. Lin, A. Walsh, J. L. Breslow, and A. Tall. (1993). Down-regulation of mRNA for the low density lipoprotein receptor in trnasgenic mice containing the gene for human cholesteryl ester transfer protein. Mechanism to explain accumulation of lipoprotein B particles. *J. Biol. Chem.* **268:** 27406-27412.

Jiang, X.-C., C. Bruce, T. Cocke, S. Wang, M. Boguski, and A. R. Tall. (1995). Point mutagenesis at positively charged amino acids of cholesteryl ester transfer protein: conserved residues within the lipid transfer/lipopolysaccharide binding protein gene family essential for function. *Biochemistry.* **24**: 7258-7263.

Jian, B., M. de la Llera-Moya, Y. Ji, N. Wang, M. C. Phillips, J. B. Swaney, A. R. Tall, and G. H. Rothblat. (1998). Scavenger receptor class B type I as a mediator of cellular cholesterol efflux to lipoproteins and phospholipid acceptors. *J. Biol. Chem.* **273:** 5599-5606.

Jin, L., Y.-P. Lee, and A. Jonas. Biochemical and biphysical characterization of human recombinant lecithin:cholesterol acyltransferase. (1997). *J. Lipid Res.* **38**: 1085-1093.

Johanson, B. G. (1972). Agarose gel electrophoresis. *J. Clin. Lab. Invest.* **124 (suppl. 29)**: 7-19.

Johnson, W. J., E. P. C. Kilsdonk, A. van Tol, M. C. Phillips, G. H. Rothblat. (1991). Cholesterol efflux from cells to immunopurified subfractions of human high density lipoprotein: LpA-I and LpA-I:A-II. *J. Lipid. Res.* **32**: 1992-2000.

Jonas, A. (1991). Lecithin:cholesterol acyltransferase in the metabolism of high density lipoproteins. *Biochim. Biophys. Acta.* **1084**: 205-225.

Jonas, A. (1998). Regulation of lecithin:cholesterol acyltransferase activity. *Prog. Lipid Res.* **37**: 209-234.

Kane, J. P. (1983). Apolipoprotein B: structural and metabolic heterogeneity. *Annu. Rev. Physiol.* **45**: 637-650.

Kanellis, P., A. Y. Romars, B. J. Johnson, H. Keruet, R. Chioretti Jr., T. M. Allen, and J. P Segrest. (1980). Studies of synthetic peptide analogs of the amphipathic helix. Effect of charged amino acid residue topography on lipid affinity. *J. Biol. Chem.* **255:** 11464-11472.

Karathanasis, K. S., V. I Zannis, and J. L. Breslow. (1983). Isolation and characterization of the human apolipoprotein A-I gene. *Proc. Natl. Acad, Sci. USA.* **80:** 6147-6151.

Karathanasis, S. K. (1985). Apolipoprotein multigene family and tandem organization of human apolipoportien A-I, C-III, and A-IV genes. *Proc. Natl. Acad. Sci. USA.* **82**: 6374-6378.

Khoo, J. C., E. Miller, P. mcLoughlin, and D. Steinberg. (1988). Enhanced macrophage uptake of low density lipoprotein after self-aggregation. *Arteriosclerosis.* **8:** 348-358.

Klein, H. G., S. Santamarina-Fojo, N. Duverger, M. Clerc, M.-F. Dumon, J.J. Albers, S. Marcovina, H. B. Brewer Jr.. (1993). Fish-eye syndrome: a molecular defect in the lecithin:cholesterol acyltransferase (LCAT) gene associated with normal α-LCAT specific activity. *J. Clin. invest.* **92**: 479-485.

Klein, H. G., N. Duverger, J. J. Albers, S. Marcovina, H. B. Brewer Jr., and S. Santamarina-Fojo. (1995). In vitro expression of structural defects in the lecithin-cholesterol acyltransferase gene. *J. Biol. Chem.* **270:** 9443-9447.

Knipping, G., A. Hermetter, and M. R. Hubmann. (1993). Interaction of N-methyl-anthraniloyl-labelled porcine apolipoprotein A-I with porcine lecithin: cholesterol acyltransferase: an energy transfer study. *Int. J. Biochem.* **25:** 505-511.

Koizumi, J., H. Mabuchi, A. Yoshimura, I ichishita, M. Takeda, H. Itoh, Y. Sakai, Y. Sakai, K. Ueda, R. Takeda. (1985). Deficiency of serum cholesteryl ester transfer activity in patients with familial hyperalphalipoproteinemia. *Atherosclerosis.* **58:** 175-186.

Kotake, H., L. B. Agellon, and S. Yokoyama. (1997). Modification of the N-terminal cysteine of plasma cholesteryl ester transfer protein selectively inhibits triglyceride transfer activity. *Biochim. Biophys. Acta.* 1**347**: 69-74.

Krieger, M. and J. Herz. (1994). Structures and functions of multiligand lipoprotein receptors: macropjage scavenger receptors and LDL receptor-related protein (LRP). *Annu. Rev. Biochem.* **63**: 601-637.

Kunitake, S. T., K. J. La Sala, and J. P. Kane. (1985). Apolipoprotein A containing lipoproteins with prebeta electrophoretic mobility. *J. Lipid. Res.* **26**: 549-555.

Kunitake, S. T., C. M. Mendel, and L. K. Hennessy. (1992). Interrconversion between apolipoprotein A-I containing lipoproteins of pre-beta and alpha electrophoretic mobilities. *J. Lipid Res.* **33**:1807-1816.

Kuksis, A., J. J. Myher, K. Geher, A. G. D. Hoffman, W. C. Breckenridge, G. J. L. Jones, and J. A. Little. (1978). Comparative determination of plasma cholesterol and triglycerides by automated gas-liquid chromatographic and autoanalyzer methods. *J. Chromtogr.* **146**: 393-412.

Kushwaha, R. S., S. Q. Hasan, H. C. McGill Jr., G. S. Getz, R. G. Dunham, and P. Kanda. (1993). Characterization of cholesteryl ester transfer protein inhibitor from plasma of baboons (Papio sp.) *J. Lipid Res.* **34:** 1285-1297.

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

Kuivenhoven, J. A., A. F. H. Stalenhoef, J. S. Hill, N. M. Demacker, A. Errami, J. J. P. Kastelein, and P. H. Pritchard. (1996). Two novel molecular defects in the LCAT gene associated with fish-eye disease. *Arterioscler. Thromb. Vasc. Biol.* **16**: 294-303.

Lacko, A. G., A. J. Reason, C. Nuckolls, B. J. Kudchodkar, M. P. Nair, G. Sundarrajan, P. H. Pritchard, H. R. Morris, and A. Dell. (1998). Characterization of recombinant human plasma lecithin:cholesterol acyltransferase (LCAT): N-linked carbohydrate stuctures and catalytic properties. *J. Lipid Res.* **39**: 807-820.

Laccotripe, M., S. C. Makrides, A. Jonas, and V. I. Zannis. (1997). The carboxyl-terminal hydrophobic residues of apolipoprotein A-I affect its rate of phospholipid binding and its association with high density lipoprotein. *J. Biol. Chem.* **272:** 17511-17522.

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.

Lee, Y.-P., S. Adimoolam, M. Liu, P. V. Subbaiah, K. Glenn, and A. Jonas. (1997). Analysis of human lecithin-cholesterol acyltransferase activity by carboxyl-terminal truncation. *Biochim. Biophys. Acta.* **1344**: 250-261.

Li, W. S., M. Tanimura, C. -C. Luo, S. Datta, and L. Chan. (1988). The apolipoprotein multigene family: biosynthesis, structure, structure-function relationships, and evolution. *J. Lipid Res.* **29:** 245-271.

Li W.-H., M. Tanimura, C.-C. Luo, S. Datta, L. Chan. (1988). The apolipoprotein multigene family: biosynthesis, structure, structure-function relationships, and evolution. *J. Lipid Res.* **29**: 245-271.

Liu, M., and P. V. Subbaiah. (1994). Hydrolysis and transesterification of platelet-activating factor by lecithin-cholesterol acyltransferase. *Proc. Natl. Acad. Sci. USA.* **91:** 6035-6039.

Liu, M. J., D. Bagdade, and P. V. Subbaiah. (1995). Specificity of lecithin:cholesterol acyltransferase and atherogenic risk: comparative studies on the plasma composition and in vitro synthesis of cholesteryl esters in 14 vertebrate species. *J. Lipid Res.* **36:** 1813-1824.

Lohse, P., S. Chahrokh- Zadeh, P. Lohse, and D. Seidel (1997). Human lysosomal acid lipase / choelsteryl ester hydrolase and human gastric lipase: identification of the catalystically active serine, aspartic acid and histidine residues. *J. Lipid Res.* **38:** 892-903.

Lowe, M. E.(1997). Site-specific mutagenesis of human pancreatic lipase. *In* Methods in Enzymology. B. Rubin and E. A. Dennis (eds) Academic Press, N. Y. **284**: 157-170.

Lusis, A. J., C. Heinzmann, R. S. Sparks, J. Scott, T. J., Knott, R. Geller, M. C. Sparkes, and T. Mohandas. (1986). Regional mapping of human chromosome 19: organization of genes for plasma lipid transport (APOC1, -C2, and –E and LDLR) and the genes C3, PEPD, and GPI. *Proc. Natl. Acad. Sci. USA.* **83**: 3929-3933.

Lusis, A. J., S. Zollman, R. S. Sparkes, I. Klisak, T. Mohandas, D. Drayna, and R. M. Lawn. (1987). Assignment of the human gene for cholesteryl ester transfer protein to chromosome 16q12-16q21. *Genomics.* **1:** 232-235.

Mackness, M. I., S. Arrol, C. Abbott, and P. N. Durington. (1993). Protection of low density lipoprotein against oxidative modification by high density lipoprotein associated paraoxonase. *Atherosclerosis.* **104:** 129-135.

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:** 2059-2066.

Maguire, G. F., and W. C. Breckenridge. (1975). Agarose gel elctrophoresis of plasma lipoproteins using durrum cell. *Clin. Biochem.* **8:** 161-168.

Marcel, Y. L. (1982). Lecithin:cholesterol acyltransferase and intravascular cholesterol transport. *Adv. Lipid Res.* **19:** 85-136.

Marcel, Y. L., C. A Vezina, P. K. Weech, F. Terce, and R. W. Milne. (1987). Lecithin:cholesterola cyltransferase: a review and immunochemical studies. *Adv. Expt. Med. Bio.* **210:** 163-179.

Markwell, M. A., S. M. Haas, L. L. Bieber, and N. E. Tolbert. (1978). A modification of the Lowry procedure to simplify protein determination in membrane and lipoprotein samples. *Anal. Biochem.* **87**: 206-210.

Marotti, K. R., C. K. Castle, T. P. Boyle, A. H. Lin, R. W. Murray, and G. W. Melchoir. (1993). Severe atherosclerosis in transgenic mice expressing simian cholesteryl ester transfer protein. *Nature.* **364:** 73-75.

Marquez-Vidal, P., C. Azema, X. Collet, C. Vieu, H. Chap, B, Perret, (1994). Hepatic lipase promotes the uptake of HDL esterified cholesterol by the perfused rat liver: a study using reconstituted HDL particles of defined phospholipid composition. *J. Lipid Res.* **35**: 373-384.

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

Masucci-Magoulas, L., P. Moulin, X.-C. Jiang, H. Richardson, A. M. Walsh et al. (1995). Decreased cholesteryl ester transfer protein (CETP) mRNA and protein and increased high density lipoprotein following lipopolysaccharide administration in human CETP transgenic mice. *J. Clin. Invest.* **95:** 1587-1594.

Masucci-Magoulas, L., A. Plump, X.-C. Jiang, A. Walsh, J. L. Breslow, A. R. Tall (1996). Profound induction of hepatic cholesteryl ester transfer protein transgene expression in apolipoprotein E and low density lipoprotein receptor gene knockout mice; a novel mechanism signal changes in plasma cholesterol levels. *J. Clin Invest.* **97**: 1-9.

Matz, C. E., 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.

McLean, J., K. Wion, D. Drayna, C. J. Fielding, and R. Lawn. (1986). Human Lecithin:cholesterol acyltransferase gene sequence and sites of expression. *Nuc. Acid. Res.* **14**: 9387-9406.

McLean, J. W. (1992). Molecular defects in the lecithin:cholesterol acyltransferase gene. In High density lipoproteins and atherosclerosis III. N. E. Miller, and A. R. Tall. (eds). Elsiever Science Publishers B. V. Amsterdam. 59-65.

Mehlum, A., B. Staele, and N. Duverger, A. Tailleux, G. Castro,C. Fievet, G. Luc, J. C. Fruchart, G. Olivecrona, G. Skretting, J. Auwerx, and H. Prydz. (1995). Tissue-specific expression of the human gene for lecithin: cholesterol acyltransferase in transgenic mice alters blood lipids, lipoproteins and lipases towards a less atherogenic profile. *Eur. J. Biochem.* **230:** 567-275.

Melchoir G. W., K. A. Greenlee, C. K. Castle, M. J. Prough, R. W. Milne, K. R. Marotti, F. J. Kezdy. (1995). Evodence that cynomolgus monkey cholesteryl ester transfer protein has two neutral lipid binding sites. *J. Biol. Chem.* **270:** 21068-21074.

Meroni, G., N. Malgaretti, P. Magnaghi, R. Taramelli. (1990). Nucleotide sequence of the cDNA for lecithin-cholesterol acyl transferase (LCAT) from the rat. *Nuclei Acids. Res.* **18:** 5308.

Meroni, G., N. Malgaretti, M. Pontoglio, S. Ottolenghi, and R. Taramelli. (1991). Functional analysis of the human lecithin cholesterol acyl transferase gene promoter. *Biochem. Biophys. Res. Commun.* **180**: 1469-1475.

Miller, S. A., D. D. Dykes, and H. F. Polesky. (1988). A simple salting out procedure for extracting DNA from human nucleated cells. *Nuclei Acid Res.* **16**: 1215.

Miller, K. R., J. Wang, M. Sorci-Thomas, R. A. Anderson, and J. S. Parks. (1996). Glycosylation structure and enzyme activity of lecithin:cholesterol acyltransferase from human plasma, HepG2 cells, and baculovirus and Chinese hamster ovary cll expression systems. *J. Lipid Res.* **37**: 551-561.

Miller, M., and K. Zeller. (1997). Alternative splicing in lecithin:cholesterol acyltransferase mRNA: an evolutionary paradigm in humans and great apes. *Gene.* **190**: 309-313.

Minnich, A., X. Collet, A. Roghani, C. Cladaras, R. L. Hamilton, C. J. Fielding, and V. I. Zannis. (1992). Site-directed mutagenesis and structure-function analysis of the human apoliporpotein A-I. *J. Lipid Res.* **267:** 16553-16560.

Morton, R. E., and D. B. Zilversmit. (1981). A plasma inhibitor of triglyceride and cholesteryl ester transfer activities. *J. Biol. Chem.* **256:** 11992-11995.

Morton, R. E., and D. B. Zilversmit. (1982). Purification and characterization transfer protein(s) from human lipoprotein-deficient plasma. *J. Lipid Res.* **23:** 1058-1067.

Morton, R. E. (1985). Binding of plasma-derived lipid transfer protein to lipoprotein substrates. *J. Biol. Chem.* **260**: 12593-12599.

Morton, R. E. (1988). Free cholesterol is a potent regulator of lipid transfer protein. *J. Biol. Chem.* **263**: 12235-12241.

Morton, R. E., and J. V. Steinbrunner. (1990). Concentration of neutral lipids in the phospholipid surface of substrate particles determines lipid transfer activity. *J. Lipid Res.* **31**: 1559-1567.

Morton, R. E., and D. J. Greene. (1994). Regulation of lipid transfer between lipoproteins by an endogenous plasma protein: selective inhibition among lipoprotein classes. *J. Lipid Res.* **35:** 836-847.

Morrison, W. R., and L. M. Smith. (1964). Preparation of fatty acid methyl esters and dimethylacetals from lipids with boron-fluoride-methanol. *J. Lipid Res.* **5**: 600-608.

Mowri, H.-O., W. Patsch, L. C. Smith, A. Gotto Jr., J. R. Patsch. (1992). Different reactivities of high density lipoprotein 2 subfractions with hepatic lipase. *J. Lipid Res.* **33:** 1269-1279.

Murayama, N., Y. Asano, S. Hosoda, M. Maesawa, M. Saito, F. Takaku, T. Sugihara, K. Miyashima, Y. Yawata. (1984a). Decrease sodium influx and abnormal red cell membrane lipids in a patient with familial plasma lecithin:cholesterol acyltransferase deficiency. *Am. J. Hematology.* **16**: 129-137.

Murayama, N., Y. Asano, K. Kato, Y. Sakamoto, S. Hosoda, N. Yamada, T. Kodama, T. Murase, and Y. Akanuma. (1984b). Effects of plasma infusion on plasma lipids, apoproteins and plasma enzyme activities in gamilial lecithin:cholesterol acyltransferase deficiency. *Eur. J. Clin. Invest.* **14:** 122-129.

Murata, Y., E. Maeda, G. Yoshina, and M. Kasuga. (1996). Cloning of rabbit LCAT cDNA: increase in LCAT mRNA abundance in the liver of cholesterol-fed rabbits. *J. Lipid Res.* **37:** 1616-1622.

Nagashima, M., J. W. McLean, R. M. Lawn. (1988). Cloning and mRNA tissue distribution of rabbit cholesteryl ester transfer protein. *J. Lipid Res.* **29**: 1643-1649.

Nishide, T., J. H. Tollefson, and J. J. Albers. (1989). Inhibition of lipid transfer by a unique high density lipoprotein subclass conatining an inhibitor protein. *J. Lipid Res.* **30:** 149-158.

Nishida, H. I., H. Arai, and T. Nishida. (1993). Cholesteryl ester transfer mediated by lipid transfer protein as influenced by changes in the charge characteristics of plasma lipoproteins. *J. Biol. Chem.* **268**: 16352-16360.

Ng, D. S., O. L. Francone, T. M. Forte, J. Zhang, M. Haghpassand, and E. M. Rubin. (1997). Disruption of the murine lecithin:cholesterol acyltransferase gene causes impairment of adrenal lipid delivery and up-regulation of scavenger receptor class B type I. *J. Biol. Chem.* **272:** 15777-15781.

Norum, K. R., and E. Gjone. (1967). Familial plasma lecithin:cholesterol acyltransferase deficiency. *Scand. J. Clin. Lab Invest.* **20**: 231-243.

Norum, K. R., E. Gjone, and J. A. Glomset. (1989). Familial lecithin:cholesterol acyltransferase deficiency, including Fish Eye Disease. *In* The Metabolic basis of Inherited Disease. C. R. Scriver, A. L. Beaudet, W. S. Sly, and D. Valle, editors. McGraw-Hill Inc., New York. 1181-1194

Norum, R. A, J. B. Lakier, S. Goldstein, A. Angel, R. B. Goldberg, W. D. Block, D. K.Noffzee, P. J. Dolphin, J. Edelglass, D. D. Bogorad, and P. Alaupovic. (1982). Familial Deficiency of Apolipoproteins A-I and C-III and Precocious Coronary Artery Disease. *New England J. Med.* **306:** 1513-1519.

O, K., J. S. Hill, and P. H. Pritchard. (1995). Role of N-linked glycosylation of lecithin:cholesterol acyltransferase in lipoprotein substrates specificity. *Biochim. Biophys. Acta.* **1254**: 193-197.

O, K., J. S. Hill, X. Wang, R. McLeod, and P. H. Pritchard. (1993). Lecithin:cholesterol acyltransferase: role of N-linked glycosylation in enzyme function. *Biochem. J.* **294**: 879-884.

Ohnishi T., and A. Yokoyama. (1993). Activation of human plasma lipid transfer protein by apolipoproteins. *Biochemistry.* **32**: 52029-52035.

Ollis, D. L., E. Cheah, M. Cygler, B. Dijkstra, F. Frolow, S. M. Franken, M. Harel, S. J. Remington, I. Silman, J. Schraj, J. L. Sussman, K. H. G. Verschueren, and A. Goldman. (1992). The alpha/beta hydrolase fold. *Protein Engng.* **5:** 197-211.

Olivera, H. C. F., R. A. Chouinard, L. B. Agellon, C. Bruce, L. Ma, A. Walsh, J. L. Breslow, and A. R. Tall. (1996). Human cholesteryl ester transfer protein gene proximal promoter contains dietary cholesterol positive response elements and mediates expression in small intestine and periphery while predominant liver and spleen expression is controlled by 5'-distal sequences. *J. Biol. Chem.* **271**: 31831-31838.

Olivera, H. C., L. Ma, R. Milne, S. M. Marcovina, A. Inazu, H, Mabuchi, and A. R. Tall. (1997). Cholesteryl ester transfer protein activity enhances plasma cholesteryl ester

formation. Studies in CETP transgenic mice and human genetic CETP deficiency. *Arterioscler. Thromb. Vasc. Biol.* **17:** 1045-1052.

Oikawa, S. A. J. Mendez, J. F. Oram, E.L. Bierman, M. C. Cheung. (1993). Effects of high density lipoprotein particles containing apoA_I, with or without apoA-II, on intracellular cholesterol efflux. *Biochim. Biophys. Acta.* **1165**: 327-334.

Oram, J. F., A. J. Mendez, J. P. Slotte, and T. F. Johnson. (1991). HDL apolipoproteins mediate removal of sterol from intracellular pools but not form plasma membranes of cholesterol-loaded fibroblast. *Arterioscler. Thromb.* **11**: 403-414.

Owen, J. S., H. Wiebusch, P. Cullen, G. F. Watts, V. L. M. Lima, H. Funke, and G. assmann. (1996). Complete deficiency of plasma lecithin:cholesterol acyltransferase (LCAT) activity due to a novel homozygous mutation (Gly-30-Ser) in the LCAT gene. *Human Mutat.* **8:** 79-82.

Pape, M. E., E. F. Rehberg, K. R. Marotti, and G. W. Melchior. (1991). Molecular cloning, sequencing, and expression of cynomolgus monkey cholesteryl ester transfer protein. Inverse correlation between hepatic cholesteryl ester transfer protein mRNA levels and plasma high density lipoprotein levels. *Arterioscler. Thromb.* **11**: 1759-1771.

Parra, H. J., H. Mezdour, N. Ghalim, J. M. Bard, and J. C. Fruchart. (1990). Differential immunoassay on ready-to-use plates for human LpA-I lipoprotein particles. *Clin. Chem.* **36**: 1431-1435.

Pattnaik, N. M., and D. B. Zilversmit. (1979). Interaction of cholesteryl ester exchange protein with human plasma lipoproteins and phospholipid vesicles. *J. Biol. Chem.* **254:** 2782-2786.

Patsch, W., G. Schonfeld, A. M. Gotto, and J. R. Patsch. (1980). Characterization of human high density lipoproteins by zonal ultracentrifugation. *J. Biol. Chem.* **255**: 3178-3185.

Patsch, J. R., S. Prasad, A. M. Gotto, and G. Bengtsson-Olivecrona. (1984). Postprandial lipemia. A key for the conversion of high density lipoprotein2 into high density lipoprotein3 by hepatic lipase. *J. Clin. Invest.* **74**: 2017-2023.

Peelman, F., N. Vinaimont, A. Verhee, B. Vanloo, J-L. Verschelde, C. Labeur, S. Seguret-Mace, N. Duverger, G. Hutchinson, J. Vandekerckhove, J. Tavernier, and M. Rosseneu. (1998). A proposed architecture for lecithin cholesterol acyl transferase (LCAT): Identification of the catalytic triad and molecular modeling. *Protein Sci.* **7**: 587-599.

Peelman, F., M. Goethals, B. Vanloo, C. Labeur, R. Brasseur, J. Vandekerckhove, and M. Rosseneu. (1997). Structural and functional properties of the 154-171 wild-type and

variant peptides of human lecithin-cholesterol acyltransferase. *Eur. J. Biochem.* **249**: 708-715.

Peelman, F., J-L. Verschelde, B. Vanloo, C. Ampe, C. Labeur, J. Tavernier, J. Vandekerckhove, and M. Rosseneu. (1999). Effects of natural mutations in lecithin:cholesterol acyltransferase on the enzyme structure and activity. *J. Lipid Res.* **40**: 59-69.

Peelman, F., B. Vanloo, O. Perez-Mendez, A. Decout, J.-L. Verschelde, C. Labeur, N. Vinaimont, A. Verhee, N. Duverger, R. Brasseur, J. Vandekerckhove, J. Tavernier, and M. Rosseneu. (1999a). Characterization of functional residues in the interfacial recognition domain of lecithin:cholesterol acyltransferase (LCAT). *Protein Engineering.* **12**: 71-78.

Perez-Mendez, O., B. Vanloo, A. Decout, M. Goethals, F. Peelman, J. Vandekerchove, R. Brasseur, and M. Rossenue. (1998). Contribution of the hydrophobicity gradient of an amphipathic peptide to its mode of association with lipids. *Eur J Biochem.* **256:** 570-579.

Pieters, M. N., G. R. Castro, D. Schouten, P. Duchateau, J.-C. Fruchart, and T. J. C. van Berkel. (1993). Cholesterol esters selectively delivered in vivo by high density lipoprotein subclass LpA-I to rat liver are processed faster into bile acids than are LpA-I/A-II derived choelsterol esters. *Biochem. J.* **292:** 819-823.

Piran, U., and T. Nishida. (1976). Release of fatty acids from phosphatidylcholine by lecithin-cholesterol acyltransferase. *J. Biochem.* **80:** 887-889.

Piran, U., and T. Nishida (1979). Utilization of various sterols by lecithin-cholesterol acyltransferase as acyl acceptors. *Lipids.* **14:** 478-482.

Portman, O. W., and M. Sugano. (1964). Factors influencing the level and fatty acid specificity of the cholesterol esterification activity in human plasma. *Arch. Biochem. Biophys.* **105:** 532-540.

Pownall, H. J., Q. Pao, J. B. Massey. (1985). Acyl chain and headgroup specificity of human plasma lecithin:cholesterol acyltransferase. Separation of matrix and molecular specificities. *J. Biol. Chem.* **260:** 2146-2152.

Pritchard, P. H. and J. S. Hill. (1993). Genetic disorders of lecithin:cholesterol acyltransferase: clinical findings and molecular pathology. *In* Lipoproteins in health and disease. 1-56.

Puchois, P., A. Kindoussi, P. Fievet, F. L. Fourrier, M. Bertrand, E. Koren, J. C. Fruchart. (1987). Apolipoprotein A-I-containing lipoproteins in coronary artery disease. *Atherosclerosis.* **68**: 35-40.

Qu, S.-J., H.-Z. Fan, F. Blanco-Vaca, H. J. Pownall. (1993). Roles of cyseines in human lecithin:cholesterol acyltransferase. *Biochemistry*. **32**: 3089-3094.

Qu, S.-J., H.-Z. Fan, F. Blanco-Vaca, H. J. Pownall. (1994). Effects of site-directed mutagenesis on the serine residues of human lecithin:cholesterol acyltransferase. *Lipids*. **29**: 803-809.

Qu, S.-J., H. Z. Fan, F. Blanco-Vaca, H. J. Pownall. (1995). In vitro expression of natural mutants of human lecithin:cholesterol acyltransferase. *J. Lipid Res.* **36:** 967-74.

Quinet, E.M., L. B. Agellon, P. A. Kroon, Y. L. Marcel, Y. C. Lee, M. E. Whitlock, and A. R. Tall. (1990). Atherogenic diet increase cholesteryl ester transfer messenger RNA levels in rabbit liver. *J. Clin Invest.* **85**: 357-363.

Quinet, E., A. Tall, R. Ramakrishnan, L. Rudel. (1991). Plasma lipid tranfer protein as a determinant of the atherogenicity of monkey plasma lipoproteins. *J. Clin Invest.* **87:** 1559-1566.

Quinet, E., T. P. Yang, C. Marinos, and A, Tall. (1993). Inhibition of the cellular secretion of cholesteryl ester transfer protein by a variant protein formed by alternative splicing of mRNA. *J. Biol. Chem.* **268**: 16891-16894.

Rader, D. J., G. Castro, L. A. Zech, J. C. Fruchart, and H. Brewer Jr. (1991). In vivo metabolism of apolipoprotein A-I on high density lipoprotein particles LpA-I and LpA-I:A-II. *J. Lipid Res.* **32**: 1849-1859.

Rader, D. J., K. Ikewaki, N. Duverger, H. Schmidt, H. Pritchard, J. Frohlich, M. Clerc, M.–F. Dumon, T. Fairwell, L. Zech, S. Santamarina-Fojo, and H. B. Brewer Jr. (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:** 321-330.

Rothblat, G. H., and M. C. Phillips. (1982). Mechanism of cholesterol efflux from cells. Effects of acceptor structure and concentration. *J. Biol. Chem.* **257:** 4775-4782.

Roy, P., R. MacKenzie, T. Hirama, X.-C. Jiang, P. Kussie, A. Tall, E. Rassart, and R. Milne. (1996). Structure-function relationships of human choleseryl ester transfer protein: analysis using monoclonal antibodies. *J. Lipid Res.* **37**: 22-34.

Rye, K.-A., and P. J. Barter. (1992). Lipid transfer activities and apolipoproteins. *In* structure and function of apolipoprotein. M. Rosseneu. (ed.). CRC Press Inc. Boca Raton, FL. 401-426.

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:** 215-224.

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:** 613-622.

Sakai, N., Y. Matsuzawa, K. Hirano, S. Yamashita, S. Nozaki, Y. Ueyama, M. Kubo, S. Tarui. (1991). Detection of two species of low density lipoprotein particles in cholesteryl ester transfer protein deficiency. *Arterioscler. Thromb.* **11:** 71-79.

Sakai, N., S. Yamashita, K. Hirano, M. Ishigami, T. Arai, K. Kobayashi, T. Funahashi, and Y. Matsuzawa. (1995). Decreased affinity of low density liporpotein (LDL) particles for LDL receptors in patients with cholesteryl ester transfer protein deficiency. *Eur. J. Clin. Invest.* **25:** 332-339.

Sakai, N., S. Santamarina-Fojo, S. Yamashita, Y. Matsuzawa, and H. B. Brewer, Jr. (1996). Exon 10 skipping caused by intron 10 splice donor site mutation in cholesteryl ester transfer protein gene results in abnormal downstream splice site selection . *J. Lipid Res.* **37**: 2065-2073.

Sakai, N., B. L. Vaisman, C. A. Koch, R. F. Hoyt Jr., S. M. Meyn, G. D. Talley, J. A. Paiz, H. B. Brewer Jr., and S. Santamarina-Fojo. (1997). Targeted disruption of the new mouse lecithin:cholesterol acyltransferase (LCAT) gene. Generation of a new model for human LCAT deficiency. *J. Biol. Chem.* **272:** 7506-7510.

Sambrook, J., Fritsch, E. F., Maniatis, T. (eds.) (1989). Molecular cloning: a laboratory guide, second edition. Cold Spring Harbour, New York; Cold Spring Harbour University press. E.5-E.7

Sammett, D., and A. R. Tall. (1985). Mechanisms of enhancement of cholesteryl ester transfer activity by lipolysis. *J. Biol. Chem.* **260**: 6687-6697.

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**: 1002-1013.

Scanu, A. M., P. Lagocki, J. Chung. (1980). Effect of apolipoprotein A-II on the structure of high-density lipoproteins: relationship to the activity of lecithin:cholesterol acyl transferase in vitro. *Ann. N. Y. Acad. Sci. USA.* **348:** 160-173.

Schindler, P. A., C. A. Settineri, X. Collet, C. J. Fielding, and A. L. Burlingame. (1995). Site-specific detection and structural characterization of the glycosylation of human plasma proteins lecithin:cholesterol acyltransferase and apolipoprotein D using HPLC/electrospray mass spectrometry and sequential glycosidase digestion. *Prot. Sci.* **4:** 791-803.

Schneider, W. J. (1996). Removal of lipoproteins from plasma. *In* Biochemistry of lipids, lipoproteins and membranes. D. E. Vance, and J. Vance, eds. Elsevier Press, Amsterdam. **19:** 517-543.

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**: 247-258.

Segrest, J. P., B. H. Chang, C. G. Brauilette, P. Kanellis, and F. McGahan. (1983). Studies of synthetic peptide analogs of the amphipathic helix. Competitive displacement of exchangeable apolipoproteins from native lipoproteins. *J. Biol. Chem.* **258:** 2290-2295.

Serdyuk, A. P., and R. E. Morton. (1999). Lipid transfer inhibitor protein definces the participation of lipoproteins in lipid transfer reactions. CETP has no preference for cholesteryl esters in HDL versus LDL. *Arterioscler. Thromb. Vasc. Biol.* **19:**718-726.

Skretting, G., and H. Prydz. (1992). An amino acid exchange in exon 1 of the human lecithin:cholesterol acyltransferase (LCAT) gene is associated with FED. *Biochem. Biophys. Res. Commun.* **182:** 583-587.

Son, Y.-S. C. and D. B. Zilversmit. (1984). Purification and characterization of human plasma proteins that inhibit lipid transfer activities. *Biochim. Biophys. Acta.* **795:** 473-480.

Sorci-Thomas, M., J. Babiak, and L. L. Rudel. (1990). Lecithin-cholesterol acyltransferase (LCAT) catalyzes the transacylation of intact cholesteryl esters. Evidence for the partial reversal of the forward LCAT reaction. *J. Biol. Chem.* **265**: 2665-2670.

Sorci-Thomas,M., M. W. Kearns, and J. P. Lee. (1993). Apolipoprotein A-I domains in lecithin-cholesterol acyltransferase activation. Structure:function relationships. *J. Biol. Chem.* **268:** 21403-21409.

Soutar, A. K., C. W. Garner, H. N. Baker, J. T. Sparrow, R. L. Jackson, A. M. Gotto Jr., and L. C. Smith. (1975). Effect of the human plasma apolipoproteins and phosphatidylcholine acyl donor on the activity of lecithin:cholesterol acyltransferase. *Biochemistry.* **14:** 3057-3064.

Sparks, D. L., and P. H. Pritchard. (1989). Transfer of cholesteryl ester into high density lipoprotein by cholesteryl ester transfer protein: effect of HDL lipid and apoprotein content. *J. Lipid Res.* **30:** 1491-1498.

Sparrow, D. A., P. M. Laplaud, M. Saboureau, G. Zhou, P. J. Dolphin, A. M. Gotto, and J. T. Sparrow. (1995). Plasma lipid transport in the hedgehog: partial characterization of structure and function of apolipoprotein A-I. *J. Lipid Res.* **36:** 485-495.

Stahnke, G., R. Sprengel, J. Augustin, and H. Will. (1987). Human hepatic triglyceride lipase: cDNA cloning, amino acid sequence and expression in a cultured cell line. *Differentiation.* **35**: 45-52.

Steinberg, D. (1997). A critical look at the evidence for the oxidation of LDL in atherogenesis. *Atherosclerosis.* **131(Suppl S):** 5-7.

Stevenson, S. C., S. Wang, L. Deng, and A. R. Tall. (1993). Humna plasma cholesteryl ester transfer protein consists of a mixture of two forms reflecting variable glycosylation at asparagine 341. *Biochemistry.* **32**: 5121-5126.

Stokke, K. T., and K. R. Norum. (1971). Determination of lecithin:cholesterol acyltransfer in human blood plasma. *Scand. J. Clin Lab Invest.* **27:** 21-27.

Styerer, E., and G. M. Kostner. (1988). Activation of lecithin-cholesterol acyltransferase by apolipoprotein D: comparison of proteoliposomes containing apolipoprotein D, A-I or C-I. *Biochim. Biophys. Acta.* **958:** 484-491.

Subbaiah, P. V., J. J. Albers, C. H. Chen, and J. D. Bagdade. (1980). Low density lipoprotein-activated lysolecithin acylation by human plasma lecithin-cholesterol acyltransferase. Identity of lysolecithin acyltransferase and lecithin-cholesterol acyltrasnferase. *J. Biol. Chem.* **255:** 9275-9280.

Subbaiah, P. V., M. Liu, P. J. Bolan, and F. Paltauf. (1992). Altered positional specificity of human plasma lecithin-cholesterol acyltransferase in the presence of sn-2 arachidonoyl phosphatidyl cholines. Mechanism of formation of saturated cholesteryl esters. *Biochim. Biophys. Acta.* **1128:** 83-92.

Subbaiah, P.V., M. Liu, and F. Patlauf. (1994a). Role of sn-2 acyl group of phosphatidylcholine in determining the positional specificity of lecithin-cholesterol acyltransferase. *Biochemistry.* **33:** 13259-13266.

Subbaiah, P. V., M. Liu, J. Senz, X. Wang, and P. H. Pritchard. (1994b). Substrate and positional specificities of human and mouse lecithin-cholesterol acyltransferases. Studies with wild type recombinant and chimeric enzymes expressed in vitro. *Biochim. Biophys. Acta.* **1215:** 150-156.

Suzukawa, M., T. Ishikawa, H. Yoshida, H. Nakamura. (1995). Effect of in-vivo supplementation with low-dose vitamin E on susceptibility of low density density lipoprotein to oxidative modification. *J. Am. Coll. Nutr.* **14:** 46-52.

Swenson, T. L., J. S. Simmons, C. B. Hesler, C. Bisgaier, and A. R. Tall. (1987). Cholesterol ester transfer protein is secreted by HepG2 cells and contains asparagine-linked carbohydrate and sialic acid. *J. Biol. Chem.* **262**: 16271-16274.

Swenson T. L., C. B. Hesler, M. L. Brown, E. Quinet, P. P. Trotta, M. F. Haslanger, F. C. A. Gaeta, Y. L. Marcel, R. W. Milne, and A. R. Tall. (1989). Mechanism of cholesteryl ester transfer protein inhibition by a neutralizing monoclonal antibody and mapping of the monoclonal antibody epitope. *J. Biol. Chem.* **264**: 14318-14326.

Takahashi, K., X.-C. Jiang, N. Sakai, S. Yamashita, K. Hirano, H. Bujo, H. Yamazaki, J. Kusunoki, T. Muira, P. Kussie, Y. Matsuzawa, Y. Saito, and A. Tall. (1993). A missense mutation in the cholesteryl ester transfer protein gene with possible dominant effects on plasma high density lipoproteins. *J. Clin. Invest.* **92**: 2060-2064.

Tall, A. R., D. Sammett, and E. Granot. (1986). Mechanisms of enhanced cholesteryl ester transfer from high density lipoproteins to apolipoprotein B-containing lipoproteins during alimentary lipemia. *J. Clin. Invest.* **77:** 1163-1172.

Tall, A. R. (1990). Plasma high density lipoprotein. Metabolism and relationship to atherogenesis. *J. Clin Invest.* **86:** 379-384.

Tall, A. (1993). Plasma cholesteryl ester transfer protein. *J. Lipid Res.* **34**: 1255-1272.

Tall, A. (1995). Plasma lipid transfer protein. *Annu. Rev. Biochem.* **64**: 235-257.

Tata, F., M. E. Chaves, A. F. Markham, G. D. Scrace, M. D. Waterfield, N. McIntyre, R. Willaimson, and S. E. Humpries. (1987). The isolation and characterisation of cDNA and genomic clones for human lecithin: cholesterol acyltransferase. *Biochim. Biophys. Acta* **910**: 142-148.

Teh, E. M., P. J. Dolphin, W. C. Breckenridge, and M.-H. Tan. (1998). Human plasma CETP deficiency: identification of a novel mutaion in exon 9 of the CETP gene in a Caucasian subject from North America. *J. Lipid Res.* **39:** 442-456.

Teh, E. M., J. W. Chisholm, P. J. Dolphin, Y. Pouliquen, M. Savoldelli, J. L. de Gennes, and P. Benlian. (1999). Classical LCAT deficiency resulting forma a novel homozygous dinucleotide deletion in exon 4 of the human lecithin:cholesterol acyltransferase gene causing a frameshift and a stop codon at residue 144. *Atherosclerosis.* **146:** 141-151.

Thomsen, D. R., R. M. Steinberg, W. F. Goins, and M. F. Stinski. (1984). Promoter-regulatory region of the major immediate early gene of human cytomegalovirus. *Proc. Natl. Acad. Sci.* **81:** 659-663.

Tollefson, J. H., S. Ravnik, and J. J. Albers. (1988). Isolation and characterization of a phospholipid transfer protein (LTP-II) from human plasma. *J. Lipid Res.* **36**: 975-985.

Tu, A.-Y., H. I. Nishida, and T. Nishida. (1993). High density lipoprotein conversion mediated by plasma phospholipid transfer protein.*J. Biol. Chem.* **268**: 23098-23105.

Vaisman, B. L., H.-G. Klein, M. Rouis, A. M. Bérard, M. R. Kindt, G. D. Talley, S. M. Meyn, R. F. Hoyt, Jr., S. M. Marcovina, J. J. Albers, J. M. Hoeg, H. B. Brewer, Jr., and S. Santamarina-Fojo. (1995). Overexpression of human lecithin cholesterol acyltransferase leads to hyperalphalipoproteinemia in transgenic mice. *J. Biol. Chem.* **270**: 12269-12275.

van Tilbeurgh, H., M. P. Egloff, C. Martinez, N. Rugani, R. Verger, and C. Cambillau. (1993). Interfacial activation of the lipase-procolipase complex by mixed micelles revealed by X-ray crystallography. *Nature.***362:** 814-820.

von Eckardstein, A., Y. Huang, and G. Assmann. (1994). Physiological role and clinical relevance of high-density lipoprotein subclasses. *Curr. Opin. Lipidol.* **5:** 404-416.

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:** 255-262.

Wang, J., A. K. Gebre, R. A. Anderson, and J. S. Parks. (1997). Amino acid residue 149 of lecithin:cholesterol acyltransferase determines phospholipase A_2 and transacylase fatty acyl specificity. *J. Biol. Chem.* **272**: 280-286.

Wang, J., J. A. Delozier, A. K. Gebre, P. J. Dolphin, and J. S. Parks. (1998). Role of glutamic acid residues 154, 155, and 165 of lecithin:cholesterol acyltransferase in cholesterol esterfication and phospholipase A_2 activities. *J. Lipid Res.* **39**:51-58.

Wang, S., L. Deng, M. L. Brown, L. B. Agellon, and A. R. Tall. (1991). Structure-function studies of human cholesteryl ester transfer protein by linker scanning insertion scanning mutagenesis. *Biochemistry.* **30**: 3484-3490.

Wang, S., L. Deng, R. W. Milne, and A. R. Tall. (1992). Identification of a ssequence within the C-terminal 26 amino acids of cholesteryl ester trasnfer protein responsible for binding a neutralizing monoclonal antibody and necessary for neutral lipid transfer activity. *J. Biol. Chem.* **267**: 17487-17490.

Wang, S., X. Wang, L. Deng, E. Rassart, R. W. Milne, and A. R. Tall. (1993). Point mutagenesis of carboxyl-terminal amino acids of cholesteryl ester transfer protein. Opposite faces of an amphipathic helix important for cholesteryl ester transfer or for binding neutralizing antibody. *J. Biol. Chem.* **268**: 1955-1959.

Wang X, D. M. Driscoll, R. E. Morton. (1999). Molecular cloning and expression of lipid transfer inhibitor protein reveals its identity with apolipoprotein F. *J Biol Chem.* **274:** 1814-1820

Warden, C. H., C. A. Langner, J. I. Gordon, B. A. Taylor, J. W. McLean, and A. J. Lusis. (1989). Tissue-specific expression, developmental regulation, and chromosomal

mapping of the lecithin: cholesterol acyltransferase gene. Evidence for expression in brain and testes as well as liver. *J. Biol. Chem.* **264**: 21573-21581.

Watson, A.D., J. A. Berliner, S. Y. Hama, B. N. La Du, K. F. Faull, A. M. Fogelman. (1995). protective effect of high density lipoprotein associated paraoxonase. Inhibition of the biological activity of minimally oxidized low density lipoprotein. *J. Clin Invest.* **96:** 2882-2891.

Wong, H., R. C. Davis, J. S. Hill, D. Yang, and M. C. Schotz. (1997). Lipase engineering: a window into structure-function relationships. *In* Methods in Enzymology. B. Rubin and E. A. Dennis (eds) Academic Press, N. Y. **284**: 171-184.

Wiesbusch, H., P Cullen, J. S. Owen, D. Collins, P. S. Sharp, H. Funke and G. Assmann. (1995). Deficiency of lecithin:cholestrerol acyltransferase due to compound heterozygosity of two novel mutations (Gly33Arg and 30 bp ins) in the LCAT gene. *Hum. Molec. Genet.* **4**: 143-145.

Willnow, T.E., Z. Sheng, S. Ishibashi, and J. Herz. (1994). Inhibition of hepatic remnant uptake by gene transfer of a receptor antagonist. *Science.* **264:** 1471-1474.

Winder, A. F., A. Garner, G. A. Sheraidah, and P. Barry. (1985). Familial lecithin:cholesterol acyltrasnferase deficiency. Biochemistry of the cornea. *J. Lipid Res.* **26:** 283-287.

Winkler, K. E., and J. B. Marsh. (1989). Metabolism of triglyceride-rich nascent rat hepatic high density lipoproteins. *J. Lipid Res.* **30:** 979-996.

Winkler, F. K., A. D'Arcy, and W. Hunziker. (1990). Structure of human pancreatic lipase. *Nature.* **343**: 771-774.

Yamashita, S., Y. Matsuzawa, M. Okazaki, H. Kako, T. Yasugi, H. Akioka, K. Hirano, S. Tarui. (1988). Small polydispersed low density lipoproteins in familial hyperalphaliporpoteinemia with complete deficiency of choelsteryl ester transfer activity. *Atherosclerosis.* **70:** 7-12.

Yang, C. Y., D. Manoogian, Q. Pao, K. S. Lee, R. D. Knapp, A. M. Gotto, and H. J. Pownall. (1987). Lecithin:cholesterol acyltransferase. Functional regions and a structural model of the enzyme. *J. Biol. Chem.* **262:** 3086-3091.

Yang, T.-P., L. B. Agellon, A. Walsh, J. L. Breslow, and A. R. Tall. (1996). Alternative splicing of the human cholesteryl ester transfer protein gene in transgenic mice; exon exclusion modulates gene expression in response to dietary or developmentl changes. *J. Biol. Chem.* **271:** 12603-12609.

Yang, X.-P., A. Inazu, A. Honjo, I. Koizumi, K. Kajinami, J. Koizumi, S. M. Marcovina, J. J. Albers, and H. Mabuchi. (1997). Catalytically inactive lecithin: cholesterol acyltransferase (LCAT) caused by a Gly 30 to Ser mutation in a family with LCAT deficiency. *J. Lipid Res.* **38**: 585-591.

Yen, F. Y., R. J. Deckelbaum, C. J. Mann, Y. L. Marcel. R. W. Milne, and A. R. Tall. (1989). Inhibition of cholesteryl ester transfer protein activity by monoclonal antibody; effects on cholesteryl ester formation and neutral lipid mass transfer in human plasma. *J. Clin. Invest.* **83**: 2018-2024.

Zhong, S., I. J. Goldberg, C. Bruce, E. Rubin, J. L. Breslow, and A. Tall. (1994). Human apoA-II inhibits the hydrolysis of HDL triglyceride and the decrease of HDL size induced by hypertriglyceridemia and cholesteryl ester transfer protein in transgenic mice. *J. Clin. Invest.* **94:** 2457-2467.

Zhong, S., D. S. Sharp, J. S. Grove, C. Bruce, K. Yano, J. D. Curb, and A. R. Tall. (1996). Increased coronary heart disease in Japanese-American men with mutations in the cholesteryl ester transfer protein gene despite increased HDL levels. *J. Clin. Invest.* **97:** 2917-2923.

Zorich, N., A. Jonas, and H. J. Pownall. (1985). Activation of lecithin cholesterol acyltransferase by human apolipoprotein E in discoidal complexes with lipids. *J. Biol. Chem.* **260:** 8831-8837.