UNIVERSITY OF CRETE

SCHOOL OF MEDICINE

MASTER THESIS

FUNCTIONAL CHARACTERIZATION OF NATURALLY OCCURING APOA-I MUTATIONS FOUND IN THE COPENHAGEN CITY HEART STUDY: A SUBSTITUTION L144R IN APOA-I RESULTS IN ABNORMAL HDL PHENOTYPE THAT CAN BE CORRECTED WITH TREATMENT WITH LCAT

by

ANDREAS KATEIFIDES

MD School of Medicine, University of Crete, Greece, 2005

Submitted in partial fulfillment of the requirements for the degree of

Master in Sciences in the graduate program

MOLECULAR BASIS OF HUMAN DISEASE

2008

ΠΑΝΕΠΙΣΤΗΜΙΟ ΚΡΗΤΗΣ

ΤΜΗΜΑ ΙΑΤΡΙΚΗΣ

ΧΑΡΑΚΤΗΡΙΣΜΟΣ ΔΥΟ ΦΥΣΙΚΩΝ ΜΕΤΑΛΛΑΞΕΩΝ ΤΗΣ ΑΠΟΑ-Ι ΠΟΥ ΕΝΤΟΠΙΣΤΗΚΑΝ ΚΑΤΑ ΤΗΝ ΕΠΙΔΗΜΙΟΛΟΓΙΚΗ ΜΕΛΕΤΗ ΤΟΥ ΠΛΗΘΥΣΜΟΥ ΤΗΣ ΚΟΠΕΝΧΑΓΗΣ: ΑΝΤΙΚΑΤΑΣΤΑΣΗ ΤΗΣ ΛΕΥΚΙΝΗΣ 144 ΜΕ ΑΡΓΙΝΙΝΗ ΣΤΗΝ ΑΠΟΑ-Ι ΔΗΜΙΟΥΡΓΕΙ ΠΑΘΟΛΟΓΙΚΟ ΦΑΙΝΟΤΥΠΟ ΣΤΗΝ HDL ΠΟΥ ΜΠΟΡΕΙ ΝΑ ΔΙΟΡΘΩΘΕΙ ΜΕ ΧΟΡΗΓΗΣΗ ΤΟΥ ΕΝΖΥΜΟΥ LCAT

ΑΝΔΡΕΑΣ ΚΑΤΕΪΦΙΔΗΣ

ΜΕΤΑΠΤΥΧΙΑΚΟ ΠΡΟΓΡΑΜΜΑ

ΜΟΡΙΑΚΗ ΒΑΣΗ ΤΩΝ ΑΣΘΕΝΕΙΩΝ ΤΟΥ ΑΝΘΡΩΠΟΥ

2008

Approved by:

First reader _______________________________

Vassilis I. Zannis, Ph.D.

Professor of Biochemistry

Second reader _______________________________

Dimitris Kardassis, Ph.D.

Assistant Professor of Biochemistry

To my family

for their continuous support

ACKNOWLEDGEMENTS

With the completion of my master thesis I would like to truthfully thank my advisor Professor Vassilis I. Zannis for all his support and his guidance through the last five years of my life. With his encouragement I have started pursuing my dreams and started getting to know what is to be a scientist.

I would also like to thank Dr. Dimitris Kardassis for providing me the chance to work in his lab during my first rotation and also for the financial support. I would also like to thank George Koukos for all the help that he provided me during my first year in Dr. Zannis lab. I am also grateful to Effie Thymiakou, as I call her my first teacher, for the guidance that she provided me during my rotation in Dr. Kardassis lab. My thanks also go to the wonderful people in Dr. Zannis lab, including Dr. Kyriakos Kypreos, Dr. Costas Drosatos, Adelina Duka and Gayle Forbes. I also thank Alex Vezerides, it was really nice to interact with him in the lab and a reason for me to work more so I would leave from the lab later than him. I also thank Anne Plunkett for all the support that she always provided.

I am also very thankful to Mary Adamaki, she has been always there to help me whenever I had a problem and without her help I would not have been able to complete my commitments to the program.

Finally I want to thank all my friends and especially Nicholas P. and Nektaria Goulidaki that were available whenever I asked for their help.

ΠΕΡΙΛΗΨΗ

Η απολιποπρωτεΐνη Α-Ι (αποΑ-Ι) είναι η κύρια πρωτεΐνη των λιποπρωτεϊνών υψηλής πυκνότητας (HDL) και παίζει σημαντικό ρόλο στην βιογένεση, τη δομή, την λειτουργία και την συγκέντρωση της HDL στο πλάσμα. Επιδημιολογικές μελέτες και κλινικές δοκιμές φανερώνουν ότι τα επίπεδα της HDL είναι αντιστρόφως ανάλογα του κινδύνου εμφάνισης στεφανιαίας νόσου στον άνθρωπο. Δύο μεταλλαγμένες μορφές της αποΑ-Ι ανιχνεύθηκαν στην μελέτη του πληθυσμού της πόλης της Κοπεγχάγης που είχε στόχο την εύρεση γενετικών παραγόντων που συμβάλουν στην καρδιαγγειακή νόσο. Για να μελετήσουμε τις συνέπειες αυτών των μεταλλάξεων στη βιογένεση της HDL κατασκευάστηκαν ανασυνδιασμένοι αδενοϊοί που εκφράζουν δύο μεταλλαγμένες μορφές της αποΑ-Ι, αποΑ-Ι(L144R) και αποΑ-Ι(A164S). Μελέτες σε κύτταρα HTB-13 που μολύνθηκαν με τους ανασυνδιασμένους αδενοϊούς που εκφράζουν τις εν λόγω πρωτεΐνες, έδειξαν ότι τόσο η αποΑ-Ι αγρίου τύπου (WT) όσο και η μεταλλαγμένες μορφές της αποΑ-Ι εκκρίνονται με τον ίδιο ρυθμό στο θρεπτικό υλικό των κυττάρων. Γονιδιακή μεταφορά σε ποντίκια με ανεπάρκεια της αποΑ-Ι (αποΑ-Ι$^{-/-}$) έδειξε ότι η αποΑ-Ι(L144R) οδήγησε σε χαμηλά επίπεδα ολικής χοληστερόλης και μειωμένο λόγο εστέρων χοληστερόλης προς ολική χοληστερόλη (CE/TC) σε σχέση με αποΑ-Ι$^{-/-}$ ποντίκια που έλαβαν τον αδενοϊό που εκφράζει την αποΑ-Ι αγρίου τύπου ή την αποΑ-Ι(A164S). Η διαφορά στο λόγο CE/TC μεταξύ της αποΑ-Ι αγρίου τύπου και αποΑ-Ι(L144R) δεν μετεβλήθει όταν τα επίπεδα έκφρασης της μεταλλαγμένης μορφής (με βάση τα επίπεδα

ηπατικού RNA) ήταν αυξημένα. Κλασμάτωση του πλάσματος με FPLC έδειξε ότι το κλάσμα της HDL σε ποντίκια που εκφράζουν την αποA-I(L144R) ήταν παρόμοιο με το κλασμα της HDL σε ποντίκια που εκφράζουν την πρωτεΐνη ελέγχου GFP. Κλασμάτωση του πλάσματος με υπερφυγοκέντρηση σε βαθμίδωση KBr έδειξε ότι η αποA-I στα ποντίκια που εκφράζουν την αποA-I(L144R) ήταν μειωμένη και μετατοπισμένη προς την HDL3 συγκριτικά με ποντίκια που εκφράζουν την WT αποA-I ή αποA-I(A164S). Ανάλυση με ηλεκτρονική μικροσκοπία του κλάσματος της HDL έδειξε ότι η αποA-I(L144R) οδήγησε στο σχηματισμό λίγων δισκοειδών σωματιδίων καθώς και μικρού μεγέθους σωματιδίων που ήταν όμοια με τα σωματίδια που παρατηρήθηκαν σε ποντίκια που εκφράζουν την πρωτεΐνη ελέγχου GFP. Αντίθετα η WT αποA-I και η αποA-I(A164S) οδήγησαν στο σχηματισμό σφαιρικών σωματιδίων. Ηλεκτροφόρηση δύο διαστάσεων έδειξε ότι η αποA-I(L144R) οδήγησε στο σχηματισμό preβ- και α4-HDL σωματιδίων, ενώ η WT αποA-I και η αποA-I(A164S) οδήγησε στο σχηματισμό κανονικών πληθυσμών preβ- και α-HDL σωματιδιων (α1, α2, α3, α4). Ταυτόχρονη χορήγηση αδενοϊών που εκφράζουν την αποA-I(L144R) και την ανθρώπινη LCAT απεκατέστησε τα επίπεδα χοληστερόλης και το λόγο CE/TC. H χορήγηση LCAT επίσης αύξησε σε κανονικά επίπεδα το κλάσμα της HDL. Κλασμάτωση του πλάσματος με υπερφυγοκέντρηση σε βαθμίδωση KBr έδειξε ότι η αποA-I στα ποντίκια που εκφράζουν την αποA-I(L144R) επανήλθε στα φυσιολογικά επίπεδα μετα τη χορήγηση της LCAT. Επίσης η συγχορήγηση της LCAT οδήγησε στο σχηματισμό σφαιρικών σωματιδίων και απεκατέστησε

κανονικούς πληθυσμούς preβ- και α-HDL σωματιδίων. Τα ευρήματα αυτά φανερώνουν ότι η αποA-I(L144R) έχει παθολογικό και η αποA-I(A164S) ομαλό φαινότυπο HDL. Τα πειράματα αυτά επίσης υποδεικνύουν ότι τα χαμηλά επίπεδα HDL στα ποντίκια που εκφράζουν την αποA-I(L144R) οφείλονται σε ανεπάρκεια στην LCAT που αναστέλλει την ομαλή μετατροπή της λιπιδιωμένης αποA-I σε δισκοειδή και εν συνεχεία σε σφαιρικά σωματίδια HDL. Η αποκατάσταση κανονικού φαινοτύπου HDL σε ποντίκια που εκφράζουν την αποA-I(L144R) μετά από χορήγηση LCAT υποδεικνύει πιθανή θεραπεία συνδρόμων που οδηγούν σε ανώμαλους φαινοτύπους HDL και οφείλονται σε συγκεκριμένες μεταλλάξεις της αποA-I.

ABSTRACT

ApoA-I is an essential protein for the formation and the physiological functions of HDL particles. Epidemiological and genetic data have shown that low or high levels of HDL or apoA-I are associated with increased or decreased risk of developing atherosclerosis, respectively, indicating the importance of apoA-I in cellular cholesterol homeostasis. Two mutants were identified by screening the population of the Copenhagen City Heart Study (CCHS). I have generated recombinant adenoviruses expressing these two naturally occurring apoA-I mutants, apoA-I(L144R) and apoA-I(A164S). Cell culture studies showed that when HTB-13 cells were infected with adenoviruses expressing the wild type or the mutant apoA-I forms in all cases apoA-I was secreted efficiently in to the culture medium. Adenovirus mediated gene transfer in apoA-I-deficient mice showed that administration of the adenovirus expressing ApoA-I(L144R) resulted in low total cholesterol levels and decreased cholesterol esters to total cholesterol ratio (CE/TC) as compared to mice that received the adenovirus expressing the wild type apo A-I or the apoA-I(A164S). The difference in total cholesterol and CE/TC ratio between WT and apoA-I(L144R) persisted even when the level of expression of the mutant, as determined by the steady state hepatic apoA-I mRNA levels, were increased. FPLC fractionation of plasma showed that the HDL cholesterol peak of mice expressing the apoA-I(L144R) was very small and resembled the HDL fraction cholesterol peak of mice expressing the control protein (GFP). Fractionation of plasma by density gradient ultracentrifugation

showed that apoA-I in mice expressing apoA-I(L144R) was greatly reduced and was distributed mainly in the HDL-3 region as compared to mice expressing the wild type apoA-I or apoA-I(A164S). Electron microscopy of the HDL fraction showed that apoA-I(L144R) promoted the formation of few discoidal particles as well as small size particles similar to those observed in mice expressing the control protein (GFP), in contrast wild type apoA-I and apoA-I(A164S) promoted the formation of spherical particles. Two-dimensional gel electrophoresis showed that the mutant apoA-I(L144R) promoted the formation of preβ- and α4-HDL particles, whereas WT apoA-I and the mutant apoA-I(A164S) promoted the formation of normal preβ and α HDL subpopulations of different sizes (α1, α2, α3 and α4). Simultaneous treatment of the mice with the virus expressing the apoA-I(L144R) and human LCAT normalized the total cholesterol level and the CE/TC ratio. The LCAT treatment restored the HDL cholesterol peak, promoted the formation of spherical HDL and restored normal preβ- and α-HDL subpopulations. The findings establish that apoA-I(L144R) has an aberrant and apoA-I(A164S) has a normal HDL phenotype. The present study also suggest that the low HDL levels of mice expressing the apoA-I(L144R) results from LCAT insufficiency that inhibits the efficient conversion of the lipidated apoA-I to discoidal and subsequently spherical HDL particles. The correction of the aberrant HDL phenotypes by treatment with LCAT suggest a potential therapeutic intervention for HDL abnormalities that result from specific mutation in apoA-I.

TABLE OF CONTENTS

TITLE i

APPROVAL PAGE iii

ACKNOWLEDGEMENTS v

ABSTRACT vi

TABLE OF CONTENTS xi

LIST OF TABLES xviii

LIST OF FIGURES xix

LIST OF ABBREVIATIONS xxi

INTRODUCTION 1

ApoA-I primary structure and importance 1

Genetics and gene regulation 1

3D Structure of apoA-I in solution 2

Structure of apoA-I on lipoprotein particles 3

Role of apoA-I in the biogenesis of HDL 4

Interactions of apoA-I with ABCA1 5

Interactions of lipid-bound apoA-I with LCAT 13

Mutations in apoA-I that cause dyslipidemia 15

Interactions of lipid-bound apoA-I with SR-BI 17

Remodeling of HDL by the action of ABCG1 19

HDL subpopulations 20

Naturally occurring apoA-I mutations 20

Leucine 144 to arginine mutation 21

Physiological functions of apoA-I and HDL that may be relevant to their atheroprotective properties 23

Role of apoA-I in atheroprotection 23

Effects of HDL on eNOS protein levels 24

Role of lipid moieties of HDL 25

Effect of HDL on eNOS activity in humans 26

Role of HDL in endothelial cell apoptosis 26

Role of HDL in endothelial cell proliferation and migration 27

Antithrombotic effects of HDL 28

Role of HDL in the initiation of the thrombus 29

HDL, activated protein C, and coagulation factors 29

The antithrombotic properties of HDL sphingolipids 30

HDL and fibrinolysis 30

Interactions of HDL with platelets 31

Molecules involved in HDL signaling 31

MATERIALS AND METHODS 33

MATERIALS 33

EXPERIMENTAL PROCEDURES 35

DNA electrophoresis on agarose gel 35

Reaction with restriction enzymes (digest) 35

Ligation reaction 36

Transformation of bacterial *E.coli* DH5a cells 36

Mini scale preparation (miniprep) for plasmid purification 36

Large scale preparation (Maxiprep) for plasmid purification 37

Cell cultures 38

SDS-polyacrylamide gel electrophoresis (SDS-PAGE) 39

Analysis by Western blot 40

Generation of mutations on apoA-I gene 41

Transformation of *E.coli* BJ5183-AD1 cells by electroporation 43

Generation of recombinant adenoviruses 44

Plaque assay 45

Expression and secretion of the wild type (WT) and the mutated forms of apoA-I 46

Animal studies 47

Plasma isolation from mice blood 47

Plasma lipid levels 48

Fast protein liquid chromatography 48

Fractionation of plasma by density gradient Ultracentrifugation 48

Electron microscopy analysis of the apoA-I containing fractions 49

Non-denaturing two-dimensional gel electrophoresis 50

RNA isolation and hybridization analysis 50

RESULTS 53

Generation of the mutated pAdTrack-CMV-apoA-I(L144R) and pAdTrack-CMV-apoA-I(A164S) shuttle vectors 53

Generation of recombinant adenoviruses that express naturally occurring mutated forms of apoA-I 55

Expressing the WT apoA-I and the two mutants apoA-I(L144R) and apoA-I(A164S) following infection of HTB-13 cells with the recombinant adenoviruses 57

mRNA analysis 57

Plasma lipid levels of apoA-I$^{-/-}$ mice that were infected with recombinant adenoviruses that express the WT or the mutated forms of apoA-I 59

Plasma lipid profile after FPLC fractionation 61

Distribution of the various apoA-I forms after fractionation by density gradient ultracentrifugation 61

Electron microscopy analysis 64

Non-denaturing two dimensional gel electrophoresis 64

LCAT corrects the abnormal phenotype produced
by the apoA-I(L144R) mutation 67

Lipid profile of plasma obtained from mice co-infected
with adenoviruses expressing apoA-I(L144R)
and hLCAT after FPLC fractionation 67

Distribution of apoA-I(L144R) obtained from mice
that were treated with adenoviruses co-expressing
apoA-I(L144R) and hLCAT after fractionation of
plasma by density gradient ultracentrifugation and
electron microscopy analysis of the HDL fraction 69

Non-denaturing two dimensional gel electrophoresis of plasma
obtained from mice expressing apoA-I(L144R) and hLCAT 72

DISCUSSION 74

Background 74

The mutated form apoA-I(L144R) 77

The mutated form apoA-I(A164S) 78

The administration of hLCAT in mice can correct the
aberrant HDL phenotype that is caused by apoA-I(L144R) 79

The amino acid Leu144 of apoA-I affects the interaction
of apoA-I with LCAT 80

CONCLUSIONS 81

FUTURE DIRECTIONS 82

Literature cited 83

LIST OF TABLES

TABLE 1 Oligonucleotide sequence of primers used in PCR amplifications 42

TABLE 2 Hepatic apoA-I mRNA, total cholesterol and CE/TC ratio of mice expressing WT and mutant apoA-I forms 62

LIST OF FIGURES

Figure 1. Agarose gel electrophoresis of Shuttle Vectors prior to or after digestion with BglII and EcoRV 54

Figure 2. Agarose gel electrophoresis of DNA obtained from BJ-5183-AD1 cells following transformation by electroporation with the plasmids pAdTrack-CMV-apoA-I(L144R) and pAdTrack-CMV-apoA-I(A164S) 56

Figure 3. Secretion of apoA-I(L144R), apoA-I(A164S) and WT apoA-I following infection of HTB-13 cells with recombinant adenoviruses expressing these apoA-I forms 58

Figure 4. Representative northern blot analysis of hepatic mRNA 60

Figure 5. FPLC profiles of total cholesterol of apoA-I$^{-/-}$ mice infected with adenoviruses expressing the WT apoA-I, apoA-I(L144R), apoA-I(A164S) or the control protein GFP 63

Figure 6. Separation of plasma of apoA-I$^{-/-}$ mice infected with adenoviruses by density gradient ultracentrifugation 65

Figure 7. Electron microscopy pictures of HDL fractions 66

Figure 8. Analysis of mice plasma following 2D gel electrophoresis and Western blotting 68

Figure 9. FPLC profiles of total cholesterol of apoA-I$^{-/-}$ mice co-infected with adenoviruses expressing the apoA-I(L144R) and hLCAT compared with mice expressing WT apoA-I and apoA-I(L144R) 70

Figure 10. Separation of plasma of ApoA-I$^{-/-}$ mice by density gradient ultracentrifugation. Electron microscopy picture of HDL fraction 71

Figure 11. Analysis of plasma of mice expressing apoA-I(L144R) and hLCAT following 2D gel electrophoresis and Western blotting 73

Figure 12. Simplified schematic presentation of the biogenesis of HDL and HDL functions 75

LIST OF ABBREVIATIONS

ABCA1	ATP Binding Cassette transporters
Ad	Adenovirus
Apo	Apolipoprotein
BSA	Bovine Serum Albumin
CE	Cholesteryl Ester
CETP	Cholesteryl Ester Transfer Protein
CHD	Coronary Heart Disease
CHO	Chinese Hamster Ovary (cells)
EM	Electron Microscopy
FPLC	Fast Protein Liquid Chromatography
HDL	High Density Lipoprotein
IDL	Intermediate Density Lipoprotein
LB	Luria-Bertani (medium)
LCAT	Lecithin Cholesterol Acyl Transferase
LDL	Low Density Lipoprotein
moi	multiplicity of infection
PCR	Polymerase Chain Reaction
PLTP	Phospholipid Transfer Protein
SDS	Sodium Dodecyl Sulphate
SR-BI	Scavenger Receptor, Class B Type I
VLDL	Very Low Density Lipoprotein

INTRODUCTION

ApoA-I Primary Structure and Importance

Human apolipoprotein A-I (apoA-I) is a 243-aa plasma protein that is derived from a 249-aa precursor. It is synthesized and secreted predominantly by the liver and the intestine (1). ApoA-I is an essential protein for the formation and the physiological functions of HDL particles (1;2). ApoA-1 constitutes ~70% of the apolipoprotein content of HDL particles. Thus mice or humans that do not express apoA-I do not have the ability to form HDL (2;3).

Elevated levels of HDL in apoA-I transgenic mice are correlated with protection against atherosclerosis (4), proving in principle that apoA-I overexpression can positively influence both plasma HDL concentrations as well as atherosclerosis progression (5). Epidemiological and genetic data have shown that low (6;7) or high (8;9) levels of HDL or apoA-I are associated with increased or decreased risk of developing atherosclerosis, respectively, indicating the importance of apoA-I in cellular cholesterol homeostasis.

Genetics and Gene Regulation

The apoA-I gene is a 3kb gene that is located on chromosome 11q23 (10). The apoA-I gene is closely linked to the apoCIII gene. The genes have opposite orientation (11). Studies in transgenic mice that carry the apoA-I, apoCIII cluster

and all the regulatory sequences have shown that hormone nuclear receptors and specificity protein 1 (SP1) family are important for the transcription of the apoA-I gene (12).

Regulation of the apoA-I gene occurs primarily at the transcriptional level, mediated by the *cis*-acting sequences in the proximal promoter of the apoA-I gene, and partly at the post-transcriptional level by increasing the stability of the partially spliced and unspliced nuclear apoA-I mRNA (12;13). Dietary fat, alcohol, estrogen, androgens, thyroid hormone, retinoids, glucocorticoids, fibrates, niacin, and HMG-CoA reductase inhibitors are some of the many nutritional, hormonal, and pharmacological factors known to influence transcriptional induction of the apoA-I gene (13;14). Promotion of apoA-I gene transcription and biosynthesis is an attractive therapeutic target for drug development. Based on animal studies, upregulation of apoA-I expression in humans would be expected to raise HDLc concentrations and provide protection against atherosclerosis (15).

3D Structure of ApoA-I in Solution

In a N-terminal deletion mutant of apoA-I, apoA-I[Δ(1-43)], that was crystallized its structure was determined at 4 Å resolution. In this structure with the continuous amphipathic α-helical sequence that is punctuated by small or pronounced kinks the molecule is allowed to adopt a horseshoe shape with dimensions of 125x80x40 Å (16-21).

A new crystal structure of the human apoA-I has been published recently. In this 2,4 Å resolution model lipid free apoA-I has 6 helices (22). In this structure helix D can be exposed in order to interact with LCAT (22). In previous models it was proposed that it had 10 helices (23-25) but later it was shown that the region 122-144 (proposed helix 5) does not have a defined secondary structure (26). These amphipathic α-helices are formed by 22- and 11- amino acid repeats (23;27).

In another study it was shown that human apoA-I and mouse apoA-I have 70% similarity in the N-terminal domains where in the C-terminal domains the similarity is only 46% (28). The N-terminal it has been shown both in human and mouse apoA-I that adopts a helix bundle and the C-terminal forms a separate domain (28). These two domains have different functionality. The isolated mouse N-terminal domain can bind lipids but the isolated human N-terminal domain can not function efficiently. In hybrid molecules it was shown that both molecules can function efficiently in lipid binding indicating that these two domains have certain functions (28).

Structure of apoA-I on Lipoprotein Particles

Two general models have been proposed to account for the conformation of apoA-I on the discoidal particles, the picket fence model in which two

molecules form tandem antiparallel helices perpendicular to the plane of the disc and the belt model in which two apoA-I molecules form essentially continuous amphipathic α-helices that are parallel to the plane of the disc (18).

In the belt model of HDL with the antiparallel orientation two apoA-I molecules are wrapped around the edge of a discoidal lipid bilayer containing 160 lipid molecules (18). When phospholipids are removed gradually some intermediates are formed that resemble the 95 Å and 78 Å rHDL particles which have a molar phospholipid to apoA-I ratio of 100:2 and 50:2 respectively. As the rHDL is further delipidated the structure of the lipid- bound apoA-I approximates the x-ray crystal structure of lipid-free apoA-I (29).

Role of ApoA-I in the Biogenesis of HDL

HDL is synthesized through a complex pathway (30). The first step is mediated by an ABCA1 transfer of cellular phospholipids and cholesterol to lipid poor apoA-I extracellularly. The next step is the remodelling of HDL particles. It takes place in the plasma and involves the esterification of cholesterol by the enzyme lecithin: cholesterol acyl transferase (LCAT) (31). The spherical HDL that is formed is further remodelled by the exchange between HDL and other lipoproteins of apolipoproteins and lipids and as well by the putative transfer of additional cellular cholesterol to the growing particles by the scavenger receptor class B, type I (SR-BI) (32) and possibly the cell surface transporter ABCG1 (33).

Hydrolysis of lipid moieties of HDL can occur by the action of various lipases (lipoprotein lipase, hepatic lipase, endothelial lipase), and exchange of lipids by the phospholipid transfer protein (PLTP) and by the cholesteryl ester transfer protein (CETP).

Interactions of ApoA-I with ABCA1

ABCA1 is a member of the ABC family of transporters. It is a ubiquitous protein expressed abundantly in the liver, macrophages, brain and various other tissues (34;35). ABCA1 that is expressed only on the basolateral surface of the hepatocytes (36) is associated with endocytic vesicles and travels between late endocytic vesicles and the cell surface (37). ABCA1 promotes the efflux of cellular phospholipids and cholesterol to lipid free apoA-I and other apolipoproteins and amphipathic peptides, but not to spherical HDL particles (38-41). Cellular cholesterol efflux is severely reduced by inactivating mutations in ABCA1, both in patients with Tangier disease and in ABCA1$^{-/-}$ (42-48). These patients and ABCA1$^{-/-}$ mice do not form discoidal or spherical HDL, but form preβ1 HDL particles (49;50), have very low total plasma cholesterol and exhibit abnormal lipid deposition in various tissues (47;48;51-53).

Fluorescence microscopy of HeLa cells that expressed an ABCA1 green fluorescence fusion protein has shown the intracellular trafficking of apoA-I/ABCA1 complexes (36;37). In macrophages it has been observed that apoA-I

binds to ABCA1 in the coated pits, it is internalized and then interacts with intracellular lipid pools and then it is re-secreted as a lipidated particle (54;55). A similar pathway is followed in endothelial cells (56). This transcytosis of apoA-I and its release in the form of HDL may explain some of the anti-atherogenic properties of HDL.

Recent experiments investigated the in vitro interactions between apoA-I and ABCA1. A series of apoA-I mutants were generated and their ability to promote ABCA1-mediated cholesterol efflux was analyzed. These mutants had amino terminal deletions, carboxy terminal deletions that removed the 220-231 region, carboxy terminal deletions (232-243) that left the 220-231 region, double deletions of the amino- and carboxy- terminal region (57).

For these efflux studies two types of cell cultures were used. The one was J774 macrophages in which expression of ABCA1 can be induced by cAMP analogues. The other was Human Embryonic Kidney (HEK) 293 cells transfected with an ABCA1-expressing plasmid. These studies showed that WT ABCA1-mediated cholesterol and phospholipid efflux was not affected by amino-terminal deletions, it was diminished by carboxy-terminal deletions in which residues 220-231 were removed and it was restored to 80% of WT control by double deletions of both the amino and carboxy termini (30;31;57;58). This finding is consistent with direct ABCA1/apoA-I interactions that involves the central helices

of apoA-I. Lipid efflux was either unaffected or moderately reduced by a variety of point mutations or deletions of internal helices 2-7 (59;60). The data indicated that different combinations of central helices can promote lipid efflux (59;60). Chemical cross-linking/ immunoprecipitation studies showed that the ability of apoA-I mutants to promote ABCA1 depended lipid efflux is correlated with the ability of these mutants to be cross-linked efficiently to ABCA1 (60). Other studies showed that some other synthetic peptides of L or D conformations can promote ABCA1-mediated cholesterol efflux in vitro (57). These findings do not necessarily imply that following efflux in vivo these peptides can proceed to form HDL type particles (39;60-62).

In vitro studies also showed that in some cases apoA-I and ABCA1 bound efficiently but this interaction did not lead to the synthesis of HDL particles. In this case the binding was characterized a "non-productive" binding (62). ABCA1 W590S mutant that was found in a Tangier patient was studied. This mutant can even cross-link stronger to apoA-I than the WT ABCA1, but the Tangier patients that carry that carry this mutation do not form HDL (43;63;64). When the cross-linking properties of several apoA-I mutants to cells that expressed either the WT ABCA1 or the W590S were compared significant differences in binding were found. This might happen because this mutation may have altered the environment of the binding site of ABCA1 in such a way that it prevents efficient

lipid efflux (60). Based on this finding the authors suggested that efficient apoA-I/ABCA1 interaction is not enough by itself for sufficient lipid efflux.

A series of experiments were performed to see how apoA-I interacts with ABCA1 in vivo in order to form HDL. In these experiments, mice were infected with 1 to $2x10^9$ plaque forming units (pfu) of an adenovirus expressing WT apoA-I or the apoA-I mutants that were previously studied in the in vitro studies. Four to five days after the gene transfer the plasma of the apoA-$I^{-/-}$ mice that expressed the WT or the mutant apoA-I was drown and plasma lipid analyses, fast protein liquid chromatography (FPLC) and two dimensional gel electrophoretic analyses were performed (57;58). Also the hepatic mRNA levels of apoA-I were determined to make sure that there was comparable expression of the WT and the mutant apoA-I forms. These experiments showed that the WT apoA-I and the amino terminal mutation created HDL particles in vivo. The formation of HDL was assessed by FPLC fractionation to detect the size of the HDL peak as well as by electron microscopy. The two-dimensional gel electrophoresis showed that most of WT apoA-I had particles with α electrophoretic mobility and a small fraction had β electrophoretic mobility (30;49;57;58). In the double deletion mutant it was shown that discoidal particles were formed. The mutants with the carboxy terminal deletion generated very little HDL, which all of it was preβ1-HDL particles (49). Some spherical particles were observed in the carboxy terminal apoA-I deletion mutants but these particles were enriched in apoE.

These experiments showed that the first step of biogenesis of HDL can be blocked by carboxy terminal deletions in apoA-I gene (49;57). The preβ-migrating particles that were found in the carboxy terminal deletions mutants can be also found in the plasma of ABCA1$^{-/-}$ mice and in the plasma of Tangier disease patients (50;60;62). This shows that these particles are created by mechanisms that do not involve the ABCA1/apoA-I interaction.

Based on this data a two step model of lipid efflux was suggested. In the first step the formation of a tight complex between ABCA1 and apoA-I takes place (60;62). This step seems to be necessary but not sufficient for lipid efflux (62;63). In the second step the ABCA1-mediated transfer of lipids from the cell to apoA-I takes place. This step requires the formation of a productive complex between these two molecules in order to be achieved.

Several studies indicate that there are two sites of interaction of apoA-I on the cell membrane (65;66). The first site is a low capacity apoA-I binding site on the ABCA1 molecule. This interaction appears to stabilize ABCA1 on the cell membrane and protects it from proteolytic degradation (67-69). The second site is a high capacity apoA-I binding site and may be created by the phospholipid translocase activity of ABCA1 (66). It has been proposed that following the initial transient interaction of apoA-I with ABCA1, apoA-I is inserted in the high

capacity binding site, and thus extract PL and FC. The lipidated apoA-I can then be released in the form of nascent HDL particles (62;70).

It has been reported that intestinal ABCA1 mediated production of HDL accounts for the 30% of HDL in mice (71). When the liver and intestinal ABCA1 genes were deleted there was no production of HDL indicating that the liver and the intestine are the only sites that contribute to the production of HDL cholesterol. HDL produced in the intestine is secreted directly into the plasma, whereas the HDL found in the lymph originates from pre-existing plasma HDL (71). Thus in mice that do not express intestinal ABCA1 there was no transport of luminal cholesterol to the plasma, whereas there was no impairment in the cholesterol concentration of lymph. In contrast in mice that do not express hepatic ABCA1 the HDL concentration in the lymph was nearly abolished (71).

In recent studies it has been shown that the liver interactions of ABCA1/apoA-I are essential for the initial lipidation of apoA-I and also determine the subsequent maturation of nascent preβ-HDL to spherical αHDL particles (53;72). When ABCA1 is inactivated in the liver, preβ HDL fails to mature and is catabolized rapidly by the kidney (53;72). These data indicate that the ABCA1/apoA-I or ABCA1/preβ HDL interactions in the peripheral tissues seem to enrich the initially lipidated particle with cholesterol and increase its stability (73).

In another study it was shown that vascular endothelial cells transcytose apoA-I. This action is mediated via interaction of apoA-I with ABCA1 and does not involve SR-BI (74). It was proposed that lipid efflux in macrophages involves internalization of apoA-I, interaction with intracellular lipid pools, and resecretion in a minimal lipidated form (54). In various studies it has been shown that macrophages can bind HDL and apoA-I in a specific manner (38;41;75-77). In a recent study its was even more shown that HDL can compete for apoA-I binding in macrophages whereas apoA-I can not compete for HDL binding indicating that apoA-I and HDL have a minimum of two different binding sites on macrophages (78). The same study has shown that lipid free apoA-I can enter the macrophages in a saturable specific way. This process can have a relation to cholesterol efflux. The findings of this study are consistent with the prevailing concept that lipid-free apoA-I binds to ABCA1 and HDL binds to SR-BI. The existance of two distinct binding sites that are regulated independently makes sure that there is enough removal of the excess cholesterol from the macrophages (78).

It was found that binding of lipid free apoA-I to ABCA1 in human fibroblasts initiates signaling events that include the activation of Cdc42 and subsequently the phosphorylation of PAK-1 and $p54^{JNK}$ that leads to the polymerization of actin (79). Confocal microscopy showed that ABCA1 colocalizes with Cdc42 intracellularly (80). Furthermore ABCA1 coimmunoprecipitates with

Cdc42 in cells expressing the WT but not the mutant ABCA1 forms. It is unclear weather the signaling events are initiated through the ABCA1 and Cdc42 interaction. However, mutations in ABCA1 or inhibition of ABCA1 with glyburide, abrogate this signaling pathway (79). When apoA-I binds to ABCA1 the interaction of ABCA1 with Cdc42 is amplified (79).

It is documented by two studies that the expression of ABCA1 in macrophages in ABCA1 deficient mice is atheroprotective (81;82). In another study it was shown that overexpression of ABCA1 in liver in LDLr$^{-/-}$ mice is proatherogenic (83). The authors suggested that overexpression of ABCA1 in the liver leads to increased loading of HDL with liver cholesterol which is then transferred to the apoB containing lipoproteins. The increase in cholesterol loaded apoB containing lipoproteins is accompanied by decreased clearance as well as increase in hepatic cholesterol levels. As a result various mechanisms are initiated in order to reduce the hepatic cholesterol. These include increase in biliary cholesterol and bile acid secretion and increase in the intestinal cholesterol and this change contributes to intestinal sterol absorption and the accumulation of plasma cholesterol (83). Another study showed that intestinal ABCA1 can mediate cholesterol efflux from enterocytes directly into plasma HDL fractions as well as to contribute to the plasma pool of chylomicrons (71). The authors indicated that liver ABCA1 can modulate both HDL and non-HDL cholesterol levels.

Interactions of Lipid-Bound ApoA-I with LCAT

Plasma LCAT is a 416 amino acid long protein that is synthesized and secreted by the liver. LCAT reacts with discoidal and spherical HDL by transferring the 2-acyl group of lecithin or phosphatidylethanolamine to the free hydroxyl residue of cholesterol to form cholesteryl esters. Following esterification, cholesterol esters become part of the HDL particle (84).

ApoA-I in nascent discoidal HDL is the most potent apolipoprotein activator of plasma enzyme LCAT. Esterification of free cholesterol of HDL in vivo corrects the discoidal to mature spherical HDL (85;86).

Studies on apoA-I mutants, synthetic peptides, and monoclonal antibodies that will be discussed later have identified that residues 143–186 of apoA-I may interact with LCAT. Most of these residues are in helix D (residues 146–187 of apoA). Residues Val-156, Leu-159, and Pro-165 of this helix form a part of the hydrophobic core of the lipid-free apoA-I and would be unavailable for interaction with LCAT in this conformation. Upon lipid binding a change in conformation would expose these residues for interaction with LCAT (22).

The in vivo interactions of apoA-I with LCAT were studied by adenovirus-mediated gene transfer in apoA-I deficient mice. These studies investigated the ability of the naturally occurring mutants apoA-I(Leu141Arg)$_{Pisa}$, apoA-

I(Leu159Arg)$_{FIN}$, apoA-I(Arg151Cys)$_{Paris}$, apoA-I(arg160Leu)$_{Oslo}$ as well as the apoA-I(Arg149Ala) mutant that was generated by in vitro mutagenesis. The first two mutants are associated with very low HDL and apoA-I levels in humans (87-90) and premature atherosclerosis (87;91) and the second two are associated with low HDL but milder phenotype in humans (92-94). In vitro studies showed that all five mutants had reduced capacity to activate LCAT (95;96). The gene transfer studies showed that all the mutants generated aberrant HDL phenotypes (95;96). The mutants apoA-I(Leu141Arg)$_{Pisa}$, apoA-I(Leu159Arg)$_{FIN}$ produced only small amounts of HDL that formed mostly preβ1 HDL particles. The apoA-I(Arg151Cys)$_{Paris}$, apoA-I(arg160Leu)$_{Oslo}$ formed discoidal HDL particles. These studies indicate that apoA-I(Leu141Arg)$_{Pisa}$ and apoA-I(Leu159Arg)$_{FIN}$ mutation inhibit an early step in the biogenesis of HDL due to insufficient esterification of the cholesterol of the preβ1-HDL particles by the endogenous LCAT. The LCAT insufficiency appears to result for depletion of the plasma LCAT mass (96). It was suggested that the mutations in apoA-I promote rapid catabolism of the newly lipidated apoA-I as well as the LCAT that is associated with these particles. The resulting depletion of LCAT prevents the formation of either discoidal or spherical HDL particles (96). The mutants apoA-I(Arg151Cys)$_{Paris}$, apoA-I(arg160Leu)$_{Oslo}$ and apoA-I(Arg149Ala) formed discoidal particles and this also suggests insufficiency of the endogenous LCAT, which resulted in slow conversion of discoidal HDL to spherical HDL (95). A remarkable finding was that all the aberrant phenotypes were corrected with treatment with LCAT. This

indicates that LCAT could be a potential therapeutic intervention to correct low-HDL in humans that are caused by these and other unidentified mutations.

Mutations in ApoA-I that Cause Dyslipidemia

Three mutations of apoA-I have been described which affect the overall cholesterol and triglyceride levels. Two mutants, apoA-I[Δ(62-78)] and apoA-I [Glu110Ala/Glu111Ala], caused combined hyperlipidemia, which had high plasma cholesterol and severe hypertriglyceridemia (59;97). All the triglycerides and the majority of the excess cholesterol were distributed in the apoA-I-enriched VLDL/IDL-sized lipoproteins. The findings indicate that the apoA-I mutants had increased affinity for lower density lipoprotein fractions. Other observations were that the VLDL/IDL Also this VLDL/IDL fractions of mice expressing these mutant had decreased levels of apoE and apoCII and increased level of apoB-48. The combined findings suggest that these mutations might have inhibited lipolysis in vivo. When the mice were infected with two adenoviruses the one expressing the mutant apoA-I and the other expressing human lipoprotein lipase, VLDL triglycerides were reduced (97). These studies showed for the first time that some forms of human hypertriglyceridemia or combined dyslipidemia may be due to mutations in apoA-I. Similar studies with another mutation, apoA-I[Δ(89-99)], showed that this apoA-I mutant induced hypercholesterolemia that was characterized by increased cholesterol and phospholipids in the VLDL/IDL/LDL size lipoproteins. There was also substantial decrease of the

CE/TC ratio in HDL and LDL whereas the triglycerides remained normal. This apoA-I deletion also increased apoA-I in the LDL-sized particles. Electron microscopy analysis showed accumulation of discoidal HDL and two dimension gel electrophoresis of plasma showed increased level of preβ1 relative to the αHDL subpopulation (97).

It is interesting to note that mice that expressed the apoA-I[Δ(89-99)] mutant had PLTP activity that was only 32% of that of mice that expressed the WT apoA-I. The function of PLTP is to link the donor and acceptor lipoprotein particles in order to facilitate the net transfer of phospholipids to HDL (98). It has been shown that PLTP interacts physically with apoA-I and that these interactions may facilitate the transfer of the phospholipids from the donor molecule to HDL (99). Other studies have shown that PLTP remodels the HDL and promotes the generation of preβ-HDL particles (100-102). PLTP deficient mice fed a high fat diet had increased concentration of phospholipids and cholesterol in the VLDL and LDL and promoted the formation of discoidal particles (103). The phenotype of these mice was similar type to that of mice that expressed apoA-I[Δ(89-99)].

For the biogenesis and catabolism of HDL, five steps have been defined where this pathway can be disrupted and lead to dyslipidemia:

1) lack of synthesis of HDL due to mutations in ABCA1 or mutations in apoA-I that affect the ABCA1/apoA-I interaction, 2) failure to synthesize discoidal or spherical HDL. This defect most likely results from fast catabolism of apoA-I following its lipidation by ABCA1, 3) induction of hypertriglyceridemia this defect has been observed for apoA-I[Δ(62-78)] and apoA-I[Glu110Ala/Glu111Ala] mutants, 4) accumulation of discoidal HDL associated with inhibition of PLTP and induction of hypercholesterolemia. This condition has been observed in the case of the apoA-I[Δ(89-99)] mutant, 5) accumulation of discoidal HDL. this phenotype has been generated by the mutations in the 149-160 region of apoA-I or other mutations that inhibit LCAT activation.

Interactions of Lipid-Bound ApoA-I with SR-BI

SR-BI is an 82 kDa membrane glycoprotein. The protein has a large extracellular domain and two transmembrane domains and two cytoplasmic amino and carboxy terminal domains (104). SR-BI is primarily expressed in the liver and steroidogenic tissues but is also found in other tissues (105). SR-BI binds a variety of ligands including HDL, LDL and VLDL (32;104-107). However the most important property of SR-BI is considered its ability to act as the HDL receptor. There is evidence that the interaction of SR-BI with apoA-I and apoE is important for the maturation of HDL and the generation of HDL particles (32;108-112). SR-BI also affects the cholesterol content of the adrenal gland and the bile and protects mice from atherosclerosis (110;113-118).

It has been shown that SR-BI binds to HDL and discoidal reconstituted HDL, through its apoprotein moiety (apoA-I or apoE) (32;112;119;120). When it is bound to lipoproteins, SR-BI mediates both selective cholesteryl ester (105;121-123) as well uptake from HDL (105;124) to cells (105;125;126). It also promotes bidirectional movement of unesterified cholesterol (127;128) and also cellular uptake of triglycerides, phospholipids and vitamin E (105;121-123). SR-BI mutants which display altered biological functions were generated by in vitro mutagenesis. A Met158Arg mutant does not bind HDL (128). A Gln402Arg/Gln418Arg mutant also does not bind HDL but in contrast with the first mutant it binds LDL (125;128). A Gly420His mutant has normal selective cholesteryl ester uptake but reduced cholesterol efflux to HDL and reduced hydrolysis of internalized cholesteryl esters (129). Studies were performed using rHDL containing mutated apoA-I and these SR-BI mutants. These studies showed that the greater reduction of cholesterol efflux was with mutants Asp102Ala/Asp103Ala and Arg160Val/His162Ala (21% and 49% of cholesterol efflux using cell cultures that express the WT SR-BI) (130). When the mutant SR-BI were examined all the apoA-I mutants had reduced efflux and bound less tightly compared to WT apoA-I with the exception of rHDL that contained the mutant apoA-I(Arg160Val/His162Ala). This mutant bound almost as tightly to the cells that expressed [Met158Arg] SR-BI mutant as it bound to the cells that expressed WT SR-BI (130). Based on these data the authors suggested that

efficient SR-BI mediated cholesterol efflux requires not only direct binding (128) but also the formation of a productive complex between SR-BI and the rHDL particle (130).

Remodeling of HDL by the action of ABCG1

Recently it was suggested that HDL can be remodeled following interactions with ABCG1 a 67 kDa protein which is a member of ABC family of half transporters. ABCG1 is expressed in the spleen, the thymus, the lung and the brain (131-133) and is localized on plasma membrane the Golgi and recycling endosomes. The expression of ABCG1 is induced by LXR agonists or by cholesterol loading in macrophages and in the liver (134-136). Overexpression of ABCG1 promotes cholesterol efflux from cells to HDL but not to lipid free apoA-I (33;133;136;137). ABCG1 mediated cholesterol efflux to HDL is abolished by mutations in the ATP binding motif (137). It was suggested that cholesterol efflux does not require direct binding of HDL to ABCG1 (137). In macrophages it has been suggested that ABCG1 plays an important role for cholesterol export from cells (136). It has also been shown that HDL_2 can be formed by the action of lipid free apoA-I, ABCA1 and ABCG1 (33). First ABCA1 forms the nascent or preβ-HDL and then ABCG1 mediates the export of lipids to the nascent particles to form HDL (138). It is still not known how HDL interacts with ABCG1 to promote us the efflux (139).

HDL Subpopulations

Various subpopulations of HDL have been described based on different fractionation procedures (140-142). The HDL fractions can be separated by two-dimensional electrophoresis which involves by agarose gel electrophoresis and non-denaturing polyacrylamide gradient gel electrophoresis (86;143-145). This separation reveals the presence of preβ-HDL and the αHDL subpopulations. Preβ HDL is approximately 5% of plasma HDL. It is heterogeneous and its size is 5-6 nm in diameter (146;147). The subpopulation of preβ1 is increased in large lymph vessels (148;149) and in aortic intima (150;151). The cholesterol of the preβ particles can be esterified by LCAT (152).

The origin of preβ particles is still unclear. Also the functions of preβ particles and their relation to the αHDL subpopulations is not known. From what is already published we know that there are two pathways that lead to the formation of preβ-HDL particles. The first is de novo synthesis (153-155) and the second is formation from lipid poor αHDL particles (156-158).

Naturally occurring ApoA-I mutations

Several apoA-I mutations have been described in the general population. From a total of 46 natural apoA-I mutations that have been reported, 25 are associated with low plasma HDL levels (159). Most of the mutations that affect the interaction of apoA-I with LCAT occur around helix 6. Eight more mutations,

seven between residues 26 and 107 and one on residue 173, have been associated with amyloidosis and low HDL levels (159;160).

Leucine 144 to arginine mutation

A missense mutation has been identified in a kindred in Spain. This mutation is in exon 4 of the apoA-I gene and is responsible for the substitution of leucine 144 with arginine. Heterozygotes carrying this mutation have low levels of HDL cholesterol (161). Humans carrying this mutation was showed that have reduction of apoA-I, apoA-II and HDL-C. It was also shown that the HDL-C of these subjects has different composition compared to HDL-C provided by control subjects. The study showed that there was an increase of the triglycerides and a decrease in the cholesterol esters to free cholesterol the latter indicating LCAT insufficiency (161). The study also showed that the subjects that had the mutation had increased fractional catabolic rate of apoA-I and apoA-II and the apoA-I secretion rate was also increased whereas the apoA-II secretion rate was normal (161).

A substitution, L159R, was found in a kindred in Finland (90;91;162). Heterozygous carriers of this mutation had lower HDL cholesterol and apoA-I plasma concentrations (20% and 25% respectively compared to unaffected family members) and the mutation was characterized as dominant negative (159). A recent study has shown a method using mass spectrometry that can help identify the ratio of apoA-I_{WT}/apoA-I(L159R) in plasma. With this method it may become

possible to study further the mechanism that underlies the dominant negative effect of apoA-I(L159R) on apoA-I wild-type plasma concentrations (163).

Recently a novel mutation that introduces a protein termination at the codon for amino acid 136 has been identified in French Canadians and is associated with low HDL cholesterol levels (< 5th percentile for age and gender matched controls) and is associated with CAD. This mutation is dominant negative (164).

In another study it was shown that the serum obtained from humans carrying one allele of apoA-I_{Milano} had the ability to efflux cholesterol from J774 macrophages and human fibroblasts through ABCA1 more efficiently compared to control subjects (165). It was shown that the serum contained normal amounts of small apoA-I-containing preβ-HDL particles as well as a small apoA-I_{Milano}/A-I_{Milano}-containing HDL particles that migrate between the preβ- and α-regions. When reconstituted particles were analyzed in separate experiments they had the same efficiency in promoting cholesterol efflux that authors suggested that the simultaneous presence of the two particles gives them the ability to promote efflux more effectively (166).

It was also shown that rHDL containing WT apoA-I or apoA-I Milano have the same capacity to promote cholesterol efflux in fibroblasts (167) and

macrophages (168).Also when adjusted for HDL levels the serum of mice expressing apoA-I_{MILANO} or WT apoA-I had similar ability to promote cholesterol efflux from macrophages and fibroblasts (168). It was also shown that there was no significant difference between the phospholipid or free cholesterol efflux that is mediated through apoA-I_{MILANO} and apoA-I_{WT} (168).

Physiological functions of ApoA-I and HDL that may be relevant to its atheroprotective properties

Role of apoA-I in atheroprotection

When apoA-I is over expressed in transgenic animals (mouse, rabbit) the animals were protected from atherosclerosis (169;170). Recent studies suggest that even in humans apoA-I should be measured as a component of the assessment of cardiovascular risk in humans (171)). In a clinical trial it was shown that intravenous administration of 15 mg/kg of apoA-I Milano/phospholipid complexes in five weekly doses in patients with acute coronary syndrome resulted in significant regression as it was shown by intravascular ultrasound (172).

High levels of HDL cholesterol are not always correlated with atheroprotection. There are human subjects with high HDL and coronary artery disease. It is suggested that the HDL in these subjects has pro-atherogenic properties (173). Also SR-BI-deficient mice have increased levels of HDL but

again these high levels promoted atherosclerosis (115;118). These findings suggest that interactions of HDL with SR-BI and other proteins of the HDL pathway are important for the physiological functions of HDL and for atheroprotection.

Effect of HDL on eNOS protein levels

It has been shown that HDL increases 3-fold eNOS protein in cultured endothelial cells by increasing its half life but does not affect steady state eNOS mRNA levels (53) (Fig. 1 mechanism 4).

Addition of HDL to endothelial cell cultures that contain OxLDL prevents eNOS displacement from caveolae and also prevents the depletion of cholesterol of the caveolae (174). Expression of SR-BI in endothelial cells reverses the impact of OxLDL on the localization and function of eNOS. It has also been shown that apoA-I mimetics prevent LDL from uncoupling eNOS activity to favour $O_2^{\cdot -}$ anion production as opposed to normal production of NO (174-177).

Experiments in Chinese hamster ovary (CHO) cells showed that SR-BI is involved in the activation of eNOS by HDL. HDL also promotes NO-dependent relaxation of aortic rings and this effect is lost in aortic rings of SR-BI null mice. It has been proposed that the HDL-induced eNOS activation is associated with caveolae (178).

Akt kinase activates eNOS through phosphorylation of the enzyme at Ser1179. Akt is activated by phosphorylation by the phosphoinositide 3-kinase (PI3-kinase). Several receptor and non receptor tyrosine kinases (TK) are involved in PI3-kinase/Akt-mediated eNOS activation in response to agonists. On the other hand, phosphorylation of Thr497 of eNOS attenuates its activity. The activity of eNOS is regulated positively or negatively by MAP kinases and by calmodulin binding and by the intracellular Ca^{2+} concentration (179). Existing data indicate that HDL upregulates eNOS activity through activation via phosphorylation of Src which leads to phosphorylation and activation of Akt and MAPK (174;180). Several studies (but not all) have also indicated that HDL increases the intracellular calcium stores in endothelial cells (181;182) and that calcium is required for NO production in response to HDL (181). Calcium release is mediated both by HDL and LDL and is inhibited by pertoussis toxin. Other studies in CHO cells that express SR-BI have shown that in addition to HDL intracellular ceramide levels activate eNOS.

Role of the lipid moieties of HDL

Lysophospholipids that are components of HDL such as sphigosylphosphorylcholine, spigosine-1-phospate (S1P) and lysosulphatide cause eNOS dependent relaxation of mouse aortic rings (174). However in vivo HDL stimulates myocardial perfusion to similar levels in WT and $S1P_3$ deficient mice (183), and thus questions the in vivo effect of S1P in endothelial relaxation.

The role of HDL associated estradiol to stimulate eNOS activity is unclear (181;184;185).

Effect of HDL on eNOS activity in humans

Earlier studies have shown that increased HDL levels are associated with greater vasodilator effects in humans (186-188). Niacin treatment that increased HDL by 25% also increases vasodilation of patients with coronary artery disease (189). Other studies have shown that blood flow responses to endothelium-dependent vasodilators were decreased in patients heterozygous for ABCA1 deficiency and these responses were improved by infusion of apoA-I/phosphatidylcholine disks (190). Similarly administration of apoA-I/phosphatidylcholine improved the endothelial functions of hypercholesterolemic men (191).

Role of HDL in endothelial cell apoptosis

Exposure of endothelial cells to inflammatory stimuli may disturb the endothelial monolayer integrity (192). Furthermore thrombogenic membrane particles released from apoptotic endothelial cells may lead to coronary events (193;194). Numerous proatherogenic factors that promote endothelial apoptosis have been described and include OxLDL(195;196), TNF-a (196;197), homocysteine (198), and angiotensin II (198;199). The effects of OxLDL increase intracellular calcium and result in apoptosis that can be resolved by HDL

(174;200). HDL also reverses the TNF-a induced and growth deprivation induced endothelial cell apoptosis (201;202). It has been proposed that the protective effect of HDL is mediated by S1P and its receptors which activate the PI3 and Erk kinase pathways (203).

It has been shown that SR-BI via a putative redox motive CXXS between residues 323-326 can promote a ligand depended apoptosis and this effect can be reversed by HDL and eNOS (204).

Role of HDL in endothelial cell proliferation and migration

Damage of the endothelium is associated with vascular disease (205-207) which can be blunted by reendothelialization (208;209). HDL promotes endothelial cell proliferation which is calcium depended (210). HDL also promotes endothelial cell migration (211) which could be prevented by pertussis toxin and implicating the involvement of G-protein coupled S1P receptors and activation of PI3 kinase p38MAP kinase and Rho kinases (203). Another function of HDL it is capacity to promote capillary tube formation in vitro and this function is pertussis toxin sensitive and requires p44/42MAP kinase which is downstream of Ras (212).

Recent studies indicate that interaction of SR-BI with lipid-bound HDL or rHDL stimulates endothelial cell migration in a NO independent fashion that

activates Src kinases and Rac. In vivo studies have also shown that re-endothelialization of carotid artery following injury is promoted by apoA-I expression and is inhibited in apoA-I deficient mice (213).

Antithrombotic effects of HDL

Atherosclerosis is associated with dyslipidemia and dyslipoproteinemia (214). Patients with arterial atherothrombosis have decreased concentration of the largest size HDL subpopulations (215-218). Recent studies have also shown that elevated HDL as well as increased in the large size HDL subpopulations is associated with decreased risk of venous thrombosis (219-221) (222) . Infusion of rHDL in volunteers that received low levels of endotoxin limited the prothrombotic and procoagulant effect of endotoxin (223).

In other experiments infusion of apoA-I Milano in a rat model of acute arterial thrombosis increased the time of thrombus formation and decreased the weigh of the thrombus (224). The antithrombotic effects of HDL have been attributed partially to the improvement of the endothelial functions and the restoration of normal blood flow (225-228).

It has been reported that HDL causes increased synthesis of prostacyclin in cultured endothelial cells (229) (230). Prostacyclin in combination with the NO promote smooth muscle cells relaxation inhibition of platelet activation and

local cell proliferation (231). It has been shown that HDL-3 induces expression of Cox-2 in endothelial cells and promotes release of prostacyclin via a signaling pathway that involves p38MAP kinase (232;233) (234). Infusion of isolated rabbit heart cells also results in release of prostacyclin. The HDL mediated release of prostacyclin may contribute to the antithrombotic properties of HDL.

Role of HDL in the initiation of the thrombus

It has been shown that the phospholipid components of HDL downregulate the expression of E-selectin on the surface of endothelial cells (228;235). In addition reconstituted HDL may directly or indirectly inhibit the expression of tissue factor by endothelial cells (235-238). HDL also may prevent apoptosis of endothelial cell which become source of microparticles that contribute to coagulation (239-241).

HDL, activated protein C, and coagulation factors

A study has displayed that there is a positive correlation between plasma apoA-I levels and anticoagulant response to activated protein C (APC)/protein S in vitro (242) and another has shown that the plasma thrombin activation markers like prothrombin fragments F1 + 2 have a reversed association with HDL levels in vivo (243). It has been shown that APC has been cable to inactivate factors Va and VIIIa in plasma and thus it can downregulate thrombin formation (244). It has also been displayed that when HDL is administered to cholesterol-

fed rabbits there is an increase in endothelial cell thrombodulin, which in turn can also promote the generation of APC that leads to inhibition of thrombin formation (245). Thrombodulin also has anti-inflammatory properties (246). Due to these properties, the increased expression of thrombodulin that is mediated by HDL may have various important implications regarding HDL action in humans.

The antithrombotic properties of HDL sphingolipids

It has been shown that glucosylceramide and glycosphigolipids which are present in HDL are lipid cofactors for the APC anticoagulant actions and in a significant number of patients with venous thrombosis the levels of glucosylceramides are low (219) (247). Shpingosine, another molecule present in HDL, has been shown to inhibit prothrombin activation on platelets surface and also prevents procoagulant interactions between factors Xa and Va (248). Also lysosphingolipids interact with G-protein coupled receptors (249;250) and these interactions lead to vasoactive and anti-apoptotic events (186) (251) (208) (252). Finally HDL via lysosphingolipids downregulates endothelial cell adhesive reactions (252-254).

HDL and fibrinolysis

It has been shown that HDL downregulates plasminogen activator inhibitor-I (PAI-I) and upregulates tissue plasminogen activator (t-PA) (255-257). Also oxidized HDL3 favours the expression of PAI-I (258).

Interactions of HDL with platelets

HDL levels regulate platelet aggregation inversely (259). It has been shown that HDL suppresses the activation of platelets directly (260). Moreover HDL modifies platelet functions indirectly through its actions on endothelial cells. HDL can downregurale the release of platelet activating factor and can also upregulate the synthesis and release of NO from endothelial cells (255). It has been displayed that HDL can limit the generation of thromboxane A2 (TxA2) and can increase the production of prostacyclin (255) (258). HDL2 can promote more effectively these antithrombotic effects compared to HDL3 that involve the balance of TxA2/Prostacyclin (250).

Molecules involved in HDL signaling

Short-term exposure of cultured endothelial cells to HDL or methyl-β-cyclodextrin caused similar eNOS activation, whereas methyl-β-cyclodextrin loaded with cholesterol did not. Also cholesterol free particles composed of apoA-I and phosphatidylocholine (Lp2A-I) stimulated eNOS activity but particles that contain cholesterol did not. Moreover, native HDL caused poorer activation of eNOS compared to Lp2A-I and when cholesterol efflux was blocked with an antibody for SR-BI again there was no activation of eNOS. In Cos-M6 cells SR-BI can mediate eNOS activation via HDL and small unilamellar vesicles. When the

cells expressed the SR-BI mutant AVI, which can not efflux to unilamellar vesicles, the signal was present only in the presence of HDL (261).

In the same study it was also shown by using SR-BII, which is a splice variant of SR-BI, mutant, and chimeric class B scavenger receptors that the C-terminal cytoplasmic PDZ-interacting domain and the transmembrane domain of SR-BI were both required for the signaling initiated by HDL (261). Also a photoactive derivative of cholesterol binds in the same region indicating that this region of SR-BI is implicated in the signaling mediated by HDL.

In a study where limb ischemia was induced in C3H/He mice, intravenous injection of rHDL twice per week increased blood flow recovery compared to mice that were treated with PBS. It was shown in the same study that rHDL can promote endothelial progenitor cell differentiation via PI3 kinase/Akt signaling. The rHDL mediated increased blood flow recovery was severely impaired in eNOS deficient mice, suggesting that eNOS promotes the formation of the new vessels after the hind-limp ischemia (262).

MATERIALS AND METHODS

MATERIALS

The restriction enzymes, the buffers and the DNA polymerases that were used for the construction of plasmids were purchased from New England Biolabs. The dideoxynucleotides (dNTPs) that were used for the polymerase chain reactions (PCR) were purchased from Promega and the primers were constructed by Invitrogen. For the cloning of the mutated forms of apoA-I we used the plasmid vector pCDNA3.1 from Invitrogen. The molecular weight marker "1 kb DNA Ladder" for DNA and the Presteained Protein Marker, Broad Range (6-175kDa) for proteins were bought from New England Biolabs. The culture medium Luria Bertani (LB) with or without agar that was used for the solid and liquid cultures of bacteria, respectively, was purchased from American Bioanalytical. The agarose (Ultrapure Agarose®) was purchased from Invitrogen, we also used various chemical compounds bought from Fisher Scientific and Sigma. For the DNA isolation in big scale from bacterial culture the High Purity Plasmid Maxiprep System (Marligen Biosciencces, Inc.) was used. For the isolation and purification of plasmid DNA fractions up to 10kb from agarose gel we used the Rapid Gel Extraction System bought from Marligen Biosciences, Inc., whereas for bigger size plasmid DNA fractions we used the QIAEX II Gel Extraction kit bought from Qiagen. The Hybond nitrocellulose membranes were bought from General Electric and the Immobilon™-P PVDF (polyvinylidene

fluoride) membranes were bought from Millipore. For the detection of proteins by Western we used the enhanced chemiluminescence (ECL) system of General Electric. The materials that were used in the cell cultures and specifically the culture media Dulbecco's Modified Eagles Medium (DMEM) and Leibovitz's L-15 Medium (L-15), the buffer dilution Phosphate Buffered Saline (PBS), the enzyme Trypsin-EDTA as well as the antibiotics Penicillin-Streptomycin were bought from Cellgro, whereas the Fetal Bovine Serum (FBS) from Biomeda. The acrylamide gels with gradient concentration 4-20% Tris-HCL, IPR COMB were bought from BIO-RAD. For the radioactive blotting of the probes we used the Rediprime II Random Prime Labelling System and the radioactive nucleotides Redivue [^{32}P]dCTP that were bought from Amersham Biosciences. The column Superose 6 PC 3.2/30 was bought from General Electric. Salmon Sperm DNA (10mg/ml) and 50X Denhart's Solution were bought from Invitrogen. For the Northern and Western analyses we used BioMax MS and X-Omat LS films (Kodak) respectively. The rest of the chemical compounds that were used were obtained from common commercial sources in the clearest possible form.

EXPERIMENTAL PROCEDURES

DNA electrophoresis on agarose gel

For the nucleic acid electrophoresis agarose gels of 0.5% to 1% concentration were used. The procedure that was used is the following: In a conical flask of 250 ml we add 120 ml of TAE 1x (50x TAE; 2M Tris-HCL pH 7.5, 2mM EDTA, acetic acid for pH equilibration) and 1 g agarose. The mixture is heated up to boiling point until the agarose is dissolved. When the temperature of the mixture goes down to around 50°C we add 7.5 μl of ethidium bromide (10 mg/ml), then we pour the mixture in an appropriate apparatus (cast) and we place the combs that will form the sample loading wells. When the gel sets we transfer it to a tank that contains 1x TAE buffer. Usually the electrophoresis is performed at 80 Volt (V) and for the isolation of DNA fragments from gel it is performed at 50 V until the samples are efficiently analyzed.

Reaction with restriction enzymes (digest)

The plasmids and the products of the PCRs that were used for cloning were incubated with restriction enzymes according to the instructions of the manufacturer (New England Biolabs). Most digestions were performed using 15 μg of nucleic acid at 37°C, for 2 hours.

Ligation reaction

The ligation reactions were performed at 16°C, for 16 hours in total volume of 20 μl. The amount of DNA that was used was approximately 200 ng. The reaction had plasmid DNA and DNA of the insert in a ratio of 1:100, T4 DNA ligase and the appropriate buffer according to the manufacturer (New England Biolabs).

Transformation of bacterial *E.coli* DH5a cells

We transfer 100 μl of DH5a bacterial competent cells and 10 μl of the ligation reaction or 20-100 ng of the plasmid that we want to amplify. Cells were incubated on ice for 30 min followed by heat-shock for 45 seconds in a 42°C water bath. The reaction then is placed on ice for 2 minutes. Subsequently, we add 0.9 ml of S.O.C. medium (Invitrogen) and we incubate in shaker at 225 rpm at 37°C for 1 hour. Then 100 μl of the transformed cells are spread on LB plates with the appropriate antibiotic. The plates are incubated at 37°C for 16-18 hours.

Mini scale preparation (miniprep) for plasmid purification

After bacteria have grown on agar plates overnight, a single colony of bacterial clone is picked and it is transferred in 5 ml of LB broth containing 250 or 500 μg of kanamycin or ampicillin, respectively. The culture is incubated overnight at 37°C in a shaking incubator. The bacteria are centrifuged at 3000 rpm for 10 min. The supernatant is removed and the pellet is resuspended in 1 ml

STE buffer [0.1 M NaCl, 0.01M Tris-HCL pH: 8.0]. The cells are transferred in to a 1.5 ml microcentrifuge tube and are centrifuged at 13000 rpm for 30 sec. The supernatant is removed and the cells are resuspended in 200 µl of Cell Suspension Buffer [50 mM Tris-HCl (pH 8.0), 10 mM EDTA, 2 mg RNase A]. Then 400 µl of Cell Lysis Solution [200 mM NaOH, 1% SDS w/v] are added and the cells are incubated on ice for 5 min. The next step is to add the Neutralization Buffer [3.1 M potassium acetate (pH5.5)] and the cells are mixed well. The lysed cells are centrifuged at 13000 rpm for 10 minutes and 500 µl of the supernatant are transferred in to a new tube. Then equal volume of chloroform is added and the contents of the tube are vortexed. The mixture is centrifuged again at 13000 rpm for 5 min and 400 µl of the supernatant are transferred in to a new tube. Then 600 µl of cold isopropanol are added, the contents are mixed well, and the DNA is centrifuged at 13000 rpm for 30 min. The supernatant is then removed and the pellet is let to dry. Finally it is resuspended in 20 µl of ddH_2O.

Large scale Preparation (Maxiprep) for plasmid purification

The "High Purity Plasmid Maxiprep System" produced by Marligen Biosciences, Inc. was used for the plasmid purification, and the directions provided by the manufacturer were followed. The bacterial cells were pelleted by centrifugation at 4000 rpm for 10 min. The bacterial pellet was resuspended in 10 ml of Cell Suspension Buffer, and lysed by the addition of Cell Lysis Solution. Precipitation of the bacterial proteins was achieved by adding Neutralization

Buffer.The mixture was centrifuged at 4000 rpm for 10 minutes and the supernatant was removed and applied on a Marligen column that was previously equilibrated with 30 ml of Equilibration Buffer [600 mM NaCl, 100 mM sodium acetate (pH 5.0), 0.15% Triton® X-100 (v/v)]. The column was washed with 60 ml of Wash Buffer [800 mM NaCl, 100 mM sodium acetate (pH 5.0)], and the DNA was eluted with 15 ml of Elution Buffer [1.25 M NaCl, 100 mM Tris-HCl (pH 8.5)]. The DNA was precipitated by adding 10.5 ml of isopropanol, mixed well and centrifuged at 9000 rpm for 30 minutes. The supernatant was discarded and the DNA was diluted in 300 µl of ddH_2O and transferred to a 1.5 ml microcentrifuge tube. It was finally precipitated with 750 µl of ethanol and 30 µl of CH_3COOH 3M (pH 5.5), mixed well and centrifuged at 13000 rpm for 30 minutes. The supernatant was discarded and the pellet was let to dry. The DNA was then resuspended in 200 µl of TE Buffer [10 mM Tris-HCl (pH 8.0), 0.1 mM EDTA].

Cell cultures

In this study we used the cell lines HTB-13 (SW1783, human astrocytes), 911 (human embryonic retinoblasts), and HEK-293 (human embryonic kidney). The cultures were performed in 25 or 175 cm^2 flasks and 6-well plates in 37^oC incubator and under condition of 5% CO_2. The stocks of the cultures are kept at -80^oC in DMEM or L15 medium with 10% FBS and 10% DMSO. The cells are placed in 37^oC water bath to thaw and then transferred in flasks with culture

medium which is replaced the next day. The medium in the flasks is replaced every 72 hours. The cells are split when the monolayer is confluent, with the use of trypsin-EDTA, to the desired concentration with the addition of culture medium (DMEM or L-15) that contains 10% FBS and 1% antibiotics (penicillin-streptomycin).

SDS-polyacrylamide gel electrophoresis (SDS-PAGE)

In every sample we add the appropriate amount of 4x SDS Loading Buffer [2.5 ml 1M Tris-HCl pH 8, 1.6 ml β-mercaptoethanol, 8 ml 20% SDS, 4 ml glycerol, 8 mg bromophenol blue]. In the experiments we used 14% polyacrylamide gels for the running gel [2.2 ml ddH_2O, 3.5 ml 30% bis-acrylamide, 1.8 ml running buffer (Tris-HCl 1.5 M, SDS 0.4%, pH 8.8), 37 μl 10% ammonium persulfate (APS), 5 μl TEMED] and 4% for the stacking gel [1.8 ml ddH_2O, 0.45 ml 30% bis-acrylamide, 0.75 ml stacking buffer (0.5M Tris-HCL, 0.4% SDS, pH 6.8, 30 μl 10% APS, 3 μl TEMED]. The electrophoreses were performed in 500 ml of 1x TGS dilution [1L 10x TGS : 30.2 g Tris-HCl, 144 g Glycine, 10 g SDS, pH 8.3], at 120 V with the use of Bio-Rad Protean electroblot.

The gels were stained and fixated with Coomasie Brilliant Blue [2.5 g Coomasie Brilliant Blue R, 50 % methanol and 10% acetic acid] for 30 minutes and then destained in destaining solution [50% methanol, 10% acetic acid] for 20 minutes or more in order to obtain a clear image of the protein bands. The gels were dried in a Bio-Rad gel dryer under vacuum at 80°C for 1 hour.

Analysis by Western blot

The proteins analyzed by SDS-PAGE are transferred to nylon PVDF membranes.. Prior transfer the membranes are incubated first in methanol for 15s, then in water for 2 min, and finally in transfer buffer [700 ml H_2O, 100 ml 10x TGS and 200 ml methanol] to equilibrate for at least 5 min.

The transfer was performed using Bio-Rad Protean electroblot apparatus in 1L transfer buffer with electrophoresis at 40 V, at 4^oC for 16 hours.

After the transfer, the membranes are washed with TBS-T [1x TBS, 0.05% Tween-20], (1L 10x TBS: 90 g NaCl, 0.5M Tris-HCl, pH 7.3) for 10 minutes at room temperature. Then the membranes are incubated with blocking buffer [1x TBS-T, 5% non-fat milk] for 1 hour at room temperature. Then the membranes are incubated for 1 hour at 37^oC with primary antibody specific for the protein we want to detect, that is diluted usually 1:1000 in blocking buffer. The membranes are washed three times for 10 minutes with TBS-T at room temperature. The secondary antibody is applied usually in 1:3000 dilution in blocking buffer. This secondary antibody recognizes the primary and it has also attached to it the enzyme horse radish peroxidase (HRP). The incubation again is for 1 hour at 37^oC. Then we wash 3 times for 10 minutes each time with TBS-T and wash for an additional 5 minutes with TBS at room temperature. The detection of the proteins is accomplished with the ECL system and by exposing the films for different time intervals (usually 30 sec to 2 min were enough).

Generation of mutations on apoA-I gene

The plasmid pCDNA3.1-apoAIg-ΔBglII was used as template for the generation of the mutations on apoA-I gene that we wanted to study in this study. For this purpose we used the QuickChange® II XL system that was bought from Stratagene. The template plasmid was generated as it is described in (95). The template DNA was incubated with the appropriate primers which are described in Table 1. The primers harbor mutations at the center of their sequence and on either side they have the sequence of the nucleotides of the wild type protein. The changes were done in order to obtain the desired amino acid changes in the final protein. The mutagenic primers were constructed according to the guidelines provided by Stratagene.

The mixture of template plasmid and primers was incubated with PfuUltra® polymerase that was provided with the QuickChange®II XL system and dNTPs in PCR program as described by the manufacturer. After 18 amplification cycles the PCR product was incubated with the restriction enzyme DpnI in order to digest the template plasmid which is methylated or semi-methylated DNA. After the incubation, the reaction that has the newly synthesized DNA which carries the apoA-I mutations was used for the transformation of XL10-Gold® (Stratagene) competent cells. Colonies resistant to ampicillin were selected. DNA was extracted and purified from these colonies, and was sequenced in order to confirm the introduction of the desired mutations. Colonies having the mutation of interest were then used to produce large

Table 1. Oligonucleotide sequence of primers used in PCR amplifications.

Name	Sequence	Location of sequence
apoA-I (L144R)F	5´-G CAA GAG AAG CTG AGC CCA C**<u>G</u>**[a]G GGC GAG GAG ATG CGC GAC CG-3´	nt 483-524[b] (sense) (aminoacids +137 to +151)[c]
apoA-I (L144R)R	5´-CG GCT GCG CAT CTC CTC GCC C**<u>C</u>**G TGG GCT CAG CTT CTC TTG C-3´	nt 524-483 (antisense) (aminoacids +151 to +137)
apoA-I (A164S)F	5´-C GCG CTG CGC ACG CAT CTG **<u>T</u>**CC CCC TAC AGC GAC GAG CTG-3´	nt 543-582 (sense) (aminoacids +157 to +170)
apoA-I (A164S)R	5´-CAG CTC GTC GCT GTA GGG GG**<u>A</u>** CAG ATG CGT GCG CAG CGC G-3´	nt 582-543 (antisense) (aminoacids +170 to +157)

[a]Mutagenized residues are marked in boldface type and are underlined. [b]Nucleotide number of the human apoA-I cDNA sequence, oligonucleotide position relative to the translation initiation ATG condon. [c]Aminoacid position (+) refers to the mature plasma apoA-I sequence.

quantities of the plasmid. The Maxi Kit manufactured by Marligen was used for this plasmid purification. The plasmids that were produced for each mutation were incubated with the restriction enzymes BglII and EcoRV and the generated DNA fragments (2.2 kb) were isolated, after analysis on agarose gel, and were ligated in the corresponding restriction sites, BglII and EcoRV, of the pAdTrack-CMV vector in order to make the recombinant adenoviruses, according to the AdEasy™ system (Sratagene).

Transformation of E.coli BJ5183-AD1 cells by electroporation

The adenovirus plasmid was generated in BJ-5183-AD1 (Strategene) bacterial cells after electroporation in the presence of the pAdTrack-CMV-X vector (where X: the mutated form of apoA-I) that was previously digested with PmeI, according to the instructions of the manufacturer. For the electropotation 40 μl of BJ-5183-AD1 cells were used for each reaction. These cells have already the pAdEasy-1 plasmid that encodes the genome of adenovirus type 5 except for transcription units E1 and E3 and they also promote the homologous recombination of plasmids. The recombination with the shuttle vector pAdTrack-CMV-X formed finally a plasmid that has the adenoviral genes and the gene that we wanted to study. The electroporation was performed under these conditions: 200 Ω, 2.5 kV, 25 μF using the Bio-Rad Gene Pulser II electroporation machine in 0.2 cm Gene Pulser Cuvettes (Bio-Rad). The cells were plated in agar plates and the correct clones were selected based on their resistance to kanamycin. DNA

was purified from the resistant clones and was analyzed on agarose gel. The clones that had the correct recombination gave a 3 or a 4.5 kb band and also had a band at 32 kb after being digested with the restriction enzyme PacI. The positive clones were amplified (DH5a transformation) and were isolated using the High Purity Plasmid Maxiprep System (Marligen Biosciences, Inc.).

Generation of recombinant adenoviruses

The recombinant vectors for each mutation of apoA-I were incubated with the restriction enzyme PacI in order to get linearized and 10 μg of this DNA were used to transfect 911 cells. For the transfection the Lipofectamin™2000 reagent (Invitrogen) was used according to the manufacture's instructions. Ten to twelve days post-transfection the viral particles that were formed caused lysis of the cells and the cell lysate was used for infection of a larger scale culture in a T_{175} flask. The infections were performed in DMEM culture medium with 2% Heat Inactivated Horse Serum (HIHS) and 1% antibiotics. Two to three days after the new infection the cells were lysed and the new lysate was used for the infection of HEK-293 cells in a larger scale. For this porpuse, HEK-293 cells were plated in T_{175} triple flasks until the monolayer was confluent. The infection with the previously mentioned lysate was done using L-15 medium with 2% HIHS and 1% antibiotics. Three days after the infection and before the cells get lysed, large amounts of recombinant viral particles were produced. The cells that carry the produced viral particles were collected with centrifugation at 1000 rpm for 10

minutes. The collected pellet was resuspended in 2 ml medium and stored at -80°C. Then the suspension was frozen and thawed (-80°C/37°C) three times so the cells would break and the viral particles would be released in the medium. Then the suspension was centrifuged at 3000 rpm for 10 min. The supernatant that contained the viral particles was subsequently centrifuged in CsCl gradient twice in order to isolate the viral particles . For the first centrifugation 2 ml of CsCl I (0.619 g/ml in TE) were transferred in a centrifuge tube, they were overlaid with 5 ml of CsCl II (0.277 g/ml in TE) and 2-3 ml of the viral particles were placed on top. They were centrifuged at 30,000 rpm for 90 minutes at 4°C. The viral particles were concentrated in a region between the two dilutions. This region was collected with the help of a syringe and was transferred in 12 ml of dilution CsCl III (0.450 g/ml in TE). It was centrifuged again at 55,000 rpm at 4°C for 16-20 hours. The viral particles were concentrated in a small ~2 mm zone. This zone was collected and was dialyzed against sucrose dilution [10 mM Tris-HCL, 2 mM $MgCl_2$, 5% Sucrose, pH; 8] in a Slide-A-Lyzer® (MWCO: 10000) (PIERCE) dialysis cassette. The viral dilution was separated in 50 μl aliquots in 1.5 ml tubes and stored at -80°C.

Plaque assay

911 cells were plated and grown to a monolayer, and then they were infected with serial dilutions of the virus. More specifically the viral particles were diluted to $5x10^4$ to $5x10^7$ times in L-15 culture medium supplemented with 2

% HIHS and 1% antibiotics, and then were used to infect 911 cells that were seeded the previous day in 6-well plates with concentration $1.5x10^6$ cells per well. The cells after 20 minutes of incubation with the virus were fixed with culture medium [2x MEM, 4% HIHS, and 25 mM $MgCl_2$] diluted with agar [1.5% agar 40 mM Hepes, pH: 7.4]. The cells were incubated at 37°C for 10 to 12 days. The plaques of lysis/infection of the cells were visual with naked eye and formed characteristic gray regions, in the case of the viruses we studied there was also expression of green fluorescence protein (GFP) and the plaques could be visualized under an optical microscope with the help of ultraviolet light. The plaques were counted for each dilution and the title of the virus was calculated.

Expression and secretion of the wild type (WT) and the mutated forms of apoA-I

To estimate the expression and secretion of the various types of apoA-I that were generated we used HTB-13 cells in 80% confluence in 6-well plates that were cultured in 2 ml of L-15 medium which had 2% HIHS and 1% antibiotics. The cells were infected with adenoviruses that express WT apoA-I and its mutant forms with multiplicity of infection (moi) 10, 15, and 20. As multiplicity of infection we define the number of viral particles per cell. Twenty four hours post infection the cells were washed with 1x PBS and were incubated in medium that did not have any serum for 2 hours. Then new medium, again without serum was added. The cells were incubated for 24 hours at 37°C and then the medium was

collected. An aliquot of the medium (100 μl) was analyzed with SDS-PAGE to estimate the expression/secretion of the apoA-I protein. The amount of protein was estimated with analyzing on the same gel a known amount of BSA protein.

Animal studies

ApoA-I$^{-/-}$ (ApoA-I^{tm1Unc}) C57BL/6J mice (3) were purchased from Jackson Laboratories (Bar Harbor, ME). The mice were maintained on a 12 h light/dark cycle and standard rodent chow diet. All procedures performed on the mice were in accordance with National Institutes of Health and institutional guidelines. Four to six apoA-I $^{-/-}$ mice 6-8 weeks of age were injected via tail vein with 2-4x10^{9} pfu of recombinant adenovirus per animal and the animals were sacrificed 4 days post-injection following a 4 h fast.

Plasma isolation from mice blood

For the collection of blood sample and the subsequent plasma isolation from the mice vein we used the Microvette CB 300 K2E (STARSTEDT) tubes. For the collection of larger volume of blood before the sacrifice of the animals we used Microtube 1.3 ml KE microcentrifuge tubes. From each mouse we collected around 750 μl of blood. Then the samples were centrifuged at 4000 rpm for 5 min and the plasma was separated. The plasma was transferred in a new microcentrifuge tube and was stored at 4°C until further analyses were performed.

Plasma lipid levels

The concentration of total cholesterol and free cholesterol of plasma drawn 4 days post-infection was determined using the cholesterol E and free cholesterol C reagent (Wako Chemicals USA, Inc.) respectively, according to the manufacturer's instructions. The concentration of cholesteryl esters was determined by subtracting the concentration of free cholesterol from the concentration of total cholesterol.

Fast protein liquid chromatography (FPLC)

For the analysis of plasma with FPLC 17 μl of plasma were used. The plasma obtained from mice infected with adenovirus-expressing WT or mutant apoA-I forms was loaded onto a Sepharose 6 PC column in a SMART microFPLC system (Amersham Biosciences) and eluted with PBS. A total of 25 fractions of 50 μl volume each were collected for further analysis. The concentration of lipids in the FPLC fractions was determined as described above.

Fractionation of plasma by density gradient ultracentrifugation

For this analysis, 300 μl of plasma obtained from adenovirus-infected mice was diluted with saline to a total volume of 0.5 ml. The mixture was adjusted to a density of 1.23 g/ml with KBr and overlaid with 1 ml of KBr solution of d = 1.21 g/ml, 2.5 ml of KBr solution of d = 1.063 g/ml, 0.5 ml of KBr solution of d = 1.019 g/ml, and 0.5 ml of normal saline. The mixture was centrifuged for

22 h in SW55 rotor at 30000 rpm. Following ultracentrifugation, 0.5 ml fractions were collected from the top for further analyses. The refractive index of the fractions was measured using a refractometer (American Optical Corp.) and it was converted to density for each sample based on a standard curve derived from solutions of known densities. The fractions were dialyzed against ammonium acetate and carbonate buffer (126 mM ammonium acetate, 2.6 mM ammonium carbonate, 0.26 mM EDTA, pH 7.4). Aliquots of the fractions were subjected to SDS-PAGE, and the protein bands were visualized by staining with Coomassie Brilliant Blue.

Electron microscopy (EM) analysis of the apoA-I containing fractions

For EM analysis, fractions 6-8 that float in the HDL (1.100 g/ml ≤ d ≤ 1.152 gr/ml) region were dialyzed against ammonium acetate and carbonate buffer. The samples were applied on carbon-coated grids, were stained with sodium phosphotungstate, were visualized in the Philips CM-120 electron microscope (Philips Electron Optics, Eindhoven, Netherlands) and photographed; these procedures were performed by Dr. Donald Gantz at the Department of Biophysics of Boston University. The photographs have been magnified 225,000 times.

Non-denaturing two-dimensional (2D) gel electrophoresis

The distribution of HDL subfractions in plasma was analyzed by 2D electrophoresis as described by Fielding and Fielding 1996 with some modifications. In the first dimension, 0.5 to 1 µl of plasma sample was separated by electrophoresis at 4°C in a 0.75% agarose gel using a 50 mM barbital buffer (pH 8.6, Sigma, St Louis, MO) until the bromophenol blue marker had migrated 5.5 cm. Agarose gel strips containing the separated lipoproteins were then transferred to a 4-20% polyacrylamide gradient gel. Separation in the second dimension was performed at 90 V for 2-3 h at 4°C using non-denaturing 1x TGS buffer [1l 10x TGS: 30.2g Tris-HCl, 144g Glycine]. The proteins were transferred to a nitrocellulose membrane and apoA-I was detected by using the goat polyclonal anti-human apoA-I antibody AB740 (Chemicon International) in a 1:1000 dilution.

RNA isolation and hybridization analysis

Total cellular RNA was isolated from the mouse liver using the Trizol® procedure (Invitrogen Corp.) as recommended by the manufacturer. More specifically approximately 5 mm^3 of hepatic tissue and 1 ml of Trizol® were used. The tissue was homogenized for 30s in Minibeadbeater homogenizer (Biospec Products). The sample was extracted with 100 µl of chlorophorm and the sample was homogenized for 10 sec. The RNA was isolated in the aqueous phase after centrifugation at 13000 rpm for 15 minutes. The aqueous phase was then

transferred in to a new microcentrifuge tube and was precipitated with equal volume of isopropanol and centrifugation at 13000 rpm for 20 min. Finally the RNA was reconstituted in DEPC (Diethylpyrocarbonate) treated water and its concentration was measured.

Fifteen micrograms of total RNA were denatured in RNA loading buffer [125 μl Deionized Formamide,40 μl Formaldehyde, 20 μl RNA running buffer and bromophenol blue], were separated on 1.0% agarose-formaldehyde gels at 20 V in RNA running buffer [20 mM MOPS, 5 mM Na-Acetate, and 1 mM EDTA], transferred to Hybond-N+ nylon membrane (Amersham Biosciences) in 10x SSC buffer [1.5 M NaCl, 0.3 M sodium citrate, pH: 7], and cross-linked to the membrane by UV irradiation (Stratalinker, Stratagene) at 0.12 J/cm^2 for 30 sec. The apoA-I probe used for hybridization contained 290 bp of exon 4 of human apoA-I and 148 bp of the intergenic sequence between the apoA-I and apoCIII genes. The mouse GAPDH probe (~1400 bp) was obtained from GAPDH digested with PstI. The probes were labelled with ^{32}P using the Multiprime DNA labeling system (Amersham Biosciences) which is based on random promotion and replication of DNA and labelling with [^{32}P]dCTP. After this step the RNA was hybridized with the labelled probes. The membranes were incubated in pre-hybridization buffer [5x Denhardt's buffer, 20% Deionized formimide, 50 mM Tris-HCl pH 7.5, 0.8M NaCl, 0.1% Sodium Phosphate, 10% Dextran Sulfate, 100 μg/ml Salmon sperm DNA, 0.5% SDS] at 55°C for 4 hours. Then new buffer was added and in this buffer the labeled probe was added too. The hybridization was

performed at 55°C for 16 hours. After hybridization the membrane was washed 2-3 times with wash buffer [2x SSC, 0.01% SDS] at room temperature until the signal from the membrane was about 5000 cpm and was around 3 times greater than the background signal. The signal was visualized on BioMax MS film for at least 2 hours at -80°C. Quantitation of the signal of the probe was performed by a phosphorimager (Molecular Dynamics) using the ImageQuant program. The apoA-I mRNA signal was normalized for the GAPDH (Glyseraldehyde-3-phosphate Dehydrogenase) mRNA signal.

RESULTS

Generation of the mutated pAdTrack-CMV-apoA-I(L144R) and pAdTrack-CMV-apoA-I(A164S) shuttle vectors

Two single site mutations were introduced in the pCDNA3.1apoA-IgWTΔBglII plasmid as described in materials and methods. The two single amino acid apoA-I mutations that I studied are leucine 144 mutated to arginine and alanine 164 mutated to serine. The QuickChange® II XL mutagenesis kit was used. The mutagenic primers were designed and generated according to the instructions of the manufacturer. The sequence of the primers is shown on table 1. The template plasmid DNA was incubated with the appropriate primers, PfuUltra® polymerase, and dNTPs in PCR and after 18 cycles the PCR product was digested with DpnI to digest the template plasmid. Cells provided by Stratagene (XL10-Gold®) were then transformed with the PCR product and 10 colonies resistant to ampicillin were selected for each mutation. DNA was extracted from these colonies and was sequenced at the Core Facility of Tuft's University. DH5a bacterial cells were transformed with the positive clones and plasmid DNA was generated in large amount. The 2.2 kb fragment that encodes the mutant apoA-I forms was digested with BglII and EcoRV, was extracted from the gel and then it was ligated to the shuttle vector pAdTrack-CMV that was previously linearized with the same restriction enzymes (Figure 1).

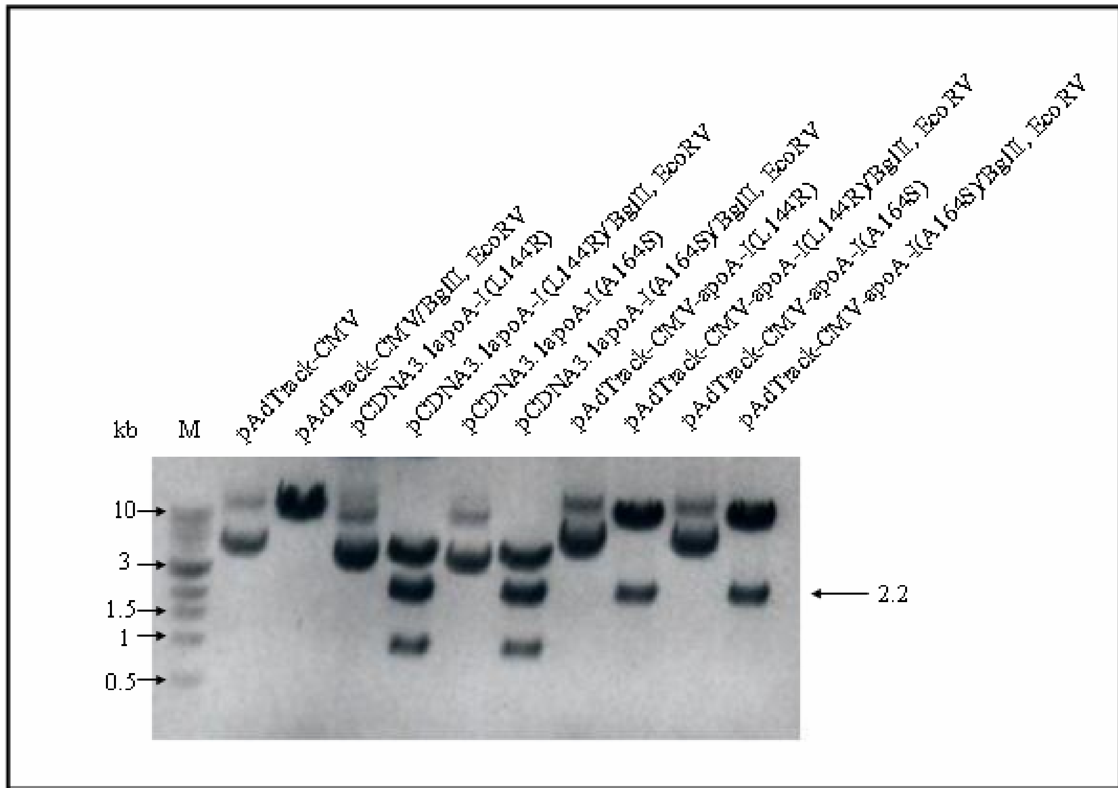

Figure 1. Agarose gel electrophoresis of Shuttle Vectors prior to or after digestion with BglII and EcoRV. The pAdTrack-CMV-apoA-I(L144R) and pAdTrack-CMV-apoA-I(A164S) vectors were generated as described in the text. The lanes contain samples that either were not treated or treated with BglII and EcoRV. The lanes that have samples that were treated with the restriction enzymes BglII and EcoRV show the presence of a 2.2 kb band that encodes for the apoA-I gene. M indicates DNA markers of different known sizes.

Generation of recombinant adenoviruses that express naturally occurring mutated forms of apoA-I

For the generation of the recombinant adenoviruses that express the two mutated forms of apoA-I the system pAdEasy® was used as described in materials and methods. The two shuttle vectors were linearized with PmeI and were used to transform electro-competent cells by electroporation. A number of colonies were selected and DNA was extracted as described in materials and methods and digested with PacI to find positive clones (Figure 2). The two positive clones (mini culture 8 for both mutants) were then transferred to DH5a cells and large amount of plasmid DNA was isolated and purified. The DNA was linearized with PacI and used to transfect 911 cells. The 911 and 293 cells are cells that can reproduce the adenovirus because they have the genes that encode for the proteins that are needed for the generation of the viral envelop. The viruses were packed in the 911 cells and then 293 cells were infected in large scale. The two recombinant viruses were then purified by two consecutive CsCl gradient ultracentrifugations. The titers of the viruses were estimated with plaque assay as described in materials and methods. The two titers were 4.4×10^8 pfu/μl for apoA-I(L144R) and 1.6×10^8 pfu/μl for apoA-I(A164S).

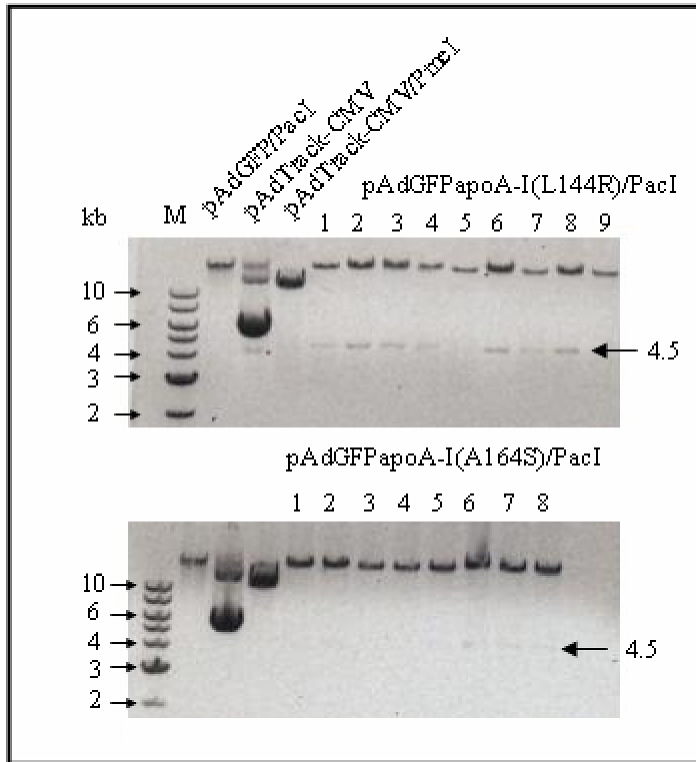

Figure 2. Agarose gel electrophoresis of DNA obtained from BJ-5183-AD1 cells following transformation by eloctroporation with the plasmids pAdTrack-CMV-apoA-I(L144R) and pAdTrack-CMV-apoA-I(A164S). The DNA obtained from the BJ-5183-AD1 cells after electroporation was digested with PacI to identify positive clones. The clones that have the recombination event produce a 3 or 4.5 kbase band (indicated by arrows).

Expressing the WT apoA-I and the two mutants apoA-I(L144R) and apoA-I(A164S) following infection of HTB-13 cells with the recombinant adenoviruses

To evaluate the level of expression of the two mutated apoA-I proteins that are expressed from the recombinant adenoviruses we used HTB-13 cells. The procedure is described in materials and methods. Briefly the cells were infected with the adenoviruses that have the genes for the WT apoA-I and the mutated forms apoA-I(L144R) and apoA-I(A164S). The infection was performed with three multiplicities of infection (moi) (10, 15, and 20). The next day the infected cells were washed and new serum free medium was added. The medium was harvested 24 hours after and an aliquot of 100 μl was analyzed with SDS-PAGE. The analysis showed that in all cases apoA-I was secreted efficiently into the culture medium at similar levels (Figure 3). From comparison with the BSA standard it is estimated that the cells, when infected with moi 20, secrete 3.6, 4.2 and, 6.5 μg/ml/24h of WT apoA-I, apoA-I(L144R), and apoA-I(A164S) respectively (for the estimation the ImageJ 1.38x software was used).

mRNA analysis

For the *in vivo* experiments I used apoA-I-deficient mice (apoA-I$^{-/-}$) (Williamson et al. 1992). These mice were injected with the recombinant adenoviruses in order to express the various forms of apoA-I in the liver. The infection dose was 2 x10^9 viral particles except in one case that the mouse was

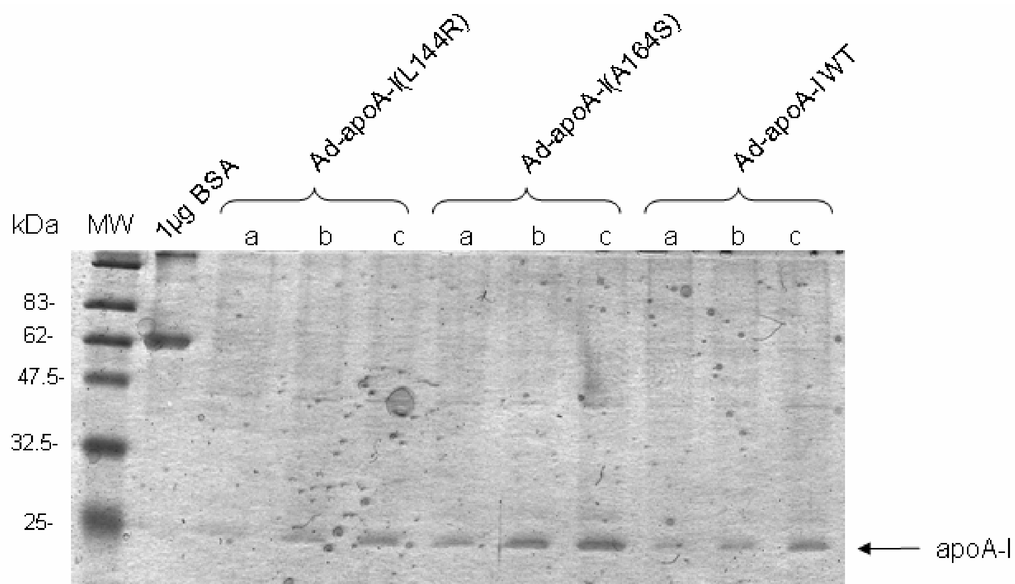

Figure 3. Secretion of apoA-I(L144R), apoA-I(A164S) and WT apoA-I following infection of HTB-13 cells with recombinant adenoviruses expressing these apoA-I forms. The panel shows SDS-PAGE analysis of 100 μl of culture medium of HTB-13 cells infected with control adenovirus expressing WT and the indicated mutant apoA-I forms, a, b and c indicate multiplicity of infection (moi) of HTB-13 cells of 10, 15 and 20 respectively.

injected the double amount of viral particles to determine if higher expression of the protein would give a different phenotype. The hepatic expression of the protein was determined by Northern analysis (Figure 4). Briefly total cellular RNA was isolated from mouse liver using the Trizol® procedure. Fifteen micrograms of total RNA from each mouse were denatured and analyzed on 1%agarose-formaldehyde gel. The RNA was transferred to Hybond-N+ nylon membrane and the apoA-I and mGAPDH mRNAs were detected with probes labelled with ^{32}P. The apoA-I probe contained 290 bp of exon 4 of human apoA-I and 148 bp of the intergenic sequence between the apoA-I and apoCII genes. The mouse GAPDH probe (~1400 bp) was obtained for GAPDH digested with PstI. All the mice had comparable hepatic apoA-I mRNA levels (Table 1) as determined by quantification by a phosphorimager (Molecular Dynamics) using the ImageQuant program.

Plasma lipid levels of apoAI$^{-/-}$ mice that were infected with recombinant adenoviruses that express the WT or the mutated forms of apoA-I

The analysis of the lipid levels in mice plasma four days post infection showed that administration of the adenovirus expressing ApoA-I(L144R) resulted in low total cholesterol levels and decreased cholesterol esters to total cholesterol ratio (CE/TC) as compared to mice that received the adenovirus expressing the wild type apoA-I. The difference in total cholesterol and CE/TC ratio between WT

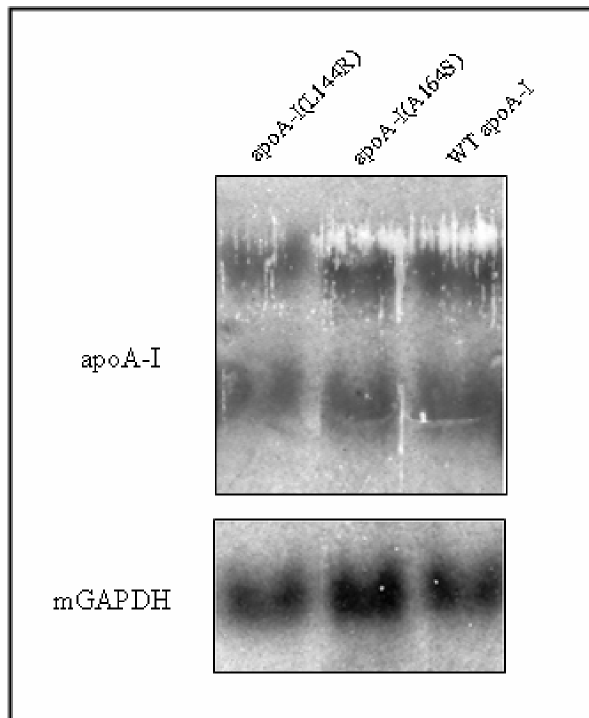

Figure 4. Representative northern blot analysis of hepatic mRNA. Hepatic mRNA was obtained four days post-infection from apoA-I deficient mice that were infected with adenoviruses expressing apoA-I(L144R) apoA-I(A164S) and WT apoA-I. The apoA-I signal was normalized for the mGAPDH mRNA signal.

and apoA-I(L144R) persisted even when the level of expression of the mutant (as determined by the steady state hepatic apoA-I mRNA levels) were increased (Table 2). These results indicate a dysfunction in the esterification of plasma cholesterol of mice that express the apoA-I(L144R) mutant protein. The plasma from mice that expressed apoA-I(A164S) had total cholesterol levels similar to the total cholesterol levels of plasma obtained from mice that expressed WT apoA-I (Table2). Also the CE/TC ratio of plasma obtained from mice expressing apoA-I(A164S) was similar to the CE/TC of plasma obtained from mice expressing WT apoA-I.

Plasma lipid profile after FPLC fractionation

A sample of mice plasma was analyzed by fast protein liquid chromatography (FPLC) as described in materials and methods. The FPLC fractionated the plasma particles according to their size in VLDL, LDL, and HDL. The HDL cholesterol fraction of mice expressing the apoA-I(L144R) was very small and resembled the HDL cholesterol fraction mice expressing the control protein (GFP). The HDL fraction of mice expressing the apoA-I(A164S) was similar to the fraction of mice expressing WT apoA-I (Figure 5).

Distribution of the various apoA-I forms after fractionation of plasma by density gradient ultracentrifugation

The distribution of the various apoA-I to the VLDL, LDL, and HDL

Table 2. Hepatic apoA-I mRNA, total cholesterol and CE/TC ratio of mice expressing WT and mutant apoA-I forms.

apoA-I	WT	(L144R)	(L144R)	(L144R)+LCAT	(A164S)
mRNA%	100	94±23	195	58±4	61±15
Total cholesterol (mg/dl)	157	29±6	41	208±11	144±17
CE/TC	0.7	0.41±0.18	0.35	0.75±0.01	0.68±0.05

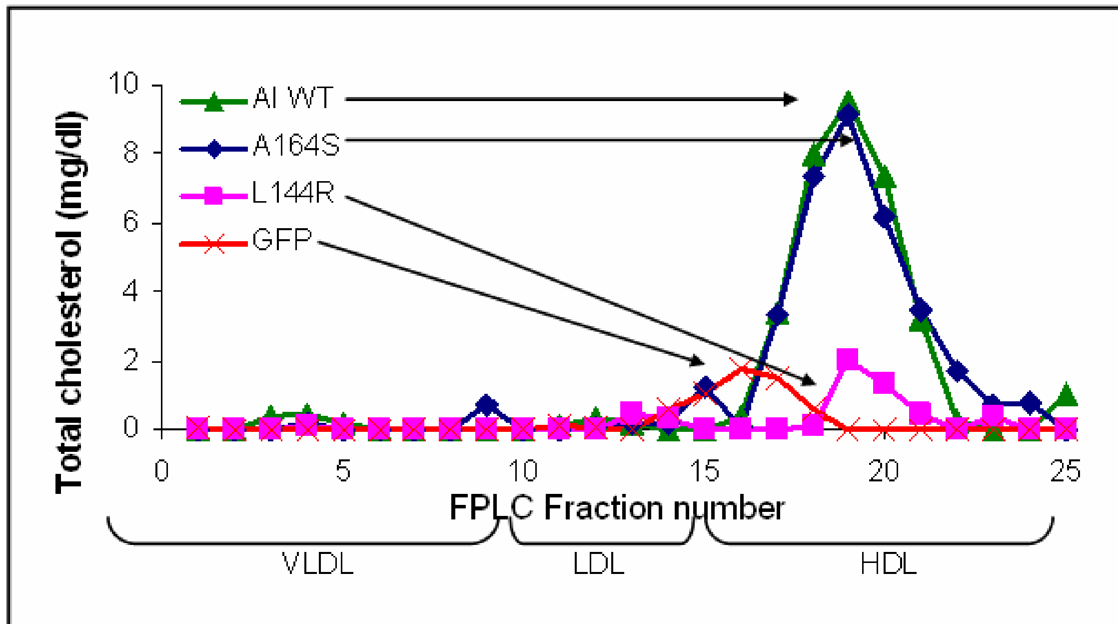

Figure 5. FPLC profiles of total cholesterol of apoA-I$^{-/-}$ mice infected with adenoviruses expressing the WT apoA-I, apoA-I(L144R), apoA-I(A164S) or the control protein GFP. Plasma samples were obtained 4 days-post infection.

lipoprotein fraction was determined by fractionation of plasma by density gradient ultracentrifugation as described in materials and methods. The analysis showed that apoA-I in mice expressing apoA-I(L144R) was greatly reduced and was distributed mainly in the HDL-3 region as compared to mice expressing the wild type apoA-I or apoA-I(A164S) where apoA-I was distributed in the HDL-2 and to a lesser extent in the HDL-3 region (Figure 6A-C).

Electron microscopy analysis

To determine the nature of the HDL particles formed by the WT and the apoA-I(L144R) and apoA-I(A164S) mutant forms, fractions 6-8 ($1.1<d<1.152$) were pooled and analyzed by electron microscopy. This analysis showed that apoA-I(L144R) promoted the formation of few discoidal particles as well as of small size particles similar to those observed in mice expressing the control protein (GFP) (Figure 7A,C). In contrast wild type apoA-I and apoA-I(A164S) promoted the formation of spherical particles (Figure 7B,D).

Non-denaturing two dimensional gel electrophoresis

Plasma obtained from mice infected with recombinant adenoviruses expressing WT and mutant forms of apoA-I was analysed by 2D gel electrophoresis. The particles of the sample were analysed on the first dimension on agarose gel according to their electrical charge and on the second on non-denaturing polyacrylamide gel according to their size. ApoA-I was detected with

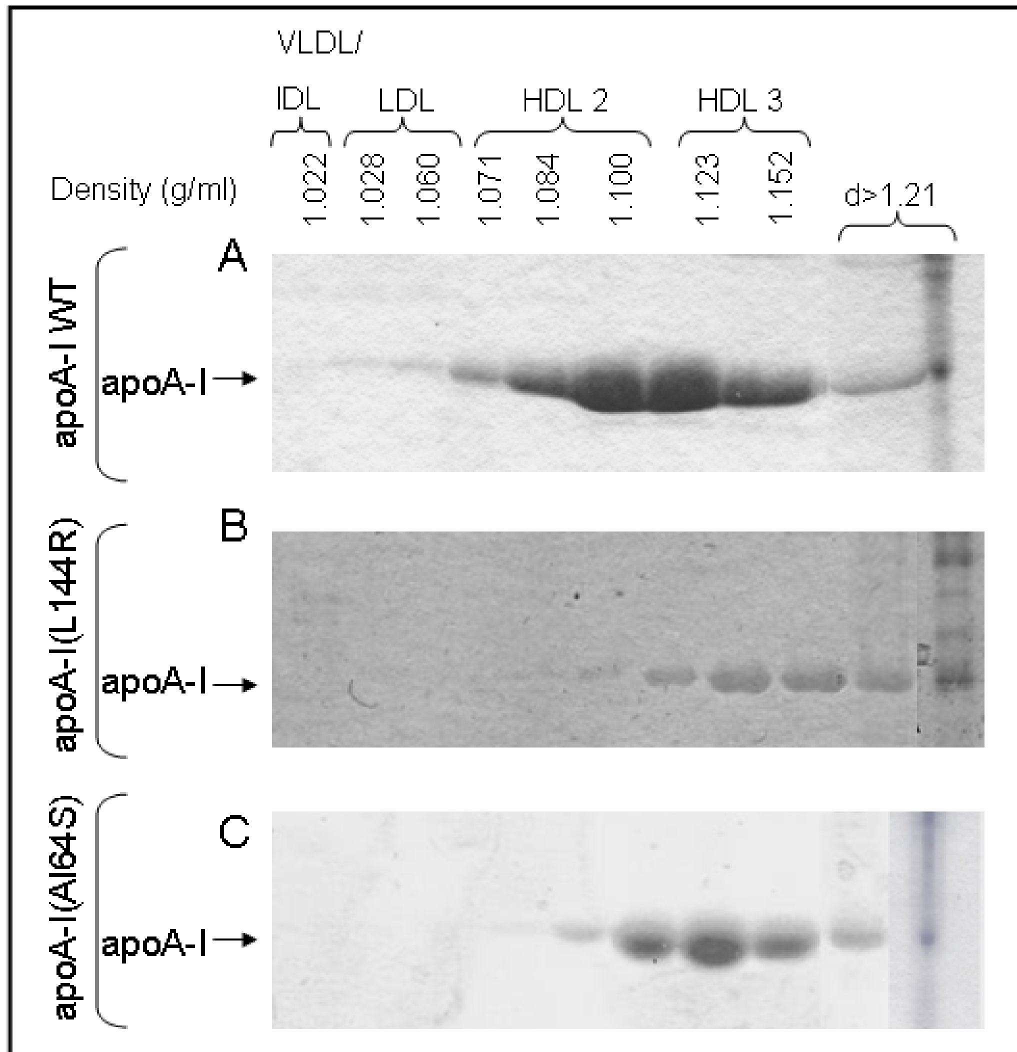

Figure 6A-C. Separation of plasma of apoA-I$^{-/-}$ mice infected with adenoviruses expressing the WT apoA-I (A), the apoA-I(L144R) (B), the apoA-I(A164S) (C) by density gradient ultracentrifugation and analysis of the fractions by SDS-PAGE.

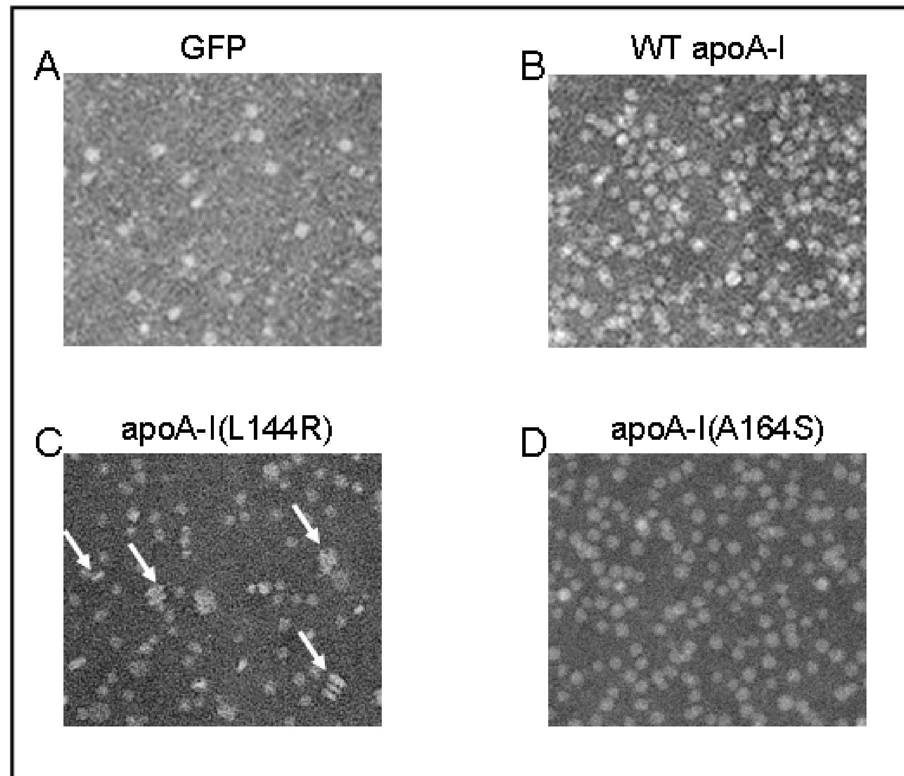

Figure 7A-D. EM pictures of HDL fractions obtained from apoA-I$^{-/-}$ mice expressing the WT apoA-I, the apoA-I(L144R), the apoA-I(A164S) or the control protein GFP following density gradient ultracentrifugation. Panel A: HDL obtained from mice infected with adenovirus expressing the GFP control protein. Panel B: HDL obtained from mice infected with adenovirus expressing apoA-I WT. Panel C: HDL obtained from mice infected with adenovirus expressing apoA-I(L144R). Panel D: HDL obtained from mice infected with the adenovirus expressing the apoA-I(A164S). The photomicrographs were taken at 75,000x magnification and enlarged 3 times. Discoidal particles in panel C are indicated by arrows.

goat anti-human-apoA-I antibody in 1:1000 dilution and the first antibody was detected with rabbit anti-goat antibody in 1:3000 dilution. The electrophoresis showed that the mutant apoA-I(L144R) promoted the formation of preβ- and α4-HDL particles (Figure 8B), whereas WT apoA-I and the mutant apoA-I(A164S) promoted the formation of normal preβ and α HDL subpopulations of different sizes (α1, α2, α3 and α4)(Figure 8A,C).

LCAT corrects the abnormal phenotype produced by the apoA-I(L144R) mutation

The aberrant phenotype produced by the apoA-I(L144R) mutant form of apoA-I based on the low CE/TC ratio suggests LCAT insufficiency. To address this abnormality we treated apoA-I deficient mice with two viruses. The first virus was expressing the apoA-I(L144R) and the second human LCAT (hLCAT). I performed the same analyses that are presented above. The analysis showed that the treatment with hLCAT increased the total cholesterol level. Also the CE/TC ratio was normalized (0.75) and became similar to the CE/TC ratio of mice expressing WT apoA-I (Table 2).

Lipid profile of plasma obtained from mice co-infected with adenoviruses expressing apoA-I(L144R) and hLCAT after FPLC fractionation

A sample of mice plasma was analyzed by fast protein liquid

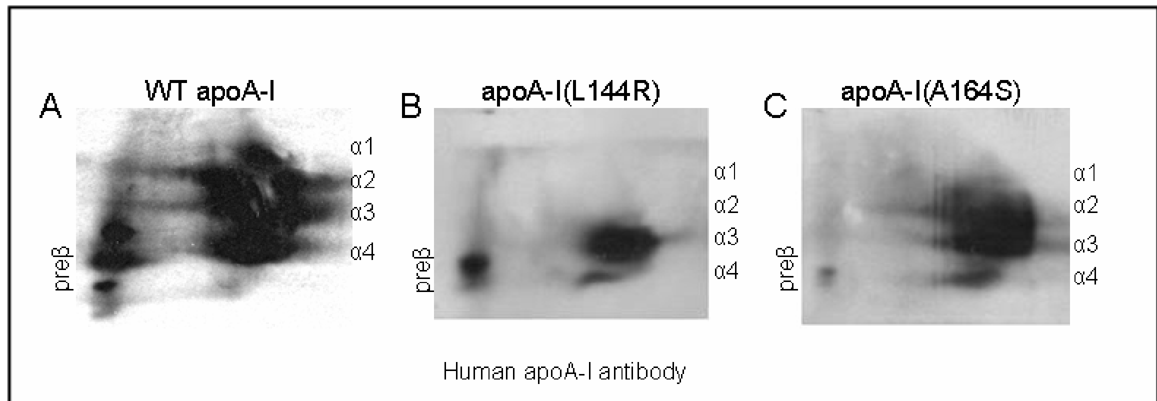

Figure 8A-C. Analysis of plasma obtained from mice expressing the WT apoA-I, the apoA-I(L144R) and the apoA-I(A164S) as indicated in the panels following 2D gel electrophoresis and Western blotting.

chromatography (FPLC) as described in materials and methods. Co-infection of apoA-I deficient mice with adenoviruses expressing apoA-I(L144R) and hLCAT normalized the HDL cholesterol peak and made it look more similar to the HDL peak obtained from mice infected with adenovirus expressing WT apoA-I as compared to the HDL peak of mice infected with adenovirus expressing apoA-I(L144R) (Figure 9).

Distribution of apoA-I(L144R) obtained from mice that were treated with adenoviruses co-expressing apoA-I(L144R) and hLCAT after fractionation of plasma by density gradient ultracentrifugation and electron microscopy analysis of the HDL fraction

The distribution of apoA-I to the VLDL, LDL, and HDL lipoprotein fraction was determined by fractionation of plasma by density gradient ultracentrifugation as described in materials and methods. The analysis showed that apoA-I levels in mice expressing apoA-I(L144R) and hLCAT were increased and apoA-I was distributed mainly in the HDL-2 region and to a lesser extent in the HDL-3 region (Figure 10A) as compared to mice expressing only apoA-I(L144R) where apoA-I was distributed in the HDL-3 region (Figure 6B). This distribution of apoA-I in mice treated with apoA-I(L144R) and hLCAT was similar with the distribution of apoA-I of mice expressing WT apoA-I (Figure 6A). The electron microscopy analysis showed that when mice expressing apoA-I(L144R) were treated with hLCAT the apoA-I(L144R) promoted the formation of

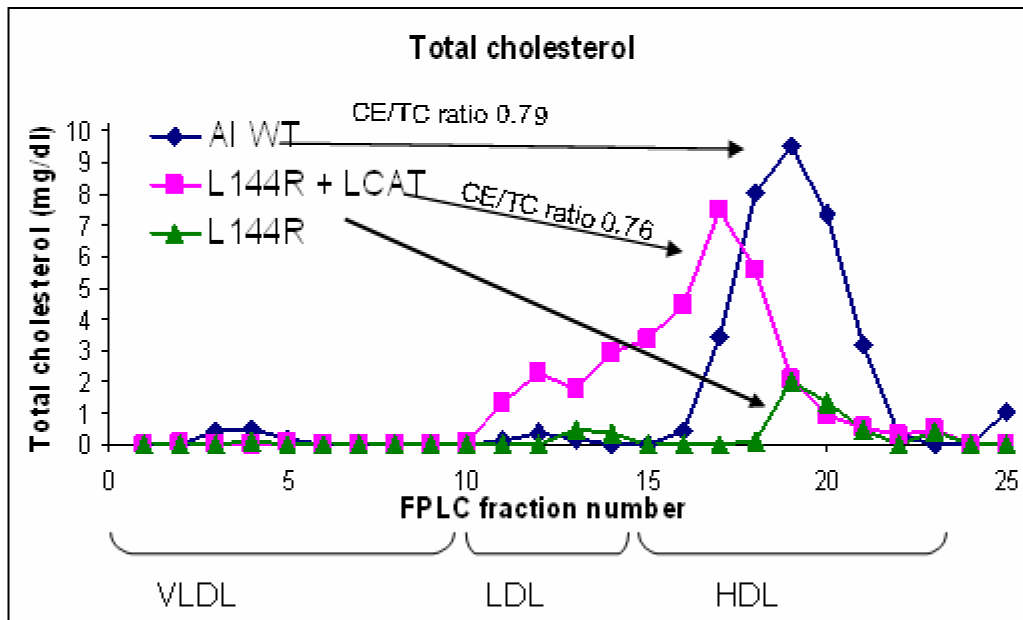

Figure 9. FPLC profiles of total cholesterol of apoA-I$^{-/-}$ mice co-infected with adenoviruses expressing the apoA-I(L144R) and hLCAT compared with mice expressing WT apoA-I and apoA-I(L144R). Plasma samples were obtained 4 days-post infection.

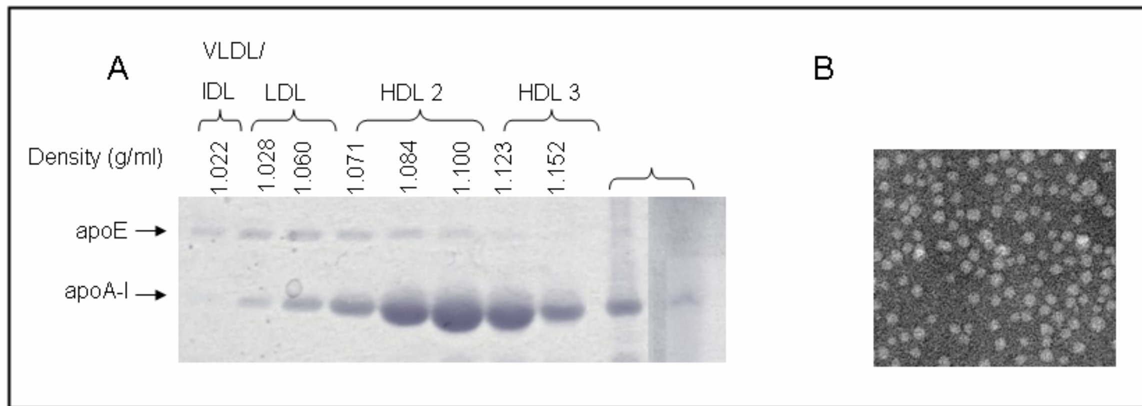

Figure 10. Separation of plasma of apoA-I$^{-/-}$ mice co-infected with adenoviruses expressing the apoA-I(L144R) and hLCAT by density gradient ultracentrifugation and analysis of the fractions by SDS-PAGE. EM picture of HDL fractions obtained from apoA-I$^{-/-}$ mice co-expressing apoA-I(L144R) and hLCAT GFP following density gradient ultracentrifugation. Panel A: HDL obtained from mice co-infected with adenovirus expressing apoA-I(L144R) and hLCAT. Panel B: HDL obtained from mice co-infected with adenoviruses expressing apoA-I(L144R) and hLCAT. The photomicrographs were taken at 75,000x magnification and enlarged 3 times. Discoidal particles in panel C are indicated by arrows.

spherical particles (Figure 10B) similar to those in mice expressing WT apoA-I (Figure 7B). This treatment corrected the presence of abnormal particles in the HDL fractions that were observed in the HDL fraction of mice expressing only apoA-I(L144R) (Figure 7C).

Non-denaturing two dimensional gel electrophoresis of plasma obtained from mice expressing apoA-I(L144R) and hLCAT

Plasma obtained from mice co-infected with recombinant adenoviruses expressing apoA-I(L144R) and hLCAT was analysed by 2D gel electrophoresis. ApoA-I was detected with goat anti-human-apoA-I antibody at a 1:1000 dilution and the first antibody was detected with rabbit anti-goat antibody at a 1:3000 dilution. The electrophoresis showed that the mutant apoA-I(L144R) when co-expressed with hLCAT promoted the formation of normal preβ and α HDL subpopulations of different sizes (α1, α2, α3 and α4) (Figure 11). This profile of HDL subpopulations is similar to the preβ and α HDL subpopulations observed in plasma obtained from mice expressing WT apoA-I (Figure 8A). This analysis showed that the treatment with hLCAT normalized the HDL subpopulations that are formed when mice are expressing apoA-I(L144R) alone (Figure 8B).

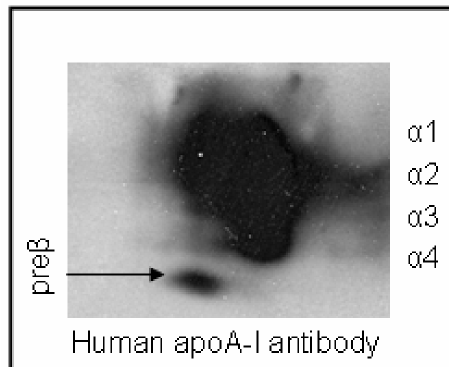

Figure 11. Analysis of plasma obtained from mice co-expressing apoA-I(L144R) and hLCAT following 2D gel electrophoresis and Western blotting. ApoA-I was detected with anti human apoA-I antibody.

DISCUSSION

Background

ApoA-I is an important apolipoprotein that is required for the biogenesis and physiological functions of different HDL species (160). The biogenesis of HDL is a continuous pathway in which apoA-I and several other proteins interact in a specific manner to form initially preβ-HDL and subsequently after further lipidation, discoidal and spherical HDL particles (31). The order of the interactions in the initial steps of this pathway is partially elucidated. The HDL that is formed can then interact with several plasma proteins and other proteins that are present on the cells surface and these interactions remodel the HDL and contribute to its physiological functions to cell signaling and to atheroprotection (Figure 12).

In the present study I introduced two mutations in apoA-I that are known to increase the risk of developing cardiovascular disease as determined in the Copenhagen City Heart Study. The Copenhagen City Heart Study is a prospective cardiovascular cohort study of the Danish general population initiated in 1976 to 1978 with follow-up examinations in 1981 to 1983, 1991, and 2001-2005. This study will determine to what extend variation to the currently known genes (i.e.

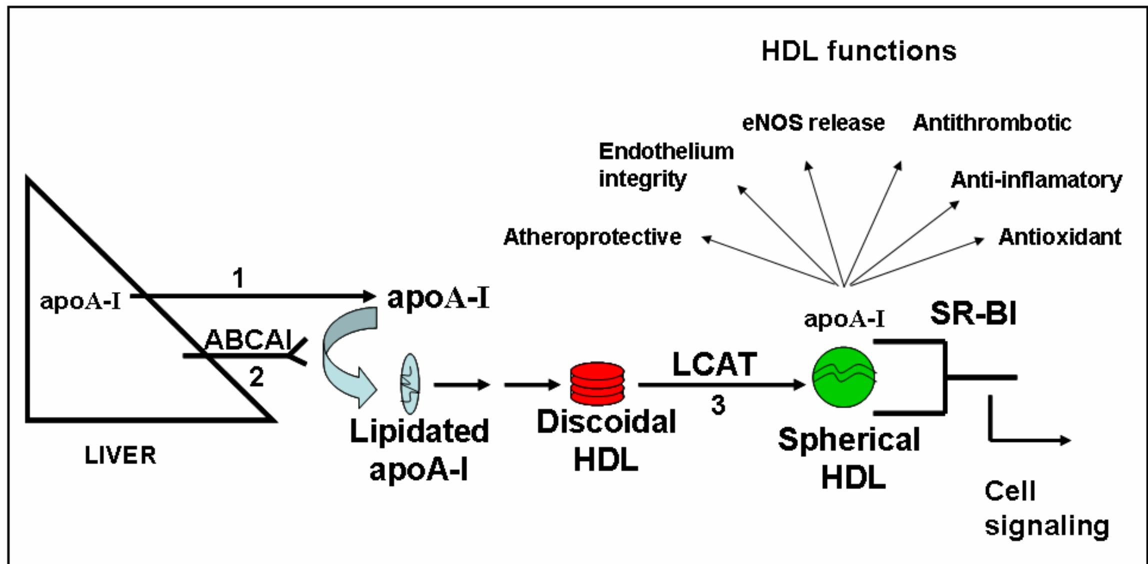

Figure 12. Simplified schematic presentation of the biogenesis of HDL and HDL functions (Modified from Zannis et al. 2007)

ABCA1, apoA-I, LCAT, CETP, PLTP, lipases and ABCGA1) (33;139) that affect the HDL pathway and few master regulatory genes of the HDL pathway (HNF-4, RXRα) determines HDL cholesterol levels and predicts risk of ischemic cardiovascular disease in the general population. Rare mutations in several of the genes encoding the above proteins are known to cause rare familiar HDL deficient syndromes (263;264). However the impact of genetic variation in these genes on the risk of CHD and ischemic cerebrovascular disease in the general population is not well understood. By studying these two naturally occurring mutations I tried to find the defect that they cause and which point of the HDL pathway they disrupt.

These genes expressing the mutated forms of apoA-I were transferred, using recombinant adenoviruses, in mice that were apoA-I deficient. This strategy has been successfully used by Dr. Zannis' lab and it has helped to identify several discreet steps in the biogenesis of HDL(57) (86) and various forms of dyslipidemias (58;97).

In this study two recombinant adenoviruses expressing two mutant forms of apoA-I (L144R and A164S) were generated. Then apoA-I deficient mice were infected, their blood was obtained, and their lipid profile was determined. Also mice were co-infected with the mutant L144R and hLCAT to determine the cause

of the aberrant phenotype and whether it could be corrected if higher doses of hLCAT were provided.

The phenotypes of the A-I deficient mice that were infected with adenoviruses were expected to be similar with the phenotypes that are present in human heterozygotes for the same mutations. For these mutations only heterozygotes patients have been described so the HDL that they produced could have only WT apoA-I, only the mutant form or both forms. This fact restricted the study of the functions of the mutated forms of apoA-I. The mice that were used in this study made it possible to study the mutated forms without the presence of WT apoA-I.

The levels and the formation of HDL were assessed by analyzing the plasma by gel filtration using FPLC, by electrophoresis in two dimensions and by using electron microscopy. Also the plasma was analyzed by density gradient ultracentrifugation in order to identify other defects in the HDL formation and distribution. Finally the total cholesterol levels and the cholesterol esters levels were measured and the CE/TC ratio was calculated.

The mutated form apoA-I(L144R)

When the L144R form of apoA-I was expressed in apoA-I deficient mice the levels of cholesterol in the plasma were decreased. Also this apoA-I formed

mainly preβ-HDL and small α4-HDL particles. The few HDL particles that were formed were discoidal or small spherical particles similar to the ones that are present in the plasma of mice expressing the control protein GFP. Moreover, the CE/TC ratio and the plasma HDL levels were greatly reduced.

A previous study in human heterozygotes for this mutation has shown that the HDL-C, apoA-I and apoA-II were reduced to 40, 60, and 50% respectively compared to control subjects (265). The same study showed that humans carrying one allele with this mutation have HDL particles that contain less esterified cholesterol compared to those obtained from control subjects.

The mutated form apoA-I(A164S)

When the apoA-I(A164S) form was expressed in apoA-I$^{-/-}$ mice the levels of cholesterol remained in the same range as the levels in apoA-I deficient mice expressing WT apoA-I. Also the apoA-I that had the A164S mutation could form α1,α2, α3, and α4-HDL particles as well as a few preβ-HDL particles similarly to the particles formed by WT apoA-I. The particles that were formed were spherical and had the same size with the particles formed by WT apoA-I. Finally the levels of HDL cholesterol and the CE/TC ratio were in the same range as compared to mice expressing WT-apoA-I. The findings suggest that the apoA-I(A164S) mutation that is associated with increased risk of CHD in the Copenhagen City Heart Study does not have abnormality in the HDL pathway. It is possible that the increased risk of CHD is due to other properties of apoA-I such as increased

intracellular cleavage that may cause amyloidosis. This hypothesis will be pursued in the future by generation of transgenic mice expressing this mutant or by long term expression of this mutant using helper dependant adenovirus or adeno-associated viruses (266;267).

The administration of hLCAT in mice can correct the aberrant HDL phenotype that is caused by apoA-I(L144R).

To obtain more information about the cause of the aberrant lipid profile phenotype caused by apoA-I(L144R) apoA-I deficient mice were co-infected with a combination of adenoviruses, the one expressing apoA-I(L144R) and the other hLCAT. The combination of these two adenoviruses corrected the levels of apoA-I and HDL in the plasma of mice; it also restored the CE/TC ratio to normal levels and leaded to the formation of spherical HDL particles with normal preβ- and α-HDL subpopulations.

The correction of the lipid profile of the mice after the expression of hLCAT indicates that the mutant form apoA-I(L144R) is expressed and secreted efficiently from the liver and that the low levels of HDL-C and apoA-I, that were observed in the plasma of the mice when the protein was expressed alone, were not involving expression or secretion defects of this protein. This finding suggests that the step that is affected is the step of esterification of cholesterol from the enzyme LCAT in the lipidated apoA-I particles that are formed by interaction of

apoA-I with LCAT. This interaction will be tested in future experiments in vivo by studying the in vitro efflux by J774 macrophages stimulated by cyclic AMP analogs or cells transfected with ABCA1 as described in (57).

The findings of this study are consistent with a previous study which showed that heterozygotes having one allele of this mutant form of apoA-I have slightly increased secretion rate of apoA-I and a two-fold higher fractional catabolic rate of apoA-I compared to controls (265). The increased catabolism most likely reflects the rapid removal of the lipid and apoA-I(L144R) mutant. Such catabolism may be mediated by the cubulin receptor in the kidney (268;269).

The amino acid Leu144 of apoA-I affects the interaction of apoA-I with LCAT

Due to the insufficiency of LCAT in plasma it seems that the newly synthesized lipid-poor apoA-I that is formed by its interactions with ABCA1 is removed from the plasma. This has been observed in a previous study (265) in plasma obtained from human heterozygotes for the apoA-I(L144R) mutation. As it is also know cubulin, a 600 kDa membrane protein, can bind apoA-I and HDL and promote its catabolism by the kidneys (268), (269). These findings follow a similar pattern with the phenotype observed in apoA-I deficient mice expressing apoA-I(L141R)$_{Pisa}$ and apoA-I(L159R)$_{FIN}$ (95). In these two naturally occurring

mutants it was also shown that there is a significant decrease of LCAT and this decrease can be attributed to the increased catabolism of LCAT that is bound to lipid-poor apoA-I (96).

CONCLUSIONS

In conclusion, the findings of this study show that in the case of apoA-I(L144R) after the initial lipidation of apoA-I, the LCAT can not esterify efficiently the cholesterol of the preβ particles. This leads to immediate removal of the preβ-HDL thus the HDL-C levels in the mice expressing apoA-I(L144R) are low.

The mutant apoA-I(A164S) which was also identified in the Copenhagen City Heart study to be connected with increased risk for CHD it has been shown from the performed studies that can form normal sized HDL that has similar sub-populations to the HDL formed by WT apoA-I. It is possible that this mutation may cause amyloidosis and thus contribute to the increased CHD risk.

The correction of the aberrant HDL phenotypes by treatment with LCAT suggest a potential therapeutic intervention for HDL abnormalities that result from specific mutations in apoA-I.

FUTURE DIRECTIONS

The study of naturally occurring mutations contributes to the clarification of the molecular pathways that are involved in the several steps of biogenesis of HDL. It also helps developing new diagnostic methods and can also help in the prognosis and even the therapy of dyslipidemias.

The experimental approaches used in the present thesis will be used in future studies to investigate the structural function of apoA-I mutants that affect the interactions of apoA-I with other important proteins of the HDL pathway such as ABCA1, LCAT, PLTP, and SR-BI.

Literature Cited

1. Zannis, V. I., Cole, F. S., Jackson, C. L., Kurnit, D. M., and Karathanasis, S. K. (1985) Distribution of apolipoprotein A-I, C-II, C-III, and E mRNA in fetal human tissues. Time-dependent induction of apolipoprotein E mRNA by cultures of human monocyte-macrophages. *Biochemistry 24*, 4450-4455.
2. Matsunaga, T., Hiasa, Y., Yanagi, H., Maeda, T., Hattori, N., Yamakawa, K., Yamanouchi, Y., Tanaka, I., Obara, T., and Hamaguchi, H. (1991) Apolipoprotein A-I deficiency due to a codon 84 nonsense mutation of the apolipoprotein A-I gene. *Proc. Natl. Acad. Sci. U. S. A 88*, 2793-2797.
3. Williamson, R., Lee, D., Hagaman, J., and Maeda, N. (1992) Marked reduction of high density lipoprotein cholesterol in mice genetically modified to lack apolipoprotein A-I. *Proc. Natl. Acad. Sci. U. S. A 89*, 7134-7138.
4. Rubin, E. M., Krauss, R. M., Spangler, E. A., Verstuyft, J. G., and Clift, S. M. (1991) Inhibition of early atherogenesis in transgenic mice by human apolipoprotein AI. *Nature 353*, 265-267.
5. Benoit, P., Emmanuel, F., Caillaud, J. M., Bassinet, L., Castro, G., Gallix, P., Fruchart, J. C., Branellec, D., Denefle, P., and Duverger, N. (1999) Somatic gene transfer of human ApoA-I inhibits atherosclerosis progression in mouse models. *Circulation 99*, 105-110.
6. Castelli, W. P., Doyle, J. T., Gordon, T., Hames, C. G., Hjortland, M. C., Hulley, S. B., Kagan, A., and Zukel, W. J. (1977) HDL cholesterol and other lipids in coronary heart disease. The cooperative lipoprotein phenotyping study. *Circulation 55*, 767-772.
7. Heiss, G. and Tyroler, H. Are apolipoproteins useful for evaluating ischemic heart disease? A brief overview of the literature. [83-1266], 7-24. 1982. Bethesda, MD, U.S. Department of Health and Human Services, National Institutes of Health. Proceedings of the Workshop on Apolipoprotein Quantification.
8. Glueck, C. J., Gartside, P., Fallat, R. W., Sielski, J., and Steiner, P. M. (1976) Longevity syndromes: familial hypobeta and familial hyperalpha lipoproteinemia. *J. Lab Clin. Med. 88*, 941-957.
9. Patsch, W., Kuisk, I., Glueck, C., and Schonfeld, G. (1981) Lipoproteins in familial hyperalphalipoproteinemia. *Arteriosclerosis 1*, 156-161.
10. Arinami, T., Hirano, T., Kobayashi, K., Yamanouchi, Y., and Hamaguchi, H. (1990) Assignment of the Apolipoprotein A-I Gene to 11Q23 Based on Rflp in A Case with A Partial Deletion of Chromosome-11, Del(11) (Q23.3-]Qter). *Human Genetics 85*, 39-40.
11. Karathanasis, S. K., McPherson, J., Zannis, V. I., and Breslow, J. L. (1983) Linkage of human apolipoproteins A-I and C-III genes. *Nature 304*, 371-373.

12. Zannis, V. I., Kan, H. Y., Kritis, A., Zanni, E. E., and Kardassis, D. (2001) Transcriptional regulatory mechanisms of the human apolipoprotein genes in vitro and in vivo. *Curr. Opin. Lipidol. 12*, 181-207.
13. Srivastava, R. and Srivastava, N. (2000) High density lipoprotein, apolipoprotein A-I, and coronary artery disease. *Molecular and Cellular Biochemistry 209*, 131-144.
14. Malik, S. (2003) Transcriptional regulation of the apolipoprotein AI gene. *Frontiers in Bioscience 8*, D360-D368.
15. Lewis, G. F. and Rader, D. J. (2005) New insights into the regulation of HDL metabolism and reverse cholesterol transport. *Circ. Res. 96*, 1221-1232.
16. Segrest, J. P., Jones, M. K., Klon, A. E., Sheldahl, C. J., Hellinger, M., De Loof, H., and Harvey, S. C. (1999) A detailed molecular belt model for apolipoprotein A-I in discoidal high density lipoprotein. *J. Biol. Chem. 274*, 31755-31758.
17. Segrest, J. P. (1977) Amphipathic helixes and plasma lipoproteins: thermodynamic and geometric considerations. *Chem. Phys. Lipids 18*, 7-22.
18. Segrest, J. P., Li, L., Anantharamaiah, G. M., Harvey, S. C., Liadaki, K. N., and Zannis, V. (2000) Structure and function of apolipoprotein A-I and high-density lipoprotein. *Curr. Opin. Lipidol. 11*, 105-115.
19. Panagotopulos, S. E., Horace, E. M., Maiorano, J. N., and Davidson, W. S. (2001) Apolipoprotein A-I adopts a belt-like orientation in reconstituted high density lipoproteins. *J. Biol. Chem. 276*, 42965-42970.
20. Marcel, Y. L. and Kiss, R. S. (2003) Structure-function relationships of apolipoprotein A-I: a flexible protein with dynamic lipid associations. *Curr. Opin. Lipidol. 14*, 151-157.
21. Martin, D. D., Budamagunta, M. S., Ryan, R. O., Voss, J. C., and Oda, M. N. (2006) Apolipoprotein A-I assumes a looped belt conformation on reconstituted high density lipoprotein. *J. Biol. Chem.*
22. Ajees, A. A., Anantharamaiah, G. M., Mishra, V. K., Hussain, M. M., and Murthy, H. M. (2006) Crystal structure of human apolipoprotein A-I: insights into its protective effect against cardiovascular diseases. *Proc. Natl. Acad. Sci. U. S. A 103*, 2126-2131.
23. Nolte, R. T. and Atkinson, D. (1992) Conformational analysis of apolipoprotein A-I and E-3 based on primary sequence and circular dichroism. *Biophys. J. 63*, 1221-1239.
24. Borhani, D. W., Rogers, D. P., Engler, J. A., and Brouillette, C. G. (1997) Crystal structure of truncated human apolipoprotein A-I suggests a lipid-bound conformation. *Proc. Natl. Acad. Sci. U. S. A 94*, 12291-12296.
25. Borhani, D. W., Engler, J. A., and Brouillette, C. G. (1999) Crystallization of truncated human apolipoprotein A-I in a novel conformation. *Acta Crystallogr. D. Biol. Crystallogr. 55 (Pt 9)*, 1578-1583.

26. Gorshkova, I. N., Liu, T., Zannis, V. I., and Atkinson, D. (2002) Lipid-free structure and stability of apolipoprotein A-I: probing the central region by mutation. *Biochemistry 41*, 10529-10539.
27. Li, W. H., Tanimura, M., Luo, C. C., Datta, S., and Chan, L. (1988) The apolipoprotein multigene family: biosynthesis, structure, structure-function relationships, and evolution. *J. Lipid Res. 29*, 245-271.
28. Tanaka, M., Koyama, M., Dhanasekaran, P., Nguyen, D., Nickel, M., Lund-Katz, S., Saito, H., and Phillips, M. C. (2008) Influence of tertiary structure domain properties on the functionality of apolipoprotein A-I. *Biochemistry 47*, 2172-2180.
29. Catte, A., Patterson, J. C., Jones, M. K., Jerome, W. G., Bashtovyy, D., Su, Z., Gu, F., Chen, J., Aliste, M. P., Harvey, S. C., Li, L., Weinstein, G., and Segrest, J. P. (2006) Novel changes in discoidal high density lipoprotein morphology: a molecular dynamics study. *Biophys. J. 90*, 4345-4360.
30. Zannis, V. I., Chroni, A., Kypreos, K. E., Kan, H. Y., Cesar, T. B., Zanni, E. E., and Kardassis, D. (2004) Probing the pathways of chylomicron and HDL metabolism using adenovirus-mediated gene transfer. *Curr Opin Lipidol. 15*, 151-166.
31. Zannis, V. I., Chroni, A., and Krieger, M. (2006) Role of apoA-I, ABCA1, LCAT, and SR-BI in the biogenesis of HDL. *J. Mol. Med. 84*, 276-294.
32. Krieger, M. (2001) Scavenger receptor class B type I is a multiligand HDL receptor that influences diverse physiologic systems. *J. Clin. Invest 108*, 793-797.
33. Wang, N., Lan, D., Chen, W., Matsuura, F., and Tall, A. R. (2004) ATP-binding cassette transporters G1 and G4 mediate cellular cholesterol efflux to high-density lipoproteins. *Proc. Natl. Acad. Sci. U. S. A 101*, 9774-9779.
34. Langmann, T., Klucken, J., Reil, M., Liebisch, G., Luciani, M. F., Chimini, G., Kaminski, W. E., and Schmitz, G. (1999) Molecular cloning of the human ATP-binding cassette transporter 1 (hABC1): evidence for sterol-dependent regulation in macrophages. *Biochem. Biophys. Res. Commun. 257*, 29-33.
35. Kielar, D., Dietmaier, W., Langmann, T., Aslanidis, C., Probst, M., Naruszewicz, M., and Schmitz, G. (2001) Rapid quantification of human ABCA1 mRNA in various cell types and tissues by real-time reverse transcription-PCR. *Clin. Chem. 47*, 2089-2097.
36. Neufeld, E. B., Demosky, S. J., Jr., Stonik, J. A., Combs, C., Remaley, A. T., Duverger, N., Santamarina-Fojo, S., and Brewer, H. B., Jr. (2002) The ABCA1 transporter functions on the basolateral surface of hepatocytes. *Biochem. Biophys. Res. Commun. 297*, 974-979.
37. Neufeld, E. B., Remaley, A. T., Demosky, S. J., Stonik, J. A., Cooney, A. M., Comly, M., Dwyer, N. K., Zhang, M., Blanchette-Mackie, J., Santamarina-Fojo, S., and Brewer, H. B., Jr. (2001) Cellular localization and trafficking of the human ABCA1 transporter. *J. Biol. Chem. 276*, 27584-27590.

38. Wang, N., Silver, D. L., Costet, P., and Tall, A. R. (2000) Specific binding of ApoA-I, enhanced cholesterol efflux, and altered plasma membrane morphology in cells expressing ABC1. *J. Biol. Chem. 275*, 33053-33058.
39. Remaley, A. T., Stonik, J. A., Demosky, S. J., Neufeld, E. B., Bocharov, A. V., Vishnyakova, T. G., Eggerman, T. L., Patterson, A. P., Duverger, N. J., Santamarina-Fojo, S., and Brewer, H. B., Jr. (2001) Apolipoprotein specificity for lipid efflux by the human ABCAI transporter. *Biochem. Biophys. Res. Commun. 280*, 818-823.
40. Schmitz, G., Assmann, G., Robenek, H., and Brennhausen, B. (1985) Tangier disease: a disorder of intracellular membrane traffic. *Proc. Natl. Acad. Sci. U. S. A 82*, 6305-6309.
41. Schmitz, G., Robenek, H., Lohmann, U., and Assmann, G. (1985) Interaction of high density lipoproteins with cholesteryl ester-laden macrophages: biochemical and morphological characterization of cell surface receptor binding, endocytosis and resecretion of high density lipoproteins by macrophages. *EMBO J. 4*, 613-622.
42. Francis, G. A., Knopp, R. H., and Oram, J. F. (1995) Defective removal of cellular cholesterol and phospholipids by apolipoprotein A-I in Tangier Disease. *J. Clin. Invest 96*, 78-87.
43. Bodzioch, M., Orso, E., Klucken, J., Langmann, T., Bottcher, A., Diederich, W., Drobnik, W., Barlage, S., Buchler, C., Porsch-Ozcurumez, M., Kaminski, W. E., Hahmann, H. W., Oette, K., Rothe, G., Aslanidis, C., Lackner, K. J., and Schmitz, G. (1999) The gene encoding ATP-binding cassette transporter 1 is mutated in Tangier disease. *Nat. Genet. 22*, 347-351.
44. Rust, S., Rosier, M., Funke, H., Real, J., Amoura, Z., Piette, J. C., Deleuze, J. F., Brewer, H. B., Duverger, N., Denefle, P., and Assmann, G. (1999) Tangier disease is caused by mutations in the gene encoding ATP-binding cassette transporter 1. *Nat. Genet. 22*, 352-355.
45. Brooks-Wilson, A., Marcil, M., Clee, S. M., Zhang, L. H., Roomp, K., van Dam, M., Yu, L., Brewer, C., Collins, J. A., Molhuizen, H. O., Loubser, O., Ouelette, B. F., Fichter, K., Ashbourne-Excoffon, K. J., Sensen, C. W., Scherer, S., Mott, S., Denis, M., Martindale, D., Frohlich, J., Morgan, K., Koop, B., Pimstone, S., Kastelein, J. J., and Hayden, M. R. (1999) Mutations in ABC1 in Tangier disease and familial high-density lipoprotein deficiency. *Nat. Genet. 22*, 336-345.
46. Lawn, R. M., Wade, D. P., Garvin, M. R., Wang, X., Schwartz, K., Porter, J. G., Seilhamer, J. J., Vaughan, A. M., and Oram, J. F. (1999) The Tangier disease gene product ABC1 controls the cellular apolipoprotein-mediated lipid removal pathway. *J. Clin. Invest 104*, R25-R31.
47. Orso, E., Broccardo, C., Kaminski, W. E., Bottcher, A., Liebisch, G., Drobnik, W., Gotz, A., Chambenoit, O., Diederich, W., Langmann, T., Spruss, T., Luciani, M. F., Rothe, G., Lackner, K. J., Chimini, G., and Schmitz, G. (2000) Transport of lipids from golgi to plasma membrane is

defective in tangier disease patients and Abc1-deficient mice. *Nat. Genet. 24*, 192-196.
48. Assmann, G., von Eckardstein, A., and Brewer, H. B. (2001) in *The Metabolic and Molecular Basis of Inherited Disease* (Scriver, C. R., Beaudet, A. L., Sly, W. S., and Valle, D., Eds.) pp 2937-2960, McGraw-Hill, New York.
49. Chroni, A., Koukos, G., Duka, A., and Zannis, V. I. (2007) The carboxy-terminal region of apoA-I is required for the ABCA1-dependent formation of alpha-HDL but not prebeta-HDL particles in vivo. *Biochemistry 46*, 5697-5708.
50. Marcil, M., Brooks-Wilson, A., Clee, S. M., Roomp, K., Zhang, L. H., Yu, L., Collins, J. A., van Dam, M., Molhuizen, H. O., Loubster, O., Ouellette, B. F., Sensen, C. W., Fichter, K., Mott, S., Denis, M., Boucher, B., Pimstone, S., Genest, J., Jr., Kastelein, J. J., and Hayden, M. R. (1999) Mutations in the ABC1 gene in familial HDL deficiency with defective cholesterol efflux. *Lancet 354*, 1341-1346.
51. McNeish, J., Aiello, R. J., Guyot, D., Turi, T., Gabel, C., Aldinger, C., Hoppe, K. L., Roach, M. L., Royer, L. J., de Wet, J., Broccardo, C., Chimini, G., and Francone, O. L. (2000) High density lipoprotein deficiency and foam cell accumulation in mice with targeted disruption of ATP-binding cassette transporter-1. *Proc. Natl. Acad. Sci. U. S. A 97*, 4245-4250.
52. Christiansen-Weber, T. A., Voland, J. R., Wu, Y., Ngo, K., Roland, B. L., Nguyen, S., Peterson, P. A., and Fung-Leung, W. P. (2000) Functional loss of ABCA1 in mice causes severe placental malformation, aberrant lipid distribution, and kidney glomerulonephritis as well as high-density lipoprotein cholesterol deficiency. *Am. J. Pathol. 157*, 1017-1029.
53. Timmins, J. M., Lee, J. Y., Boudyguina, E., Kluckman, K. D., Brunham, L. R., Mulya, A., Gebre, A. K., Coutinho, J. M., Colvin, P. L., Smith, T. L., Hayden, M. R., Maeda, N., and Parks, J. S. (2005) Targeted inactivation of hepatic Abca1 causes profound hypoalphalipoproteinemia and kidney hypercatabolism of apoA-I. *J. Clin. Invest 115*, 1333-1342.
54. Takahashi, Y. and Smith, J. D. (1999) Cholesterol efflux to apolipoprotein AI involves endocytosis and resecretion in a calcium-dependent pathway. *Proc. Natl. Acad. Sci. U. S. A 96*, 11358-11363.
55. Smith, J. D., Waelde, C., Horwitz, A., and Zheng, P. (2002) Evaluation of the role of phosphatidylserine translocase activity in ABCA1-mediated lipid efflux. *J. Biol. Chem. 277*, 17797-17803.
56. Rohrer, L., Cavelier, C., Fuchs, S., Schluter, M. A., Volker, W., and von Eckardstein, A. (2006) Binding, internalization and transport of apolipoprotein A-I by vascular endothelial cells. *Biochim. Biophys. Acta 1761*, 186-194.
57. Chroni, A., Liu, T., Gorshkova, I., Kan, H. Y., Uehara, Y., von Eckardstein, A., and Zannis, V. I. (2003) The central helices of apoA-I can promote ATP-binding cassette transporter A1 (ABCA1)-mediated lipid efflux.

Amino acid residues 220-231 of the wild-type apoA-I are required for lipid efflux in vitro and high density lipoprotein formation in vivo. *J. Biol. Chem. 278*, 6719-6730.
58. Reardon, C. A., Kan, H. Y., Cabana, V., Blachowicz, L., Lukens, J. R., Wu, Q., Liadaki, K., Getz, G. S., and Zannis, V. I. (2001) In vivo studies of HDL assembly and metabolism using adenovirus-mediated transfer of ApoA-I mutants in ApoA-I-deficient mice. *Biochemistry 40*, 13670-13680.
59. Chroni, A., Kan, H. Y., Kypreos, K. E., Gorshkova, I. N., Shkodrani, A., and Zannis, V. I. (2004) Substitutions of glutamate 110 and 111 in the middle helix 4 of human apolipoprotein A-I (apoA-I) by alanine affect the structure and in vitro functions of apoA-I and induce severe hypertriglyceridemia in apoA-I-deficient mice. *Biochemistry 43*, 10442-10457.
60. Chroni, A., Liu, T., Fitzgerald, M. L., Freeman, M. W., and Zannis, V. I. (2004) Cross-linking and lipid efflux properties of apoA-I mutants suggest direct association between apoA-I helices and ABCA1. *Biochemistry 43*, 2126-2139.
61. Remaley, A. T., Thomas, F., Stonik, J. A., Demosky, S. J., Bark, S. E., Neufeld, E. B., Bocharov, A. V., Vishnyakova, T. G., Patterson, A. P., Eggerman, T. L., Santamarina-Fojo, S., and Brewer, H. B. (2003) Synthetic amphipathic helical peptide mediated efflux of lipid from cells by an ABCA1-dependent and an ABCA1-independent pathway. *J. Lipid Res. 44*, 828-836.
62. Fitzgerald, M. L., Morris, A. L., Chroni, A., Mendez, A. J., Zannis, V. I., and Freeman, M. W. (2004) ABCA1 and amphipathic apolipoproteins form high-affinity molecular complexes required for cholesterol efflux. *J. Lipid Res. 45*, 287-294.
63. Fitzgerald, M. L., Morris, A. L., Rhee, J. S., Andersson, L. P., Mendez, A. J., and Freeman, M. W. (2002) Naturally occurring mutations in the largest extracellular loops of ABCA1 can disrupt its direct interaction with apolipoprotein A-I. *J. Biol. Chem. 277*, 33178-33187.
64. Fitzgerald, M. L., Mendez, A. J., Moore, K. J., Andersson, L. P., Panjeton, H. A., and Freeman, M. W. (2001) ATP-binding cassette transporter A1 contains an NH2-terminal signal anchor sequence that translocates the protein's first hydrophilic domain to the exoplasmic space. *J. Biol. Chem. 276*, 15137-15145.
65. Vedhachalam, C., Ghering, A. B., Davidson, W. S., Lund-Katz, S., Rothblat, G. H., and Phillips, M. C. (2007) ABCA1-Induced Cell Surface Binding Sites for ApoA-I. *Arterioscler. Thromb. Vasc. Biol.*
66. Hajj, H. H., Denis, M., Donna Lee, D. Y., Iatan, I., Nyholt, D., Ruel, I., Krimbou, L., and Genest, J. (2007) Identification of an ABCA1-dependent phospholipid-rich plasma membrane apolipoprotein A-I binding site for nascent HDL formation: Implications for current models of HDL biogenesis. *J. Lipid Res.*

67. Yokoyama, S. (2006) Assembly of high-density lipoprotein. *Arteriosclerosis Thrombosis and Vascular Biology 26*, 20-27.
68. Wang, N. and Tall, A. R. (2003) Regulation and mechanisms of ATP-Binding cassette transporter A1-mediated cellular cholesterol efflux. *Arteriosclerosis Thrombosis and Vascular Biology 23*, 1178-1184.
69. Oram, J. F. (2003) HDL apolipoproteins and ABCA1: partners in the removal of excess cellular cholesterol. *Arterioscler. Thromb. Vasc. Biol. 23*, 720-727.
70. Denis, M., Haidar, B., Marcil, M., Bouvier, M., Krimbou, L., and Genest, J., Jr. (2004) Molecular and cellular physiology of apolipoprotein A-I lipidation by the ATP-binding cassette transporter A1 (ABCA1). *J. Biol. Chem. 279*, 7384-7394.
71. Brunham, L. R., Kruit, J. K., Iqbal, J., Fievet, C., Timmins, J. M., Pape, T. D., Coburn, B. A., Bissada, N., Staels, B., Groen, A. K., Hussain, M. M., Parks, J. S., Kuipers, F., and Hayden, M. R. (2006) Intestinal ABCA1 directly contributes to HDL biogenesis in vivo. *J. Clin. Invest 116*, 1052-1062.
72. Singaraja, R. R., Stahmer, B., Brundert, M., Merkel, M., Heeren, J., Bissada, N., Kang, M., Timmins, J. M., Ramakrishnan, R., Parks, J. S., Hayden, M. R., and Rinninger, F. (2006) Hepatic ATP-binding cassette transporter A1 is a key molecule in high-density lipoprotein cholesteryl ester metabolism in mice. *Arterioscler. Thromb. Vasc. Biol. 26*, 1821-1827.
73. Singaraja, R. R., Van Eck, M., Bissada, N., Zimetti, F., Collins, H. L., Hildebrand, R. B., Hayden, A., Brunham, L. R., Kang, M. H., Fruchart, J. C., van Berkel, T. J., Parks, J. S., Staels, B., Rothblat, G. H., Fievet, C., and Hayden, M. R. (2006) Both Hepatic and Extrahepatic ABCA1 Have Discrete and Essential Functions in the Maintenance of Plasma High-Density Lipoprotein Cholesterol Levels In Vivo. *Circulation 114*, 1301-1309.
74. Cavelier, C., Rohrer, L., and von Eckardstein, A. (2006) ATP-binding cassette transporter al modulates apolipoprotein A1 transcytosis through aortic endothelial cells. *Circulation Research 99*, 1060-1066.
75. Chambenoit, O., Hamon, Y., Marguet, D., Rigneault, H., Rosseneu, M., and Chimini, G. (2001) Specific docking of apolipoprotein A-I at the cell surface requires a functional ABCA1 transporter. *J. Biol. Chem. 276*, 9955-9960.
76. Oram, J. F., Lawn, R. M., Garvin, M. R., and Wade, D. P. (2000) ABCA1 is the cAMP-inducible apolipoprotein receptor that mediates cholesterol secretion from macrophages. *J. Biol. Chem. 275*, 34508-34511.
77. Oram, J. F., Johnson, C. J., and Brown, T. A. (1987) Interaction of high density lipoprotein with its receptor on cultured fibroblasts and macrophages. Evidence for reversible binding at the cell surface without internalization. *J. Biol. Chem. 262*, 2405-2410.

78. Lorenzi, I., von Eckardstein, A., Cavelier, C., Radosavljevic, S., and Rohrer, L. (2008) Apolipoprotein A-I but not high-density lipoproteins are internalised by RAW macrophages: roles of ATP-binding cassette transporter A1 and scavenger receptor BI. *Journal of Molecular Medicine-Jmm 86*, 171-183.
79. Nofer, J. R., Remaley, A. T., Feuerborn, R., Wolinska, I., Engel, T., von Eckardstein, A., and Assmann, G. (2006) Apolipoprotein A-I activates Cdc42 signaling through the ABCA1 transporter. *Journal of Lipid Research 47*, 794-803.
80. Tsukamoto, K., Hirano, K., Tsujii, K., Ikegami, C., Zhongyan, Z., Nishida, Y., Ohama, T., Matsuura, F., Yamashita, S., and Matsuzawa, Y. (2001) ATP-Binding cassette transporter-1 induces rearrangement of actin cytoskeletons possibly through Cdc42/N-WASP. *Biochemical and Biophysical Research Communications 287*, 757-765.
81. Aiello, R. J., Brees, D., Bourassa, P. A., Royer, L., Lindsey, S., Coskran, T., Haghpassand, M., and Francone, O. L. (2002) Increased atherosclerosis in hyperlipidemic mice with inactivation of ABCA1 in macrophages. *Arterioscler. Thromb. Vasc. Biol. 22*, 630-637.
82. Van Eck, M., Singaraja, R. R., Ye, D., Hildebrand, R. B., James, E. R., Hayden, M. R., and van Berkel, T. J. (2006) Macrophage ATP-binding cassette transporter A1 overexpression inhibits atherosclerotic lesion progression in low-density lipoprotein receptor knockout mice. *Arterioscler. Thromb. Vasc. Biol. 26*, 929-934.
83. Joyce, C. W., Wagner, E. M., Basso, F., Amar, M. J., Freeman, L. A., Shamburek, R. D., Knapper, C. L., Syed, J., Wu, J., Vaisman, B. L., Fruchart-Najib, J., Billings, E. M., Paigen, B., Remaley, A. T., Santamarina-Fojo, S., and Brewer, H. B., Jr. (2006) ABCA1 overexpression in the liver of LDLr-KO mice leads to accumulation of pro-atherogenic lipoproteins and enhanced atherosclerosis. *J. Biol. Chem. 281*, 33053-33065.
84. Zannis, V. I., Chroni, A., Liu, T., Liadaki, K. N., and Laccotripe, M. (2004) (Simionescu, M., Ed.) pp 33-72.
85. Jonas, A. (2000) Lecithin cholesterol acyltransferase. *Biochim. Biophys. Acta 1529*, 245-256.
86. Chroni, A., Duka, A., Kan, H. Y., Liu, T., and Zannis, V. I. (2005) Point mutations in apolipoprotein a-I mimic the phenotype observed in patients with classical lecithin:cholesterol acyltransferase deficiency. *Biochemistry 44*, 14353-14366.
87. Miccoli, R., Bertolotto, A., Navalesi, R., Odoguardi, L., Boni, A., Wessling, J., Funke, H., Wiebusch, H., Eckardstein, A., and Assmann, G. (1996) Compound heterozygosity for a structural apolipoprotein A-I variant, apo A-I(L141R)Pisa, and an apolipoprotein A-I null allele in patients with absence of HDL cholesterol, corneal opacifications, and coronary heart disease. *Circulation 94*, 1622-1628.

88. Pisciotta, L., Miccoli, R., Cantafora, A., Calabresi, L., Tarugi, P., Alessandrini, P., Bittolo, B. G., Franceschini, G., Cortese, C., Calandra, S., and Bertolini, S. (2003) Recurrent mutations of the apolipoprotein A-I gene in three kindreds with severe HDL deficiency. *Atherosclerosis 167*, 335-345.
89. Navalesi, R., Miccoli, R., Odoguardi, L., Funke, H., von Eckardstein, A., Wiebusch, H., and Assmann, G. (1995) Genetically determined absence of HDL-cholesterol and coronary atherosclerosis. *Lancet 346*, 708-709.
90. Miettinen, H. E., Gylling, H., Miettinen, T. A., Viikari, J., Paulin, L., and Kontula, K. (1997) Apolipoprotein A-IFin. Dominantly inherited hypoalphalipoproteinemia due to a single base substitution in the apolipoprotein A-I gene. *Arterioscler. Thromb. Vasc. Biol. 17*, 83-90.
91. Miettinen, H. E., Jauhiainen, M., Gylling, H., Ehnholm, S., Palomaki, A., Miettinen, T. A., and Kontula, K. (1997) Apolipoprotein A-IFIN (Leu159-->Arg) mutation affects lecithin cholesterol acyltransferase activation and subclass distribution of HDL but not cholesterol efflux from fibroblasts. *Arterioscler. Thromb. Vasc. Biol. 17*, 3021-3032.
92. Daum, U., Langer, C., Duverger, N., Emmanuel, F., Benoit, P., Denefle, P., Chirazi, A., Cullen, P., Pritchard, P. H., Bruckert, E., Assmann, G., and von Eckardstein, A. (1999) Apolipoprotein A-I (R151C)Paris is defective in activation of lecithin: cholesterol acyltransferase but not in initial lipid binding, formation of reconstituted lipoproteins, or promotion of cholesterol efflux. *J. Mol. Med. 77*, 614-622.
93. Bruckert, E., von Eckardstein, A., Funke, H., Beucler, I., Wiebusch, H., Turpin, G., and Assmann, G. (1997) The replacement of arginine by cysteine at residue 151 in apolipoprotein A-I produces a phenotype similar to that of apolipoprotein A-IMilano. *Atherosclerosis 128*, 121-128.
94. Leren, T. P., Bakken, K. S., Daum, U., Ose, L., Berg, K., Assmann, G., and von Eckardstein, A. (1997) Heterozygosity for apolipoprotein A-I(R160L)Oslo is associated with low levels of high density lipoprotein cholesterol and HDL-subclass LpA-I/A- II but normal levels of HDL-subclass LpA-I. *J. Lipid Res. 38*, 121-131.
95. Koukos, G., Chroni, A., Duka, A., Kardassis, D., and Zannis, V. I. (2007) Naturally occurring and bioengineered apoA-I mutations that inhibit the conversion of discoidal to spherical HDL: the abnormal HDL phenotypes can be corrected by treatment with LCAT. *Biochem. J. 406*, 167-174.
96. Koukos, G., Chroni, A., Duka, A., Kardassis, D., and Zannis, V. I. (2007) LCAT can rescue the abnormal phenotype produced by the natural ApoA-I mutations (Leu141Arg)Pisa and (Leu159Arg)FIN. *Biochemistry 46*, 10713-10721.
97. Chroni, A., Kan, H. Y., Shkodrani, A., Liu, T., and Zannis, V. I. (2005) Deletions of Helices 2 and 3 of Human ApoA-I Are Associated with Severe Dyslipidemia following Adenovirus-Mediated Gene Transfer in ApoA-I-Deficient Mice. *Biochemistry 44*, 4108-4117.

98. Huuskonen, J., Olkkonen, V. M., Jauhiainen, M., and Ehnholm, C. (2001) The impact of phospholipid transfer protein (PLTP) on HDL metabolism. *Atherosclerosis 155*, 269-281.
99. Pussinen, P. J., Jauhiainen, M., Metso, J., Pyle, L. E., Marcel, Y. L., Fidge, N. H., and Ehnholm, C. (1998) Binding of phospholipid transfer protein (PLTP) to apolipoproteins A-I and A-II: location of a PLTP binding domain in the amino terminal region of apoA-I. *J. Lipid Res. 39*, 152-161.
100. Jauhiainen, M., Metso, J., Pahlman, R., Blomqvist, S., van Tol, A., and Ehnholm, C. (1993) Human plasma phospholipid transfer protein causes high density lipoprotein conversion. *J. Biol. Chem. 268*, 4032-4036.
101. Tu, A. Y., Nishida, H. I., and Nishida, T. (1993) High density lipoprotein conversion mediated by human plasma phospholipid transfer protein. *J. Biol. Chem. 268*, 23098-23105.
102. von Eckardstein, A., Jauhiainen, M., Huang, Y., Metso, J., Langer, C., Pussinen, P., Wu, S., Ehnholm, C., and Assmann, G. (1996) Phospholipid transfer protein mediated conversion of high density lipoproteins generates pre beta 1-HDL. *Biochim. Biophys. Acta 1301*, 255-262.
103. Jiang, X. C., Bruce, C., Mar, J., Lin, M., Ji, Y., Francone, O. L., and Tall, A. R. (1999) Targeted mutation of plasma phospholipid transfer protein gene markedly reduces high-density lipoprotein levels. *J. Clin. Invest 103*, 907-914.
104. Krieger, M. (1999) Charting the fate of the "good cholesterol": identification and characterization of the high-density lipoprotein receptor SR-BI. *Annu. Rev. Biochem. 68*, 523-558.
105. Acton, S., Rigotti, A., Landschulz, K. T., Xu, S., Hobbs, H. H., and Krieger, M. (1996) Identification of scavenger receptor SR-BI as a high density lipoprotein receptor. *Science 271*, 518-520.
106. Murao, K., Terpstra, V., Green, S. R., Kondratenko, N., Steinberg, D., and Quehenberger, O. (1997) Characterization of CLA-1, a human homologue of rodent scavenger receptor BI, as a receptor for high density lipoprotein and apoptotic thymocytes. *J. Biol. Chem. 272*, 17551-17557.
107. Acton, S. L., Scherer, P. E., Lodish, H. F., and Krieger, M. (1994) Expression cloning of SR-BI, a CD36-related class B scavenger receptor. *J. Biol. Chem. 269*, 21003-21009.
108. Wang, N., Arai, T., Ji, Y., Rinninger, F., and Tall, A. R. (1998) Liver-specific overexpression of scavenger receptor BI decreases levels of very low density lipoprotein ApoB, low density lipoprotein ApoB, and high density lipoprotein in transgenic mice. *J. Biol. Chem. 273*, 32920-32926.
109. Ueda, Y., Royer, L., Gong, E., Zhang, J., Cooper, P. N., Francone, O., and Rubin, E. M. (1999) Lower plasma levels and accelerated clearance of high density lipoprotein (HDL) and non-HDL cholesterol in scavenger receptor class B type I transgenic mice. *J. Biol. Chem. 274*, 7165-7171.
110. Rigotti, A., Trigatti, B. L., Penman, M., Rayburn, H., Herz, J., and Krieger, M. (1997) A targeted mutation in the murine gene encoding the high

density lipoprotein (HDL) receptor scavenger receptor class B type I reveals its key role in HDL metabolism. *Proc. Natl. Acad. Sci. U. S. A 94*, 12610-12615.

111. Webb, N. R., de Beer, M. C., Yu, J., Kindy, M. S., Daugherty, A., van der Westhuyzen, D. R., and de Beer, F. C. (2002) Overexpression of SR-BI by adenoviral vector promotes clearance of apoA-I, but not apoB, in human apoB transgenic mice. *J. Lipid Res. 43*, 1421-1428.
112. Chroni, A., Nieland, T. J., Kypreos, K. E., Krieger, M., and Zannis, V. I. (2005) SR-BI mediates cholesterol efflux via its interactions with lipid-bound ApoE. Structural mutations in SR-BI diminish cholesterol efflux. *Biochemistry 44*, 13132-13143.
113. Mardones, P., Quinones, V., Amigo, L., Moreno, M., Miquel, J. F., Schwarz, M., Miettinen, H. E., Trigatti, B., Krieger, M., VanPatten, S., Cohen, D. E., and Rigotti, A. (2001) Hepatic cholesterol and bile acid metabolism and intestinal cholesterol absorption in scavenger receptor class B type I-deficient mice. *J. Lipid Res. 42* , 170-180.
114. Ji, Y., Wang, N., Ramakrishnan, R., Sehayek, E., Huszar, D., Breslow, J. L., and Tall, A. R. (1999) Hepatic scavenger receptor BI promotes rapid clearance of high density lipoprotein free cholesterol and its transport into bile. *J. Biol. Chem. 274*, 33398-33402.
115. Trigatti, B., Rayburn, H., Vinals, M., Braun, A., Miettinen, H., Penman, M., Hertz, M., Schrenzel, M., Amigo, L., Rigotti, A., and Krieger, M. (1999) Influence of the high density lipoprotein receptor SR-BI on reproductive and cardiovascular pathophysiology. *Proc. Natl. Acad. Sci. U. S. A 96*, 9322-9327.
116. Arai, T., Wang, N., Bezouevski, M., Welch, C., and Tall, A. R. (1999) Decreased atherosclerosis in heterozygous low density lipoprotein receptor-deficient mice expressing the scavenger receptor BI transgene. *J. Biol. Chem. 274*, 2366-2371.
117. Ueda, Y., Gong, E., Royer, L., Cooper, P. N., Francone, O. L., and Rubin, E. M. (2000) Relationship between expression levels and atherogenesis in scavenger receptor class B, type I transgenics. *J. Biol. Chem. 275*, 20368-20373.
118. Huszar, D., Varban, M. L., Rinninger, F., Feeley, R., Arai, T., Fairchild-Huntress, V., Donovan, M. J., and Tall, A. R. (2000) Increased LDL cholesterol and atherosclerosis in LDL receptor-deficient mice with attenuated expression of scavenger receptor B1. *Arterioscler. Thromb. Vasc. Biol. 20*, 1068-1073.
119. Liadaki, K. N., Liu, T., Xu, S., Ishida, B. Y., Duchateaux, P. N., Krieger, J. P., Kane, J., Krieger, M., and Zannis, V. I. (2000) Binding of high density lipoprotein (HDL) and discoidal reconstituted HDL to the HDL receptor scavenger receptor class B type I. Effect of lipid association and APOA-I mutations on receptor binding. *J. Biol. Chem. 275*, 21262-21271.

120. Xu, S., Laccotripe, M., Huang, X., Rigotti, A., Zannis, V. I., and Krieger, M. (1997) Apolipoproteins of HDL can directly mediate binding to the scavenger receptor SR-BI, an HDL receptor that mediates selective lipid uptake. *J. Lipid Res. 38*, 1289-1298.
121. Urban, S., Zieseniss, S., Werder, M., Hauser, H., Budzinski, R., and Engelmann, B. (2000) Scavenger receptor BI transfers major lipoprotein-associated phospholipids into the cells. *J. Biol. Chem. 275*, 33409-33415.
122. Thuahnai, S. T., Lund-Katz, S., Williams, D. L., and Phillips, M. C. (2001) Scavenger receptor class B, type I-mediated uptake of various lipids into cells. Influence of the nature of the donor particle interaction with the receptor. *J. Biol. Chem. 276*, 43801-43808.
123. Greene, D. J., Skeggs, J. W., and Morton, R. E. (2001) Elevated triglyceride content diminishes the capacity of high density lipoprotein to deliver cholesteryl esters via the scavenger receptor class B type I (SR-BI). *J. Biol. Chem. 276*, 4804-4811.
124. Stangl, H., Hyatt, M., and Hobbs, H. H. (1999) Transport of lipids from high and low density lipoproteins via scavenger receptor-BI. *J. Biol. Chem. 274*, 32692-32698.
125. Gu, X., Lawrence, R., and Krieger, M. (2000) Dissociation of the high density lipoprotein and low density lipoprotein binding activities of murine scavenger receptor class B type I (mSR-BI) using retrovirus library-based activity dissection. *J. Biol. Chem. 275*, 9120-9130.
126. Gu, X., Trigatti, B., Xu, S., Acton, S., Babitt, J., and Krieger, M. (1998) The efficient cellular uptake of high density lipoprotein lipids via scavenger receptor class B type I requires not only receptor-mediated surface binding but also receptor-specific lipid transfer mediated by its extracellular domain. *J. Biol. Chem. 273*, 26338-26348.
127. Ji, Y., Jian, B., Wang, N., Sun, Y., Moya, M. L., Phillips, M. C., Rothblat, G. H., Swaney, J. B., and Tall, A. R. (1997) Scavenger receptor BI promotes high density lipoprotein-mediated cellular cholesterol efflux. *J. Biol. Chem. 272*, 20982-20985.
128. Gu, X., Kozarsky, K., and Krieger, M. (2000) Scavenger receptor class B, type I-mediated [3H]cholesterol efflux to high and low density lipoproteins is dependent on lipoprotein binding to the receptor. *J. Biol. Chem. 275*, 29993-30001.
129. Parathath, S., Sahoo, D., Darlington, Y. F., Peng, Y., Collins, H. L., Rothblat, G. H., Williams, D. L., and Connelly, M. A. (2004) Glycine 420 near the C-terminal transmembrane domain of SR-BI is critical for proper delivery and metabolism of high density lipoprotein cholesteryl ester. *J. Biol. Chem. 279*, 24976-24985.
130. Liu, T., Krieger, M., Kan, H. Y., and Zannis, V. I. (2002) The effects of mutations in helices 4 and 6 of apoA-I on scavenger receptor class B type I (SR-BI)-mediated cholesterol efflux suggest that formation of a productive

complex between reconstituted high density lipoprotein and SR-BI is required for efficient lipid transport. *J. Biol. Chem. 277*, 21576-21584.

131. Savary, S., Denizot, F., Luciani, M., Mattei, M., and Chimini, G. (1996) Molecular cloning of a mammalian ABC transporter homologous to Drosophila white gene. *Mamm. Genome 7*, 673-676.
132. Croop, J. M., Tiller, G. E., Fletcher, J. A., Lux, M. L., Raab, E., Goldenson, D., Son, D., Arciniegas, S., and Wu, R. L. (1997) Isolation and characterization of a mammalian homolog of the Drosophila white gene. *Gene 185*, 77-85.
133. Nakamura, K., Kennedy, M. A., Baldan, A., Bojanic, D. D., Lyons, K., and Edwards, P. A. (2004) Expression and regulation of multiple murine ATP-binding cassette transporter G1 mRNAs/isoforms that stimulate cellular cholesterol efflux to high density lipoprotein. *J. Biol. Chem. 279*, 45980-45989.
134. Klucken, J., Buchler, C., Orso, E., Kaminski, W. E., Porsch-Ozcurumez, M., Liebisch, G., Kapinsky, M., Diederich, W., Drobnik, W., Dean, M., Allikmets, R., and Schmitz, G. (2000) ABCG1 (ABC8), the human homolog of the Drosophila white gene, is a regulator of macrophage cholesterol and phospholipid transport. *Proc. Natl. Acad. Sci. U. S. A 97*, 817-822.
135. Venkateswaran, A., Repa, J. J., Lobaccaro, J. M., Bronson, A., Mangelsdorf, D. J., and Edwards, P. A. (2000) Human white/murine ABC8 mRNA levels are highly induced in lipid-loaded macrophages. A transcriptional role for specific oxysterols. *J. Biol. Chem. 275*, 14700-14707.
136. Wang, N., Ranalletta, M., Matsuura, F., Peng, F., and Tall, A. R. (2006) LXR-induced redistribution of ABCG1 to plasma membrane in macrophages enhances cholesterol mass efflux to HDL. *Arterioscler. Thromb. Vasc. Biol. 26*, 1310-1316.
137. Vaughan, A. M. and Oram, J. F. (2005) ABCG1 redistributes cell cholesterol to domains removable by HDL but not by lipid-depleted apolipoproteins. *J. Biol. Chem. 280*, 30150-30157.
138. Wang, X., Collins, H. L., Ranalletta, M., Fuki, I. V., Billheimer, J. T., Rothblat, G. H., Tall, A. R., and Rader, D. J. (2007) Macrophage ABCA1 and ABCG1, but not SR-BI, promote macrophage reverse cholesterol transport in vivo. *J. Clin. Invest 117* , 2216-2224.
139. Kennedy, M. A., Barrera, G. C., Nakamura, K., Baldan, A., Tarr, P., Fishbein, M. C., Frank, J., Francone, O. L., and Edwards, P. A. (2005) ABCG1 has a critical role in mediating cholesterol efflux to HDL and preventing cellular lipid accumulation. *Cell Metab 1*, 121-131.
140. Chung, B. H., Segrest, J. P., Ray, M. J., Brunzell, J. D., Hokanson, J. E., Krauss, R. M., Beaudrie, K., and Cone, J. T. (1986) Single vertical spin density gradient ultracentrifugation. *Methods Enzymol. 128*, 181-209.

141. Nichols, A. V., Krauss, R. M., and Musliner, T. A. (1986) Nondenaturing polyacrylamide gradient gel electrophoresis. *Methods Enzymol. 128*, 417-431.
142. Davidson, W. S., Sparks, D. L., Lund-Katz, S., and Phillips, M. C. (1994) The molecular basis for the difference in charge between pre-beta- and alpha-migrating high density lipoproteins. *J. Biol. Chem. 269*, 8959-8965.
143. Asztalos, B. F., Brousseau, M. E., McNamara, J. R., Horvath, K. V., Roheim, P. S., and Schaefer, E. J. (2001) Subpopulations of high density lipoproteins in homozygous and heterozygous Tangier disease. *Atherosclerosis 156*, 217-225.
144. Fielding, C. J. and Fielding, P. E. (1996) Two-dimensional nondenaturing electrophoresis of lipoproteins: applications to high-density lipoprotein speciation. *Methods Enzymol. 263*, 251-259.
145. Asztalos, B. F., Sloop, C. H., Wong, L., and Roheim, P. S. (1993) Two-dimensional electrophoresis of plasma lipoproteins: recognition of new apo A-I-containing subpopulations. *Biochim. Biophys. Acta 1169*, 291-300.
146. Fielding, C. J. and Fielding, P. E. (1995) Molecular physiology of reverse cholesterol transport. *J. Lipid Res. 36*, 211-228.
147. Nanjee, M. N., Cooke, C. J., Olszewski, W. L., and Miller, N. E. (2000) Concentrations of electrophoretic and size subclasses of apolipoprotein A-I-containing particles in human peripheral lymph. *Arterioscler. Thromb. Vasc. Biol. 20*, 2148-2155.
148. Reichl, D., Hathaway, C. B., Sterchi, J. M., and Miller, N. E. (1991) Lipoproteins of human peripheral lymph. Apolipoprotein AI-containing lipoprotein with alpha-2 electrophoretic mobility. *Eur. J. Clin. Invest 21*, 638-643.
149. Asztalos, B. F., Sloop, C. H., Wong, L., and Roheim, P. S. (1993) Comparison of apo A-I-containing subpopulations of dog plasma and prenodal peripheral lymph: evidence for alteration in subpopulations in the interstitial space. *Biochim. Biophys. Acta 1169*, 301-304.
150. Heideman, C. L. and Hoff, H. F. (1982) Lipoproteins containing apolipoprotein A-I extracted from human aortas. *Biochim. Biophys. Acta 711*, 431-444.
151. Smith, E. B., C.Ashall, and J.E.Walker. High density lipoprotein (HDL) subfractions in interstitial fluid from human aortic intima and atherosclerostic lesions. Biochem.Soc.Trans. 12, 843-844. 1984.
152. Nakamura, Y., Kotite, L., Gan, Y., Spencer, T. A., Fielding, C. J., and Fielding, P. E. (2004) Molecular mechanism of reverse cholesterol transport: reaction of pre-beta-migrating high-density lipoprotein with plasma lecithin/cholesterol acyltransferase. *Biochemistry 43*, 14811-14820.

153. Castro, G. R. and Fielding, C. J. (1988) Early incorporation of cell-derived cholesterol into pre-beta-migrating high-density lipoprotein. *Biochemistry 27*, 25-29.
154. Forte, T. M., Goth-Goldstein, R., Nordhausen, R. W., and McCall, M. R. (1993) Apolipoprotein A-I-cell membrane interaction: extracellular assembly of heterogeneous nascent HDL particles. *J. Lipid Res. 34*, 317-324.
155. Forte, T. M., Bielicki, J. K., Goth-Goldstein, R., Selmek, J., and McCall, M. R. (1995) Recruitment of cell phospholipids and cholesterol by apolipoproteins A-II and A-I: formation of nascent apolipoprotein-specific HDL that differ in size, phospholipid composition, and reactivity with LCAT. *J. Lipid Res. 36*, 148-157.
156. Clay, M. A., Newnham, H. H., and Barter, P. J. (1991) Hepatic lipase promotes a loss of apolipoprotein A-I from triglyceride-enriched human high density lipoproteins during incubation in vitro. *Arterioscler. Thromb. 11*, 415-422.
157. Hennessy, L. K., Kunitake, S. T., and Kane, J. P. (1993) Apolipoprotein A-I-containing lipoproteins, with or without apolipoprotein A-II, as progenitors of pre-beta high-density lipoprotein particles. *Biochemistry 32*, 5759-5765.
158. Settasatian, N., Duong, M., Curtiss, L. K., Ehnholm, C., Jauhiainen, M., Huuskonen, J., and Rye, K. A. (2001) The mechanism of the remodeling of high density lipoproteins by phospholipid transfer protein. *J. Biol. Chem. 276*, 26898-26905.
159. Sorci-Thomas, M. G. and Thomas, M. J. (2002) The effects of altered apolipoprotein A-I structure on plasma HDL concentration. *Trends Cardiovasc. Med. 12*, 121-128.
160. Zannis, V. I., Kardassis, D., and Zanni, E. E. (1993) Genetic mutations affecting human lipoproteins, their receptors, and their enzymes. *Adv. Hum. Genet. 21*, 145-319.
161. Recalde, D., Velez-Carrasco, W., Civeira, F., Cenarro, A., Gomez-Coronado, D., Ordovas, J. M., and Pocovi, M. (2001) Enhanced fractional catabolic rate of apo A-I and apo A-II in heterozygous subjects for apo A-I(Zaragoza) (L144R). *Atherosclerosis 154*, 613-623.
162. Gylling, H., Relas, H., Miettinen, H. E., Radhakrishnan, R., and Miettinen, T. A. (1996) Delayed postprandial retinyl palmitate and squalene removal in a patient heterozygous for apolipoprotein A-IFIN mutation (Leu 159-->Arg) and low HDL cholesterol level without coronary artery disease. *Atherosclerosis 127*, 239-243.
163. Owen, J. S., Bharadwaj, M. S., Thomas, M. J., Bhat, S., Samuel, M. P., and Sorci-Thomas, M. G. (2007) Ratio determination of plasma wild-type and L159R apoA-I using mass spectrometry: tools for studying apoA-IFin. *J. Lipid Res. 48*, 226-234.

164. Dastani, Z., Dangoisse, C., Boucher, B., Desbiens, K., Krimbou, L., Dufour, R., Hegele, R. A., Pajukanta, P., Engert, J. C., Genest, J., and Marcil, M. (2006) A novel nonsense apolipoprotein A-I mutation (apoA-I(E136X)) causes low HDL cholesterol in French Canadians. *Atherosclerosis 185*, 127-136.
165. Favari, E., Gomaraschi, M., Zanotti, I., Bernini, F., Lee-Rueckert, M., Kovanen, P. T., Sirtori, C. R., Franceschini, G., and Calabresi, L. (2007) A unique protease-sensitive high density lipoprotein particle containing the apolipoprotein A-I(Milano) dimer effectively promotes ATP-binding Cassette A1-mediated cell cholesterol efflux. *J. Biol. Chem. 282*, 5125-5132.
166. Favari, E., Gomaraschi, M., Zanotti, I., Bernini, F., Lee-Rueckert, M., Kovanen, P. T., Sirtori, C. R., Franceschini, G., and Calabresi, L. (2007) A unique protease-sensitive high density lipoprotein particle containing the apolipoprotein A-I-Milano dimer effectively promotes ATP-binding cassette A1-mediated cell cholesterol efflux. *Journal of Biological Chemistry 282*, 5125-5132.
167. Franceschini, G., Calabresi, L., Chiesa, G., Parolini, C., Sirtori, C. R., Canavesi, M., and Bernini, F. (1999) Increased cholesterol efflux potential of sera from ApoA-IMilano carriers and transgenic mice. *Arterioscler. Thromb. Vasc. Biol. 19*, 1257-1262.
168. Weibel, G. L., Alexander, E. T., Joshi, M. R., Rader, D. J., Lund-Katz, S., Phillips, M. C., and Rothblat, G. H. (2007) Wild-type ApoA-I and the Milano variant have similar abilities to stimulate cellular lipid mobilization and efflux. *Arterioscler. Thromb. Vasc. Biol. 27*, 2022-2029.
169. Valenta, D. T., Bulgrien, J. J., Banka, C. L., and Curtiss, L. K. (2006) Overexpression of human ApoAI transgene provides long-term atheroprotection in LDL receptor-deficient mice. *Atherosclerosis 189*, 255-263.
170. Duverger, N., Kruth, H., Emmanuel, F., Caillaud, J. M., Viglietta, C., Castro, G., Tailleux, A., Fievet, C., Fruchart, J. C., Houdebine, L. M., and Denefle, P. (1996) Inhibition of atherosclerosis development in cholesterol-fed human apolipoprotein A-I-transgenic rabbits. *Circulation 94*, 713-717.
171. Barter, P. J. and Rye, K. A. (2006) The rationale for using apoA-I as a clinical marker of cardiovascular risk. *J. Intern. Med. 259*, 447-454.
172. Nicholls, S. J., Tuzcu, E. M., Sipahi, I., Schoenhagen, P., Crowe, T., Kapadia, S., and Nissen, S. E. (2006) Relationship between atheroma regression and change in lumen size after infusion of apolipoprotein A-I Milano. *J. Am. Coll. Cardiol. 47*, 992-997.
173. Ansell, B. J., Navab, M., Hama, S., Kamranpour, N., Fonarow, G., Hough, G., Rahmani, S., Mottahedeh, R., Dave, R., Reddy, S. T., and Fogelman, A. M. (2003) Inflammatory/antiinflammatory properties of high-density lipoprotein distinguish patients from control subjects better than high-

density lipoprotein cholesterol levels and are favorably affected by simvastatin treatment. *Circulation 108*, 2751-2756.
174. Mineo, C., Deguchi, H., Griffin, J. H., and Shaul, P. W. (2006) Endothelial and antithrombotic actions of HDL. *Circ. Res. 98*, 1352-1364.
175. Ou, J., Ou, Z., Jones, D. W., Holzhauer, S., Hatoum, O. A., Ackerman, A. W., Weihrauch, D. W., Gutterman, D. D., Guice, K., Oldham, K. T., Hillery, C. A., and Pritchard, K. A., Jr. (2003) L-4F, an apolipoprotein A-1 mimetic, dramatically improves vasodilation in hypercholesterolemia and sickle cell disease. *Circulation 107*, 2337-2341.
176. Ou, J., Wang, J., Xu, H., Ou, Z., Sorci-Thomas, M. G., Jones, D. W., Signorino, P., Densmore, J. C., Kaul, S., Oldham, K. T., and Pritchard, K. A., Jr. (2005) Effects of D-4F on vasodilation and vessel wall thickness in hypercholesterolemic LDL receptor-null and LDL receptor/apolipoprotein A-I double-knockout mice on Western diet. *Circ. Res. 97*, 1190-1197.
177. Ou, Z., Ou, J., Ackerman, A. W., Oldham, K. T., and Pritchard, K. A., Jr. (2003) L-4F, an apolipoprotein A-1 mimetic, restores nitric oxide and superoxide anion balance in low-density lipoprotein-treated endothelial cells. *Circulation 107*, 1520-1524.
178. Yuhanna, I. S., Zhu, Y., Cox, B. E., Hahner, L. D., Osborne-Lawrence, S., Lu, P., Marcel, Y. L., Anderson, R. G., Mendelsohn, M. E., Hobbs, H. H., and Shaul, P. W. (2001) High-density lipoprotein binding to scavenger receptor-BI activates endothelial nitric oxide synthase. *Nat. Med. 7*, 853-857.
179. Shaul, P. W. (2002) Regulation of endothelial nitric oxide synthase: location, location, location. *Annu. Rev. Physiol 64*, 749-774.
180. Mineo, C., Yuhanna, I. S., Quon, M. J., and Shaul, P. W. (2003) High density lipoprotein-induced endothelial nitric-oxide synthase activation is mediated by Akt and MAP kinases. *J. Biol. Chem. 278*, 9142-9149.
181. Nofer, J. R., van der, G. M., Tolle, M., Wolinska, I., von Wnuck, L. K., Baba, H. A., Tietge, U. J., Godecke, A., Ishii, I., Kleuser, B., Schafers, M., Fobker, M., Zidek, W., Assmann, G., Chun, J., and Levkau, B. (2004) HDL induces NO-dependent vasorelaxation via the lysophospholipid receptor S1P3. *J. Clin. Invest 113*, 569-581.
182. Li, X. A., Titlow, W. B., Jackson, B. A., Giltiay, N., Nikolova-Karakashian, M., Uittenbogaard, A., and Smart, E. J. (2002) High density lipoprotein binding to scavenger receptor, Class B, type I activates endothelial nitric-oxide synthase in a ceramide-dependent manner. *J. Biol. Chem. 277*, 11058-11063.
183. Levkau, B., Hermann, S., Theilmeier, G., van der, G. M., Chun, J., Schober, O., and Schafers, M. (2004) High-density lipoprotein stimulates myocardial perfusion in vivo. *Circulation 110*, 3355-3359.
184. Gong, M., Wilson, M., Kelly, T., Su, W., Dressman, J., Kincer, J., Matveev, S. V., Guo, L., Guerin, T., Li, X. A., Zhu, W., Uittenbogaard, A., and Smart, E. J. (2003) HDL-associated estradiol stimulates endothelial NO synthase

and vasodilation in an SR-BI-dependent manner. *J. Clin. Invest 111*, 1579-1587.
185. Shaul, P. W. and Mineo, C. (2004) HDL action on the vascular wall: is the answer NO? *J. Clin. Invest 113*, 509-513.
186. Zeiher, A. M., Schachlinger, V., Hohnloser, S. H., Saurbier, B., and Just, H. (1994) Coronary atherosclerotic wall thickening and vascular reactivity in humans. Elevated high-density lipoprotein levels ameliorate abnormal vasoconstriction in early atherosclerosis. *Circulation 89*, 2525-2532.
187. Li, X. P., Zhao, S. P., Zhang, X. Y., Liu, L., Gao, M., and Zhou, Q. C. (2000) Protective effect of high density lipoprotein on endothelium-dependent vasodilatation. *Int. J. Cardiol. 73*, 231-236.
188. Kuvin, J. T., Patel, A. R., Sidhu, M., Rand, W. M., Sliney, K. A., Pandian, N. G., and Karas, R. H. (2003) Relation between high-density lipoprotein cholesterol and peripheral vasomotor function. *Am. J. Cardiol. 92*, 275-279.
189. Kuvin, J. T., Ramet, M. E., Patel, A. R., Pandian, N. G., Mendelsohn, M. E., and Karas, R. H. (2002) A novel mechanism for the beneficial vascular effects of high-density lipoprotein cholesterol: enhanced vasorelaxation and increased endothelial nitric oxide synthase expression. *Am. Heart J. 144*, 165-172.
190. Bisoendial, R. J., Hovingh, G. K., Levels, J. H., Lerch, P. G., Andresen, I., Hayden, M. R., Kastelein, J. J., and Stroes, E. S. (2003) Restoration of endothelial function by increasing high-density lipoprotein in subjects with isolated low high-density lipoprotein. *Circulation 107*, 2944-2948.
191. Spieker, L. E., Sudano, I., Hurlimann, D., Lerch, P. G., Lang, M. G., Binggeli, C., Corti, R., Ruschitzka, F., Luscher, T. F., and Noll, G. (2002) High-density lipoprotein restores endothelial function in hypercholesterolemic men. *Circulation 105*, 1399-1402.
192. Dimmeler, S., Haendeler, J., and Zeiher, A. M. (2002) Regulation of endothelial cell apoptosis in atherothrombosis. *Curr. Opin. Lipidol. 13*, 531-536.
193. Mallat, Z., Benamer, H., Hugel, B., Benessiano, J., Steg, P. G., Freyssinet, J. M., and Tedgui, A. (2000) Elevated levels of shed membrane microparticles with procoagulant potential in the peripheral circulating blood of patients with acute coronary syndromes. *Circulation 101*, 841-843.
194. Martinez, M. C., Tesse, A., Zobairi, F., and Andriantsitohaina, R. (2005) Shed membrane microparticles from circulating and vascular cells in regulating vascular function. *Am. J. Physiol Heart Circ. Physiol 288*, H1004-H1009.
195. Li, D., Yang, B., and Mehta, J. L. (1998) Ox-LDL induces apoptosis in human coronary artery endothelial cells: role of PKC, PTK, bcl-2, and Fas. *Am. J. Physiol 275*, H568-H576.

196. Choy, J. C., Granville, D. J., Hunt, D. W., and McManus, B. M. (2001) Endothelial cell apoptosis: biochemical characteristics and potential implications for atherosclerosis. *J. Mol. Cell Cardiol. 33*, 1673-1690.
197. Dimmeler, S., Breitschopf, K., Haendeler, J., and Zeiher, A. M. (1999) Dephosphorylation targets Bcl-2 for ubiquitin-dependent degradation: a link between the apoptosome and the proteasome pathway. *J. Exp. Med. 189*, 1815-1822.
198. Welch, G. N. and Loscalzo, J. (1998) Homocysteine and atherothrombosis. *N. Engl. J. Med. 338*, 1042-1050.
199. Strawn, W. B. and Ferrario, C. M. (2002) Mechanisms linking angiotensin II and atherogenesis. *Curr. Opin. Lipidol. 13*, 505-512.
200. Suc, I., Escargueil-Blanc, I., Troly, M., Salvayre, R., and Negre-Salvayre, A. (1997) HDL and ApoA prevent cell death of endothelial cells induced by oxidized LDL. *Arterioscler. Thromb. Vasc. Biol. 17*, 2158-2166.
201. Sugano, M., Tsuchida, K., and Makino, N. (2000) High-density lipoproteins protect endothelial cells from tumor necrosis factor-alpha-induced apoptosis. *Biochem. Biophys. Res. Commun. 272*, 872-876.
202. Nofer, J. R., Levkau, B., Wolinska, I., Junker, R., Fobker, M., von Eckardstein, A., Seedorf, U., and Assmann, G. (2001) Suppression of endothelial cell apoptosis by high density lipoproteins (HDL) and HDL-associated lysosphingolipids. *J. Biol. Chem. 276*, 34480-34485.
203. Kimura, T., Sato, K., Malchinkhuu, E., Tomura, H., Tamama, K., Kuwabara, A., Murakami, M., and Okajima, F. (2003) High-density lipoprotein stimulates endothelial cell migration and survival through sphingosine 1-phosphate and its receptors. *Arterioscler. Thromb. Vasc. Biol. 23*, 1283-1288.
204. Li, X. A., Guo, L., Dressman, J. L., Asmis, R., and Smart, E. J. (2005) A novel ligand-independent apoptotic pathway induced by SR-BI and suppressed by eNOS and HDL. *J. Biol. Chem. 280*, 19087-19096.
205. Cunningham, K. S. and Gotlieb, A. I. (2005) The role of shear stress in the pathogenesis of atherosclerosis. *Lab Invest 85*, 9-23.
206. Ross, R. (1993) The pathogenesis of atherosclerosis: a perspective for the 1990s. *Nature 362*, 801-809.
207. Gotlieb, A. I. and Silver, M. D. (2001) in *Cardiovascular Pathology* (Gotlieb, A. I., Schoen, F. J., and Silver, M. D., Eds.) Churchill Livingstone, New York.
208. Rossig, L., Dimmeler, S., and Zeiher, A. M. (2001) Apoptosis in the vascular wall and atherosclerosis. *Basic Res. Cardiol. 96*, 11-22.
209. Werner, N., Junk, S., Laufs, U., Link, A., Walenta, K., Bohm, M., and Nickenig, G. (2003) Intravenous transfusion of endothelial progenitor cells reduces neointima formation after vascular injury. *Circ. Res. 93*, e17-e24.
210. Tamagaki, T., Sawada, S., Imamura, H., Tada, Y., Yamasaki, S., Toratani, A., Sato, T., Komatsu, S., Akamatsu, N., Yamagami, M., Kobayashi, K.,

Kato, K., Yamamoto, K., Shirai, K., Yamada, K., Higaki, T., Nakagawa, K., Tsuji, H., and Nakagawa, M. (1996) Effects of high-density lipoproteins on intracellular pH and proliferation of human vascular endothelial cells. *Atherosclerosis 123*, 73-82.

211. Murugesan, G., Sa, G., and Fox, P. L. (1994) High-density lipoprotein stimulates endothelial cell movement by a mechanism distinct from basic fibroblast growth factor. *Circ. Res. 74*, 1149-1156.
212. Miura, S., Fujino, M., Matsuo, Y., Kawamura, A., Tanigawa, H., Nishikawa, H., and Saku, K. (2003) High density lipoprotein-induced angiogenesis requires the activation of Ras/MAP kinase in human coronary artery endothelial cells. *Arterioscler. Thromb. Vasc. Biol. 23*, 802-808.
213. Seetharam, D., Mineo, C., Gormley, A. K., Gibson, L. L., Vongpatanasin, W., Chambliss, K. L., Hahner, L. D., Cummings, M. L., Kitchens, R. L., Marcel, Y. L., Rader, D. J., and Shaul, P. W. (2006) High-density lipoprotein promotes endothelial cell migration and reendothelialization via scavenger receptor-B type I. *Circulation Research 98*, 63-72.
214. Durrington, P. (2003) Dyslipidaemia. *Lancet 362*, 717-731.
215. Freedman, D. S., Otvos, J. D., Jeyarajah, E. J., Barboriak, J. J., Anderson, A. J., and Walker, J. A. (1998) Relation of lipoprotein subclasses as measured by proton nuclear magnetic resonance spectroscopy to coronary artery disease. *Arteriosclerosis Thrombosis and Vascular Biology 18*, 1046-1053.
216. Jenny, N. S., Tracy, R. P., Ogg, M. S., Luong, L. A., Kuller, L. H., Arnold, A. M., Sharrett, A. R., and Humphries, S. E. (2002) In the elderly, interleukin-6 plasma levels and the-174G > C polymorphism are associated with the development of cardiovascular disease. *Arteriosclerosis Thrombosis and Vascular Biology 22*, 2066-2071.
217. Asztalos, B. F., Cupples, L. A., Demissie, S., Horvath, K. V., Cox, C. E., Batista, M. C., and Schaefer, E. J. (2004) High-density lipoprotein subpopulation profile and coronary heart disease prevalence in male participants of the Framingham Offspring Study. *Arterioscler. Thromb. Vasc. Biol. 24*, 2181-2187.
218. Asztalos, B. F., Collins, D., Cupples, L. A., Demissie, S., Horvath, K. V., Bloomfield, H. E., Robins, S. J., and Schaefer, E. J. (2005) Value of high-density lipoprotein (HDL) subpopulations in predicting recurrent cardiovascular events in the Veterans Affairs HDL Intervention Trial. *Arterioscler. Thromb. Vasc. Biol. 25*, 2185-2191.
219. Deguchi, H., Fernandez, J. A., Pabinger, I., Heit, J. A., and Griffin, J. H. (2001) Plasma glucosylceramide deficiency as potential risk factor for venous thrombosis and modulator of anticoagulant protein C pathway. *Blood 97*, 1907-1914.
220. Gonzalez-Ordonez, A. J., Fernandez-Carreira, J. M., Fernandez-Alvarez, C. R., Obaya, R. V., Macias-Robles, M. D., Gonzalez-Franco, A., and Garcia, M. A. A. (2003) The concentrations of soluble vascular cell adhesion

molecule-1 and lipids are independently associated with venous thromboembolism. *Haematologica 88*, 1035-1043.
221. Doggen, C. J. M., Smith, N. L., Lemaitre, R. N., Heckbert, S. R., Rosendaal, F. R., and Psaty, B. M. (2004) Serum lipid levels and the risk of venous thrombosis. *Arteriosclerosis Thrombosis and Vascular Biology 24*, 1970-1975.
222. Deguchi, H., Pecheniuk, N. M., Elias, D. J., Averell, P. M., and Griffin, J. H. (2005) High-density lipoprotein deficiency and dyslipoproteinemia associated with venous thrombosis in men. *Circulation 112*, 893-899.
223. Pajkrt, D., Lerch, P. G., vanderPoll, T., Levi, M., Illi, M., Doran, J. E., Arnet, B., vandenEnde, A., tenCate, J. W., and vanDeventer, S. J. H. (1997) Differential effects of reconstituted high-density lipoprotein on coagulation, fibrinolysis and platelet activation during human endotoxemia. *Thrombosis and Haemostasis 77*, 303-307.
224. Li, D. Y., Weng, S., Yang, B. C., Zander, D. S., Saldeen, T., Nichols, W. W., Khan, S., and Mehta, J. L. (1999) Inhibition of arterial thrombus formation by apoA1 Milano. *Arteriosclerosis Thrombosis and Vascular Biology 19*, 378-383.
225. Shah, P. K., Kaul, S., Nilsson, J., and Cercek, B. (2001) Exploiting the vascular protective effects of high-density lipoprotein and its apolipoproteins - An idea whose time for testing is coming, Part II. *Circulation 104*, 2498-2502.
226. Assmann, G. and Nofer, J. R. (2003) Atheroprotective effects of high-density lipoproteins. *Annual Review of Medicine-Selected Topics in the Clinical Sciences 54*, 321-341.
227. Durand, E., Scoazec, A., Lafont, A., Boddaert, J., Al Hajzen, A., Addad, F., Mirshahi, M., Desnos, M., Tedgui, A., and Mallat, Z. (2004) In vivo induction of endothelial apoptosis leads to vessel thrombosis and endothelial denudation - A clue to the understanding of the mechanisms of thrombotic plaque erosion. *Circulation 109*, 2503-2506.
228. Barter, P. J., Nicholls, S., Rye, K. A., Anantharamaiah, G. M., Navab, M., and Fogelman, A. M. (2004) Antiinflammatory properties of HDL. *Circulation Research 95*, 764-772.
229. Fleisher, L. N., Tall, A. R., Witte, L. D., and Cannon, P. J. (1983) Effects of High-Density Lipoprotein and the Apoprotein of High-Density Lipoprotein on Prostacyclin Synthesis by Endothelial-Cells. *Advances in Prostaglandin Thromboxane and Leukotriene Research 11*, 475-480.
230. Spector, A. A., Scanu, A. M., Kaduce, T. L., Figard, P. H., Fless, G. M., and Czervionke, R. L. (1985) Effect of Human-Plasma Lipoproteins on Prostacyclin Production by Cultured Endothelial-Cells. *Journal of Lipid Research 26*, 288-297.
231. Vane, J. R. and Botting, R. M. (1995) Pharmacodynamic Profile of Prostacyclin. *American Journal of Cardiology 75*, A3-A10.

232. Vinals, M., Martinez-Gonzalez, J., Badimon, J. J., and Badimon, L. (1997) HDL-induced prostacyclin release in smooth muscle cells is dependent on cyclooxygenase-2 (Cox-2). *Arteriosclerosis Thrombosis and Vascular Biology 17*, 3481-3488.
233. Escudero, I., Martinez-Gonzalez, J., Alonso, R., Mata, P., and Badimon, L. (2003) Experimental and interventional dietary study in humans on the role of HDL fatty acid composition in PGI(2) release and Cox-2 expression by VSMC. *European Journal of Clinical Investigation 33*, 779-786.
234. Martinez-Gonzalez, J., Escudero, I., and Badimon, L. (2004) Simvastatin potenciates PGI(2) release induced by HDL in human VSMC: effect on Cox-2 up-regulation and MAPK signalling pathways activated by HDL. *Atherosclerosis 174*, 305-313.
235. Wadham, C., Albanese, N., Roberts, J., Wang, L. J., Bagley, C. J., Gamble, J. R., Rye, K. A., Barter, P. J., Vadas, M. A., and Xia, P. (2004) High-density lipoproteins neutralize C-reactive protein proinflammatory activity. *Circulation 109*, 2116-2122.
236. Landmesser, U., Hornig, B., and Drexler, H. (2004) Endothelial function - A critical determinant in atherosclerosis? *Circulation 109*, 27-33.
237. Perez-Ruiz, A., Montes, R., Velasco, F., Lopez-Pedrera, C., Paramo, J. A., Orbe, J., Hermida, J., and Rocha, E. (2002) Regulation by nitric oxide of endotoxin-induced tissue factor and plasminogen activator inhibitor-1 in endothelial cells. *Thrombosis and Haemostasis 88*, 1060-1065.
238. Yang, Y. and Loscalzo, J. (2000) Regulation of tissue factor expression in human microvascular endothelial cells by nitric oxide. *Circulation 101*, 2144-2148.
239. Bombeli, T., Schwartz, B. R., and Harlan, J. M. (1999) Endothelial cells undergoing apoptosis become proadhesive for nonactivated platelets. *Blood 93*, 3831-3838.
240. Bombeli, T., Karsan, A., Tait, J. F., and Harlan, J. M. (1997) Apoptotic vascular endothelial cells become procoagulant. *Blood 89*, 2429-2442.
241. Mallat, Z. and Tedgui, A. (2001) Current perspective on the role of apoptosis in atherothrombotic disease. *Circulation Research 88*, 998-1003.
242. Griffin, J. H., Kojima, K., Banka, C. L., Curtiss, L. K., and Fernandez, J. A. (1999) High-density lipoprotein enhancement of anticoagulant activities of plasma protein S and activated protein C. *Journal of Clinical Investigation 103*, 219-227.
243. MacCallum, P. K., Cooper, J. A., Martin, J., Howarth, D. J., Meade, T. W., and Miller, G. J. (2000) Haemostatic and lipid determinants of prothrombin fragment F1.2 and D-dimer in plasma. *Thrombosis and Haemostasis 83*, 421-426.
244. Balicki, D. and Beutler, E. (2002) Gene therapy of human disease. *Medicine (Baltimore) 81*, 69-86.

245. Nicholls, S. J., Cutri, B., Worthley, S. G., Kee, P., Rye, K. A., Bao, S., and Barter, P. J. (2005) Impact of short-term administration of high-density lipoproteins and atorvastatin on atherosclerosis in rabbits. *Arteriosclerosis Thrombosis and Vascular Biology 25*, 2416-2421.
246. Boffa, M. C. and Karmochkine, M. (1998) Thrombomodulin: an overview and potential implications in vascular disorders. *Lupus 7*, S120-S125.
247. Deguchi, H., Fernandez, J. A., and Griffin, J. H. (2002) Neutral glycosphingolipid-dependent inactivation of coagulation factor Va by activated protein C and protein S. *Journal of Biological Chemistry 277*, 8861-8865.
248. Deguchi, H., Yegneswaran, S., and Griffin, J. H. (2004) Sphingolipids as bioactive regulators of thrombin generation. *Journal of Biological Chemistry 279*, 12036-12042.
249. Kobayashi, T., Tahara, Y., Matsumoto, M., Iguchi, M., Sano, H., Murayama, T., Arai, H., Oida, H., Yurugi-Kobayashi, T., Yamashita, J. K., Katagiri, H., Majima, M., Yokode, M., Kita, T., and Narumiya, S. (2004) Roles of thromboxane A(2) and prostacyclin in the development of atherosclerosis in apoE-deficient mice. *Journal of Clinical Investigation 114*, 784-794.
250. Oravec, S., Demuth, K., Myara, I., and Hornych, A. (1998) The effect of high density lipoprotein subfractions on endothelial eicosanoid secretion. *Thrombosis Research 92*, 65-71.
251. Suzuki, H., Kurihara, Y., Takeya, M., Kamada, N., Kataoka, M., Jishage, K., Ueda, O., Sakaguchi, H., Higashi, T., Suzuki, T., Takashima, Y., Kawabe, Y., Cynshi, O., Wada, Y., Honda, M., Kurihara, H., Aburatani, H., Doi, T., Matsumoto, A., Azuma, S., Noda, T., Toyoda, Y., Itakura, H., Yazaki, Y., Kodama, T., and . (1997) A role for macrophage scavenger receptors in atherosclerosis and susceptibility to infection. *Nature 386*, 292-296.
252. Kobayashi, T., Tahara, Y., Matsumoto, M., Iguchi, M., Sano, H., Murayama, T., Arai, H., Oida, H., Yurugi-Kobayashi, T., Yamashita, J. K., Katagiri, H., Majima, M., Yokode, M., Kita, T., and Narumiya, S. (2004) Roles of thromboxane A(2) and prostacyclin in the development of atherosclerosis in apoE-deficient mice. *Journal of Clinical Investigation 114*, 784-794.
253. Bartlett, S. R., Sawdy, R., and Mann, G. E. (1999) Induction of cyclooxygenase-2 expression in human myometrial smooth muscle cells by interleukin-1 beta: involvement of p38 mitogen-activated protein kinase. *Journal of Physiology-London 520*, 399-406.
254. Grewal, T., de Diego, I., Kirchhoff, M. F., Tebar, F., Heeren, J., Rinninger, F., and Enrich, C. (2003) High density lipoprotein-induced signaling of the MAPK pathway involves scavenger receptor type BI-mediated activation of Ras. *Journal of Biological Chemistry 278*, 16478-16481.
255. O'Connell, B. J. and Genest, J. (2001) High-density lipoproteins and endothelial function. *Circulation 104*, 1978-1983.

256. Eren, M., Painter, C. A., Atkinson, J. B., Declerck, P. J., and Vaughan, D. E. (2002) Age-dependent spontaneous coronary arterial thrombosis in transgenic mice that express a stable form of human plasminogen activator inhibitor-1. *Circulation 106*, 491-496.
257. Ridker, P. M., Brown, N. J., Vaughan, D. E., Harrison, D. G., and Mehta, J. L. (2004) Established and emerging plasma biomarkers in the prediction of first atherothrombotic events. *Circulation 109*, 6-19.
258. Norata, G. D., Banfi, C., Pirillo, A., Tremoli, E., Hamsten, A., Catapano, A. L., and Eriksson, P. (2004) Oxidised-HDL3 induces the expression of PAI-1 in human endothelial cells. Role of p38MAPK activation and mRNA stabilization. *British Journal of Haematology 127*, 97-104.
259. Naqvi, T. Z., Shah, P. K., Ivey, P. A., Molloy, M. D., Thomas, A. M., Panicker, S., Ahmed, A., Cercek, B., and Kaul, S. (1999) Evidence that high-density lipoprotein cholesterol is an independent predictor of acute platelet-dependent thrombus formation. *American Journal of Cardiology 84*, 1011-1017.
260. Lerch, P. G., Spycher, M. O., and Doran, J. E. (1998) Reconstituted high density lipoprotein (rHDL) modulates platelet activity in vitro and ex vivo. *Thrombosis and Haemostasis 80*, 316-320.
261. Assanasen, C., Mineo, C., Seetharam, D., Yuhanna, I. S., Marcel, Y. L., Connelly, M. A., Williams, D. L., Llera-Moya, M., Shaul, P. W., and Silver, D. L. (2005) Cholesterol binding, efflux, and a PDZ-interacting domain of scavenger receptor-BI mediate HDL-initiated signaling. *J. Clin. Invest 115*, 969-977.
262. Sumi, M., Sata, M., Miura, S. I., Rye, K. A., Toya, N., Kanaoka, Y., Yanaga, K., Ohki, T., Saku, K., and Nagai, R. (2007) Reconstituted high-density lipoprotein stimulates differentiation of endothelial progenitor cells and enhances ischemia-induced angiogenesis. *Arteriosclerosis Thrombosis and Vascular Biology 27*, 813-818.
263. Tall, A. R., Breslow, J. L., and Rubin, E. M. (2001) in *The Metabolic & Molecular Bases of Inherited Disease* (Scriver, C. R., Beaudet, A. L., Valle, D., and Sly, W. S., Eds.) pp 2915-2936, McGraw-Hill, New York.
264. Santamarina-Fojo, S., Hoeg, J. M., Assmann, G., and Brewer, H. B., Jr. (2001) in *The Metabolic & Molecular Bases of Inherited Disease* (Scriver, C. R., Beaudet, A. L., Sly, W. S., and Valle, D., Eds.) pp 2817-2834, McGraw-Hill, New York.
265. Recalde, D., Velez-Carrasco, W., Civeira, F., Cenarro, A., Gomez-Coronado, D., Ordovas, J. M., and Pocovi, M. (2001) Enhanced fractional catabolic rate of apo A-I and apo A-II in heterozygous subjects for apo A-I-Zaragoza (L144R). *Atherosclerosis 154*, 613-623.
266. Kitajima, K., Marchadier, D. H., Burstein, H., and Rader, D. J. (2006) Persistent liver expression of murine apoA-l using vectors based on adeno-associated viral vectors serotypes 5 and 1. *Atherosclerosis 186*, 65-73.

267. Pastore, L., Belalcazar, L. M., Oka, K., Cela, R., Lee, B., Chan, L., and Beaudet, A. L. (2004) Helper-dependent adenoviral vector-mediated long-term expression of human apolipoprotein A-I reduces atherosclerosis in apo E-deficient mice. *Gene 327*, 153-160.
268. Kozyraki, R., Fyfe, J., Kristiansen, M., Gerdes, C., Jacobsen, C., Cui, S., Christensen, E. I., Aminoff, M., de la, C. A., Krahe, R., Verroust, P. J., and Moestrup, S. K. (1999) The intrinsic factor-vitamin B12 receptor, cubilin, is a high-affinity apolipoprotein A-I receptor facilitating endocytosis of high-density lipoprotein. *Nat. Med. 5*, 656-661.
269. Hammad, S. M., Stefansson, S., Twal, W. O., Drake, C. J., Fleming, P., Remaley, A., Brewer, H. B., Jr., and Argraves, W. S. (1999) Cubilin, the endocytic receptor for intrinsic factor-vitamin B(12) complex, mediates high-density lipoprotein holoparticle endocytosis. *Proc. Natl. Acad. Sci. U. S. A 96*, 10158-10163.