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Review Article

Role of apolipoproteins, ABCA1 and LCAT in the biogenesis of normal and aberrant high density lipoproteins

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Abstract

In this review, we focus on the pathway of biogenesis of HDL, the essential role of apoA-I, ATP binding cassette transporter A1 (ABCA1), and lecithin: cholesterol acyltransferase (LCAT) in the formation of plasma HDL; the generation of aberrant forms of HDL containing mutant apoA-I forms and the role of apoA-IV and apoE in the formation of distinct HDL subpopulations. The biogenesis of HDL requires functional interactions of the ABCA1 with apoA-I (and to a lesser extent with apoE and apoA-IV) and subsequent interactions of the nascent HDL species thus formed with LCAT. Mutations in apoA-I, ABCA1 and LCAT either prevent or impair the formation of HDL and may also affect the functionality of the HDL species formed. Emphasis is placed on three categories of apoA-I mutations. The first category describes a unique bio-engineered apoA-I mutation that disrupts interactions between apoA-I and ABCA1 and generates aberrant preβ HDL subpopulations that cannot be converted efficiently to α subpopulations by LCAT. The second category describes natural and bio-engineered apoA-I mutations that generate preβ and small size α4 HDL subpopulations, and are associated with low plasma HDL levels. These phenotypes can be corrected by excess LCAT. The third category describes bio-engineered apoA-I mutations that induce hypertriglyceridemia that can be corrected by excess lipoprotein lipase and also have defective maturation of HDL. The HDL phenotypes described here may serve in the future for diagnosis, prognoses and potential treatment of abnormalities that affect the biogenesis and functionality of HDL.

Keywords: HDL biogenesis, HDL phenotypes, apolipoprotein A-I mutations, apolipoprotein E, apolipoprotein A-IV, ATP- binding cassette transporter A1 (ABCA1)

The pathway of the biogenesis of HDL-AI, the role of ABCA1 and LCAT and the potential functions of HDL

The first step in the biogenesis of HDL containing apoA-I (HDL-AI) is the interaction of apoA-I that is secreted by the liver and the intestine[1], with the ABCA1 transporter. This interaction transfers cellular phospholipids and free cholesterol to apoA-I in vitro. The same interaction in vivo causes gradual lipidation of apoA-I that leads to the formation of discoidal particles that are enriched in unesterified cholesterol. In

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a subsequent step, LCAT esterifies the free cholesterol[2] and converts the discoidal to spherical HDL particles (***Fig. 1A***). A similar pathway is used in vivo for the generation of HDL containing apoE and apoA-IV that are designated HDL-E and HDL-AIV.

The role of the exchangeable apolipoproteins apoA-I, apoA-IV, apoC-III and apoE in hepatic lipoprotein assembly and secretion has been reviewed elsewhere[5]. In the case of apoA-I, using recombinant adenovirus mediated gene transfer of human apoA-I in primary hepatocytes obtained from *apoA-I* $^{-/-}$ or *ABCA1* $^{-/-}$ mice, it has been shown that a portion of the apoA-I is secreted in a lipidated form and that the lipidation is partially dependent on ABCA1[6]. Two other studies in HepG2 cells and primary mouse hepatocytes support the intracellular lipidation of apoA-I[7–8]. The initial phospholipidation of apoA-I occurs in the endoplasmic reticulum (ER) and is partially ABCA1 dependent. However, the bulk of the lipidation by phospholipids and cholesterol occurs in the Golgi and pericellularly at the plasma membrane, respectively and is ABCA1 dependent[8]. Following secretion, the intracellularly lipidated apoA-I has the ability to interact with ABCA1 on the plasma membrane and contribute to the biogenesis of HDL as depicted in ***Fig. 1A***.

Following synthesis, HDL is modified by hepatic lipase (HL), endothelial lipase (EL), phospholipid transfer protein (PLTP), cholesteryl ester transfer protein (CETP), and interacts with SR-BI and ABCG1 and *via* signaling pathways exerts its biologic functions[9–10]. HDL promotes cholesterol efflux[11–12], prevents oxidation of LDL[13–14], inhibits cell apoptosis[15] as well as the expression of proinflammatory cytokines by macrophages[16–17] and the expression of adhesion molecules, chemokines and chemokine receptors by endothelial cells[18–20]. HDL cholesterol

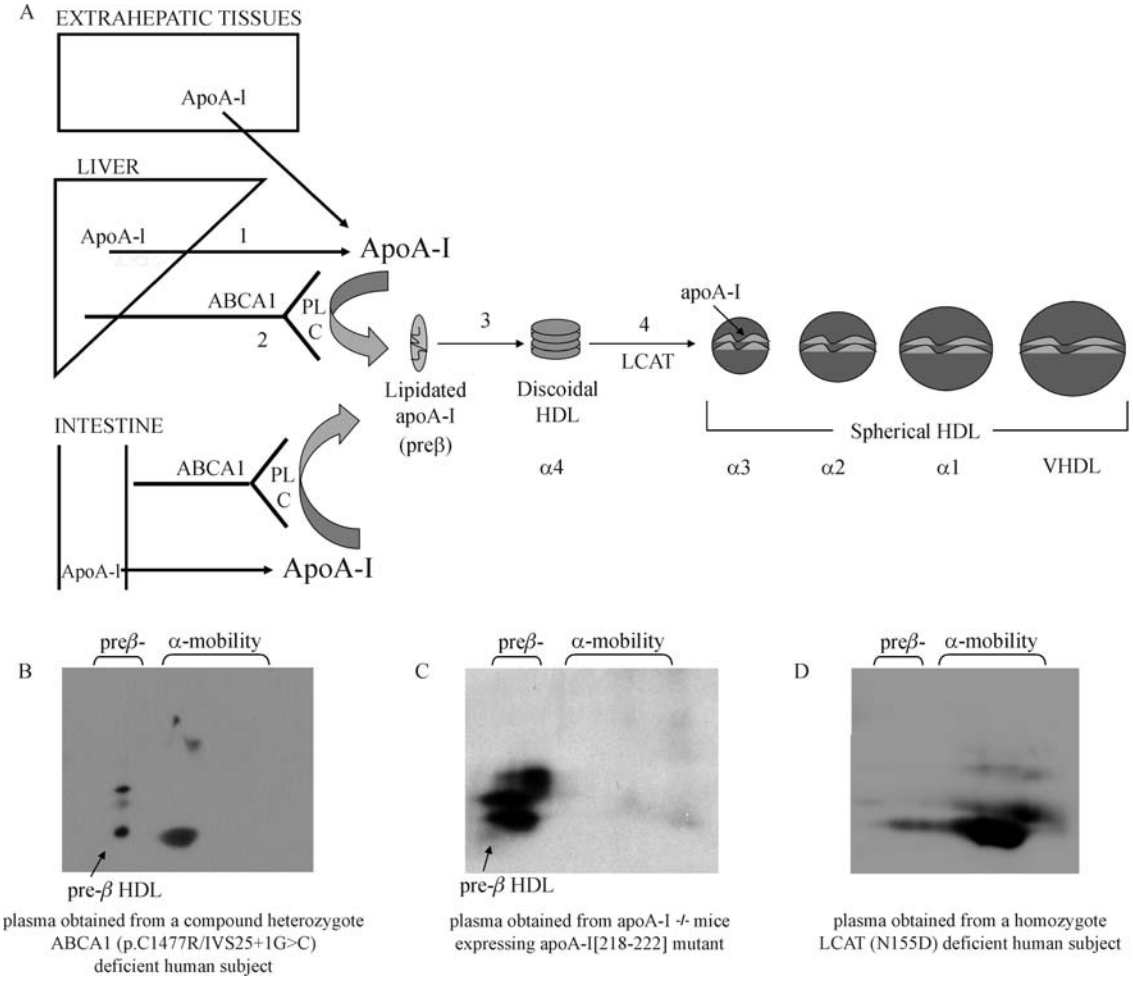

Fig. 1 **The pathway of the biogenesis of HDL and the consequence of mutations in apoA-I, ABCA1 and LCAT.** Schematic representation of the pathway of the biogenesis of HDL by the liver, intestine and extra-hepatic tissues (A). The numbers 1, 2, 3 and 4 in this panel indicate the involvement of apoA-I, ABCA1 and LCAT in the pathway of HDL biogenesis. Two dimensional gel electrophoresis of plasma obtained from the *apoA-I* $^{-/-}$ mice expressing apoA-I[L218A/L219A/V221A/L222A]. These mutations inhibit the conversion of preβ to discoidal and spherical αHDL particles[3] (C). Two dimensional gel electrophoresis of plasma obtained from a compound heterozygote (p.C1477R/IVS25 + 1G > C) deficient human subject with Tangier disease (B) and a homozygote *LCAT* (N155D) deficient human (D). Panels B and D were obtained from reference [4] with permission.

levels are positively associated with the risk of venous thrombosis[21–22], may have beneficial effects on glucose metabolism[23–24] and the function of beta pancreatic cells[25]. Interaction of HDL with the HDL receptor, SR-BI, initiates signaling pathways that promote endothelial cell proliferation and migration[26] as well as release of nitric oxide (NO) that causes vasodilation[27]. HDL also inhibits platelet aggregation and thrombosis[28] and has antibacterial, antiparasitic and antiviral activities[29–31]. Due to these properties HDL is thought to protect the endothelium and inhibit several steps in the cascade of events that lead to the pathogenesis of atherosclerosis and various other human diseases[9,32].

Structural features of apoA-I that contribute to the formation of HDL-A-I

ApoA-I contains 22- or 11-amino acid repeats, which are organized in amphipathic α-helixes[33–34]. A belt model was proposed, taking into account the 3-D structure of apoA-I in solution that was designed to explain the structure of apoA-I on discoidal HDL particles[35–37]. Based on structural work and cross-linking studies various models have been also proposed to explain the arrangement of apoA-I on spherical HDL particles[38–40].

ABCA1 and its *in vitro* interactions with apoA-I

The ABCA1 transporter is a ubiquitous protein that is synthesized by the liver and various other tissues[41–42]. It is localized only on the basolateral surface of the hepatocytes[43] and on endocytic vesicles and there is evidence that ABCA1 travels between late endocytic vesicles and the cell surface[44]. In cell cultures, ABCA1 promotes efflux of cellular phospholipids and cholesterol to lipid free or minimally lipidated apoA-I and other apolipoproteins, but not to spherical HDL particles[45–46].

Using cell culture and *in vitro* experiments, we investigated the ability of apoA-I mutants to promote ABCA1 mediated efflux of cholesterol and phospholipids and to crosslink to ABCA1.

It was found that the ABCA1 mediated efflux was diminished by C-terminal deletions in which residues 220-231 were removed, but was not affected by N-terminal deletions or deletion of the C-terminal 232-243 residues. Unexpectedly, efflux was restored to 80% of WT control by double deletions of both the amino- and carboxy-termini of apoA-I[2,47–48]. Lipid efflux was either unaffected or moderately reduced by a variety of point mutations or deletions of internal apoA-I helices. A reasonable interpretation of these findings is that different combinations of central helices can promote lipid efflux[49]. Chemical cross-linking/immunoprecipitation experiments showed that the ability of apoA-I mutants to promote ABCA1-depended lipid efflux was correlated with their ability to be cross-linked efficiently to ABCA1[49]. Other studies showed that the majority of the ABCA1 mutants that are found in patients with Tangier disease cross-link poorly to WT apoA-I and have diminished capacity to promote cholesterol efflux in vitro and HDL formation *in vivo*[50–51]. A notable exemption is the ABCA1[W590S] mutant which had diminished capacity to promote cholesterol efflux but cross-linked stronger to apoA-I than the WT ABCA1[50–52]. We suggested that in this case there is a strong but not productive binding of ApoA-I to ABCA1 that does not promote but rather prevents efficient lipid efflux[49].

In vivo interactions of ABCA1 with apoA-I lead to the biogenesis of HDL

Subjects carrying inactivating mutations in ABCA1 fail to form αHDL particles but instead they form preβ and other small size particles in their plasma[53–56] (***Fig. 1B***). Formation of only preβ particles was detected in the plasma of the experimental animals that express a specific mutant apoA-I form (***Fig. 1C***), as well as in the plasma of LCAT-deficient humans (***Fig. 1D***). The particles shown in panels 1B and 1C may be created by mechanisms that involve non-productive interactions between ABCA1 and apoA-I[3,57].

Animal studies showed that inactivation of the hepatic and intestinal *ABCA1* in mice led to the disappearance of HDL from plasma[56]. In liver-specific or whole body *ABCA1* knockout mice, plasma HDL catabolism and the fractional catabolic rate of HDL by the liver and to a lesser extent by the kidney and the adrenal is increased, resulting in the removal of the nascent HDL particles from plasma before they reach maturation[56]. Intestinal-specific inactivation of the *ABCA1* gene in mice decreased plasma HDL by 30%, but did not affect the apoA-I and cholesterol concentration in the lymph[58]. This implies that HDL that is produced in the intestine is not secreted into the lymph but is rather secreted directly into the plasma[58].

Specific mutations in apoA-I may affect apoA-I / ABCA1 interactions and inhibit the formation of spherical HDL

We have used adenovirus-mediated gene transfer of WT and mutant apoA-I to study the interactions of apoA-I with ABCA1. The adenoviruses were generated in cell cultures, purified, titrated and injected into the tail vein of apoA-I deficient or apoA-I and apoE double deficient mice. Four to five days post-infection plasma

was collected and analyzed for lipids and lipoproteins and by two-dimensional gel electrophoresis to identify the HDL subpopulations. The plasma was fractionated by density gradient ultracentrifugation and fast protein liquid chromatography (FPLC) and the HDL fraction was analyzed by electron microscopy (EM) to assess the size and shape of HDL[3,48]. Additionally, the hepatic mRNA levels of apoA-I were determined to ensure that there was comparable expression of the WT and the mutant apoA-I forms[3,47–48,59].

In the most recent studies, two sets of mutations within the 218-230 region of apoA-I, apoA-I[L218A/L219A/V221A/L222A] and apoA-I[F225A/V227A/F229A/L230A] (abbreviated apo A-I[218-222] and apoA-I[225-230]) were investigated using the methodologies described above[3,60]. Previous studies had shown that deletion of this region diminishes the ABCA1 mediated cholesterol efflux and inhibits the biogenesis of HDL[48,57].

Adenoviruses expressing the WT or the apoA-I[218-222] mutant were injected in *apoA-I* $^{-/-}$ and *apoA-I* $^{-/-}$ x *apoE* $^{-/-}$ mice and analyzed as described above[3,48]. It was found that the WT apoA-I when expressed in *apoA-I* $^{-/-}$ x *apoE* $^{-/-}$ mice floated predominantly in the HDL2/HDL3 region (***Fig. 2A***), generated spherical particles (***Fig. 2B***) and normal preβ and αHDL subpopulation (***Fig. 2C***). In contrast, the apoA-I[218-222] mutant when injected in *apoA-I* $^{-/-}$ x *apoE* $^{-/-}$ mice, the FPLC fractionation of the plasma showed the near absence of an HDL cholesterol peak[3], and density gradient ultracentrifugation of the plasma showed small amount of the apoA-I in HDL3 and in $d < 1.21$ g/mL fractions (***Fig. 2D***). EM analysis showed the presence of few discoidal particles along with larger particles corresponding in size to IDL/LDL (***Fig. 2E***) and two-dimensional gel electrophoresis showed the presence of only preβ HDL particles (***Fig. 2F***). When the WT apoA-I and the apoA-I[218-222] mutant were injected in *apoA-I* $^{-/-}$ mice, the plasma apoA-I levels of the mutant were 15% of those of the WT apoA-I[3].

Co-expression of the apoA-I[218-222] mutant and human LCAT in *apoA-I* $^{-/-}$ x *apoE* $^{-/-}$ mice did not restore the HDL cholesterol peak as determined by FPLC analysis. Density gradient ultracentrifugation showed only small amount of the apoA-I in the HDL3 fraction (***Fig. 2G***), EM analysis showed the presence of a small number of HDL size particles (***Fig. 2H***), and the two-dimensional gel electrophoresis showed the presence of small amount of preβ and α4 HDL particles (***Fig. 2I***). These findings demonstrate that the LCAT treatment failed to convert efficiently the preβ to αHDL particles or to correct this specific defect in the biogenesis of HDL.

In addition to the unique role of the L218/L219/V221/L222 residues in the biogenesis of HDL, the same residues are also required to confer trans-endothelial transport capacity[61] and bactericidal activity[62] to apoA-I. These overall properties suggest that the L218/L219/V221/L222 residues represent an effector domain for several activities of apoA-I.

The phenotype generated by the expression of the apoA-I[225-230] mutant was similar to that obtained with the apoA-I[218-222] mutant. However co-expression of the apoA-I[225-230] mutant and human LCAT corrected the abnormal HDL levels, created normal preβ and αHDL subpopulations and generated spherical HDL particles[60].

Overall, the phenotype produced by the apoA-I[218-222] mutant is distinct from all other phenotypes generated previously by apoA-I mutations. This phenotype cannot be corrected by overexpression of LCAT. Although other interpretations are possible, the in vivo and in vitro data suggest that the interaction of the apoA-I[218-222] mutant with ABCA1 results in defective lipidation of apoA-I that leads to the generation of aberrant preβ HDL particles. It appears that these particles are not a good substrate for LCAT and cannot proceed to form discoidal and spherical HDL. If this interpretation is correct, the normal lipidation of apoA-I may require a precise initial orientation of the apoA-I ligand within the binding site of ABCA1 that is similar to that described for enzyme-substrate interactions. Correct configuration would allow correct lipidation of apoA-I, which, could subsequently undergo cholesterol esterification and formation of mature spherical HDL particles. In our case the incorrectly lipidated apoA-I becomes a poor substrate for LCAT and cannot form discoidal and spherical HDL particles.

Interactions of lipid-bound ApoA-I with LCAT lead to cholesterol esterification and formation of spherical HDL

Plasma LCAT is a 416 amino acid long plasma protein that interacts with discoidal and spherical HDL and catalyzes the transfer of the 2-acyl group of lecithin or phosphatidylethanolamine to the free hydroxyl residue of cholesterol to form cholesteryl ester, using apoA-I as an activator[63,64]. It also catalyzes the reverse reaction of esterification of lysolecithin to lecithin[65]. The esterification of free cholesterol of HDL in vivo and in vitro converts the discoidal to mature spherical HDL particles[66,67]. Mutations in LCAT are associated with two phenotypes in humans. The first is the familial LCAT deficiency (FLD) which is characterized by the

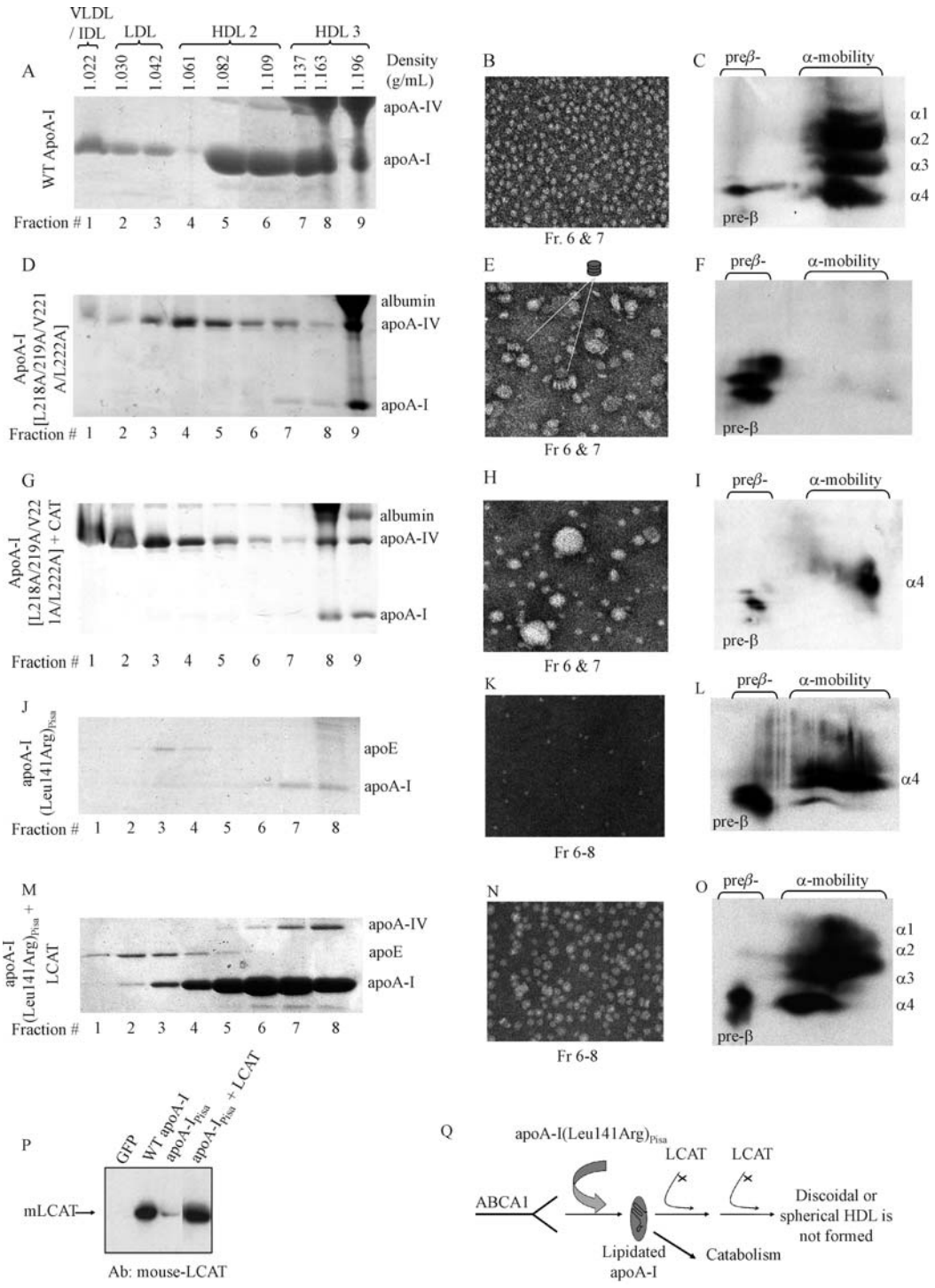

Fig. 2 **Analyses of the phenotype of the apoA-I[218-222] mutation that inhibits the conversion of the preβ to αHDL particles and the apoA-I[L141R]$_{Pisa}$ mutation that influences the activity of LCAT and is associated with low plasma HDL levels.** Analysis of plasma of *apoA-I* $^{-/-}$ x *apoE* $^{-/-}$ mice infected with adenoviruses expressing the WT apoA-I or the apoA-I[218-222] mutant alone or in combination with LCAT as indicated, by density gradient ultracentrifugation and SDS-PAGE of the resulting fractions (A, D, G). EM analysis of HDL fractions 6-7 obtained from density gradient ultracentrifugation of plasma (B, E, H). Two dimensional gel electrophoresis of plasma (C, F, I). Analysis of plasma of *apoA-I* $^{-/-}$ mice infected with adenoviruses expressing the apoA-I[L141R]$_{Pisa}$ mutant alone or in combination with LCAT by density gradient ultracentrifugation and SDS-PAGE (J, M). EM analysis of HDL fractions 6-8 obtained from density gradient ultracentrifugation of plasma (K, N). Two dimensional gel electrophoresis of plasma (L, O). Western blot analysis of plasma from *apoA-I* $^{-/-}$ mice infected with adenoviruses expressing either the control protein, GFP, or the WT apoA-I or the apoA-I[L141R]$_{Pisa}$ alone or in combination with LCAT, as indicated at the top of the figure (P). Schematic representation showing how the apoA-I(L141R)$_{Pisa}$ mutation may disrupt the pathway of biogenesis of HDL and lead to the fast catabolism of the nascent preβ HDL particles before they have the chance to proceed in the formation of discoidal and spherical HDL particles (Q). ***Fig. 2*** was obtained from reference [71] with permission

inability of the mutant LCAT to esterify cholesterol on HDL and LDL and leads to the accumulation of discoidal HDL in the plasma. The second is the fish eye disease (FED) which is characterized by the inability of mutant LCAT to esterify the cholesterol of HDL whereas it maintains its ability to esterify the cholesterol of LDL. Both diseases are characterized by low HDL levels[68] and formation of preβ and α4 HDL subpopulations.

Analysis of plasma of LCAT deficient mice by 2D gel electrophoresis showed the presence of preβ and small size α4 HDL particles. Expression of human LCAT by adenovirus mediated gene transfer in LCAT deficient mice generated large size αHDL subpopulations. In contrast, expression of the LCAT [T147I] mutant generated preβ HDL along with small size α4, α3 and α2 HDL subpopulations[69].

ApoA-I mutations that affect apoA-I/LCAT interactions

Several naturally occurring apoA-I mutations reduce the capacity of apoA-I to activate LCAT in vitro[2,70]. Two of these mutations, the apoA-I[L141R]$_{Pisa}$ and the apoA-I[L159R]$_{FIN}$, were studied in detail by adenovirus mediated gene transfer[71].

Earlier studies had shown that hemizygotes for an *apoA-I* null allele and an apoA-I[L141R]$_{Pisa}$ allele had greatly decreased plasma apoA-I levels and near absence of HDL cholesterol and abnormal HDL subpopulations.

Other studies also showed that heterozygotes for apoA-I[L159R]$_{FIN}$ mutation had greatly reduced plasma levels of HDL cholesterol and apoA-I, abnormal HDL subpopulations and increased catabolism of apoA-I[72].

In vitro studies initially showed that both mutants were secreted efficiently from cells, had normal ability to promote ABCA1-mediated cholesterol efflux but greatly diminished capacity to activate LCAT (0.4%-2% of WT apoA-I)[71]. Adenovirus-mediated gene transfer showed that compared to WT apoA-I (***Fig. 2A***), expression of either of the two mutants in apoA-I $^{-/-}$ mice greatly decreased total plasma cholesterol and apoA-I levels as well as the HDL cholesterol peak as determined by FPLC fractionation of the plasma. Density gradient ultracentrifugation of plasma showed great reduction of the amount of apoA-I that floated in the HDL region of the apoA-I[L141A]$_{Pisa}$ mutant as compared to WT apoA-I (***Fig. 2J***). EM analysis of the HDL fractions obtained by density gradient ultracentrifugation showed the presence of a large number of spherical HDL for the WT apoA-I but only a few spherical HDL particles for the apoA-I[L141A]$_{Pisa}$ mutant (***Fig. 2K***). Two-dimensional gel electrophoresis of the plasma showed the formation of small amount of preβ HDL and large amount of α1, α2, α3, and, α4 HDL subpopulations for the WT apoA-I and only preβ and small size α4-HDL subpopulations for the apoA-I [L141A]$_{Pisa}$ mutant (***Fig. 2L***).

Co-infection of *apoA-I* $^{-/-}$ mice with adenoviruses expressing either of the two mutants and human LCAT normalized the plasma apoA-I, the total plasma cholesterol levels and the CE/TC ratio, increased the HDL cholesterol peak and the amount of apoA-I that floated in the HDL region (***Fig. 2M***). It also generated large amount of spherical HDL (***Fig. 2N***) and restored the normal preβ and αHDL subpopulations (***Fig. 2O***). Similar results were obtained for the apoA-I[L159R]$_{FIN}$ mutant[71]. Analysis of the relative abundance of the endogenous mouse LCAT following gene transfer in *apoA-I* $^{-/-}$ mice showed a dramatic decrease of the mouse LCAT in mice expressing the apoA-I[L141R]$_{Pisa}$ mutant alone as compared to mice expressing the WT apoA-I. The mouse LCAT levels could be restored to normal by co-infection of *apoA-I* $^{-/-}$ mice with the apoA-I [L141R]$_{Pisa}$ mutant and human LCAT (***Fig. 2P***). The depletion of the endogenous mouse LCAT could be the result of rapid degradation of endogenous mouse LCAT bound to minimally lipidated apoA-I mutants possibly by the kidney (***Fig. 2Q***).

Another interesting naturally occurring apoA-I mutation is the apoA-I[R160L]$_{Oslo}$. Previous studies showed that heterozygotes of apoA-I[R160L]$_{Oslo}$ have approximately 60% and 70% of normal HDL and apoA-I levels respectively, form preβ1 and small size αHDL particles and have a 30% reduction in their plasma LCAT activity[73]. Gene transfer of the apoA-I[R160L]$_{Oslo}$ mutant in *apoA-I* $^{-/-}$ mice resulted in low plasma cholesterol and apoA-I levels and generated discoidal particles with α4 electrophoretic mobility. The aberrant phenotype could be corrected by co-expression of this mutant with human LCAT[67].

Similar but not identical phenotypes were produced by expressing the bioengineered apoA-I[R160V/H162A] and apoA-I[R149A] mutants and the naturally occurring mutants apoA-I[R151C]$_{Paris}$ and apoA-I [L144R]$_{Zaragosa}$[67,74–75]. These phenotypes could be corrected by co-expression of the mutant apoA-I with human LCAT.

ApoA-I mutations may induce hypertriglyceridemia and/or hypercholesterolemia

An apoA-I deletion mutant, the apoA-I[Δ89-99], when expressed in *apoA-I* $^{-/-}$ mice, increased plasma cholesterol levels, increased the plasma preβ HDL

subpopulation, generated discoidal HDL particles, but did not induce hypertriglyceridemia[76].

Furthermore, a series of apoA-I mutations that included the apoA-I[Δ(62-78)], the apoA-I [E110A/E111A], and apoA-I[D89A/E91A/E92A] in apoA-I $^{-/-}$ mice resulted in severe hypertriglyceridemia[76–78]. In the case of apoA-I[E110A/E111A] and apoA-I[D89A/E91A/E92A] mutations, negatively charged residues were substituted by alanines. In the case of the apoA-I[Δ(62-78)] deletion, there was a loss of the negatively charged residue E78. As explained below, the loss of negatively charged residues may be a determining factor for the development of hypertriglyceridemia. All apoA-I mutants of this category were studied in detail by in vivo and in vitro experiments. The studies involving the apoA-I[D89A/E91A/E92A] mutant are presented in some detail bellow[78].

The in vitro studies showed that the capacity of the apoA-I[D89A/E91A/E92A] mutant to promote ABCA1-mediated cholesterol efflux and activate LCAT was approximately 2/3 of that of WT apoA-I. However, the in vivo analyses following adenovirus-mediated gene transfer in *apoA-I* $^{-/-}$ mice showed that compared to WT apoA-I, this mutant increased plasma cholesterol, reduced the HDL cholesterol peak and the CE/TC ratio, and caused severe hypertriglyceridemia (***Table 1***)[78]. Following density gradient ultracentrifugation of plasma, approximately 40% of the apoA-I mutant was distributed in VLDL/IDL region as compared to the WT that was distributed predominantly in the HDL2/HDL3 region (***Fig. 3A, B***). EM analysis showed that the apoA-I[D89A/E91A/E92A] mutant formed mostly spherical and few discoidal HDL particles (***Figs. 3E***), whereas the WT apoA-I formed exclusively spherical particles (***Fig. 3D***). Two-dimensional gel electrophoresis showed that the apoA-I [D89A/E91A/E92A] mutant formed mostly preβ and some α4 HDL subpopulations (***Fig. 3H***) and the WT apoA-I formed small amounts of preβ and normal αHDL subpopulations (***Fig. 3G***)[78].

Overall these analyses showed that the apoA-I [D89A/E91A/E92A] mutations generated an abnormal lipid and lipoprotein profile characterized by severe hypertriglyceridemia and defective maturation of HDL. The phenotype generated by this mutant is distinct. Another apoA-I[K94A/K96A] mutant in the vicinity of residues 89-92, where the positively charged residues were changed to alanines, had similar lipid, lipoprotein and HDL profiles to those of WT apoA-I[78].

Co-expression of apoA-I[D89A/E91A/E92A] mutant and human lipoprotein lipase in *apoA-I* $^{-/-}$ mice abolished hypertriglyceridemia, redistributed apoA-I in the HDL2/HDL3 regions (***Fig. 3C***), restored in part the α1, 2, 3, and 4 HDL subpopulations (***Fig. 3I***), but did not change significantly the CE/TC ratio or the formation of discoidal HDL particles (***Fig. 3F***)[78].

The persistence of discoidal particle following the lipoprotein lipase treatment indicates a direct effect of the [D89A/E91A/E92A] mutations in the activation of LCAT in vivo.

Similarly to apoA-I[D89A/E91A/E92A], two other mutants in different regions of apoA-I, the apoA-I[Δ(62-78)] and the apoA-I [E110A/E111A][76–77], caused hypertriglyceridemia (***Table 1***). The affinity of the apoA-I[D89A/E91A/E92A], apoA-I[Δ(62-78)], and apoA-I [E110A/E111A] mutants for triglyceride (TG)-

Table 1 **Plasma lipids and hepatic mRNA levels of *apoA-I* $^{-/-}$ or *apoA-I* $^{-/-}$ x *apoE* $^{-/-}$ mice expressing WT and the mutant forms of human apoA-I or apoE4 or apoA-IV in the presence and absence of human lipoprotein lipase (hLPL) or human LCAT (hLCAT) as indicated.**

Protein expressed in apoA-I $^{-/-}$ mice	Cholesterol (mg/dL)	CE/TC	Triglycerides (mg/dL)	Relative apoA-I/E4/A-IV mRNA (%)	Plasma apoA-I/E4/A-IV (mg/dL)
apoA-I $^{-/-}$	33±6	-	42±7	-	-
WT apoA-I	268±55	0.72±0.06	70±11	100±32	283±84
apoA-I [D89A/E91A/E92A]	497±139	0.36±0.31	2106±1629	101±24	235±106
apoA-I [D89A/E91A/E92A] + hLPL	122±56	0.44±0.14	49±16	41±6	99±18
apoA-I [Δ(62-78)]	220±16	-	986±289	130±5	265±36
apoA-I [E110A/E111A]	520±45	-	1510±590	69±23	204±27
WT apoE4	561±6	-	1368±102	100	154±4
WT apoE4 + hLCAT	347±8	-	77±11	218	89±2
WT apoA-IV expressed in apoA-I $^{-/-}$ mice	52±17	-	18±12	100	-
apoA-I $^{-/-}$ x apoE $^{-/-}$	450±136	-	112±80	-	-
WT apoA-IV expressed in apoA-I $^{-/-}$ x apoE $^{-/-}$ mice	746±116	-	449±79	70	-

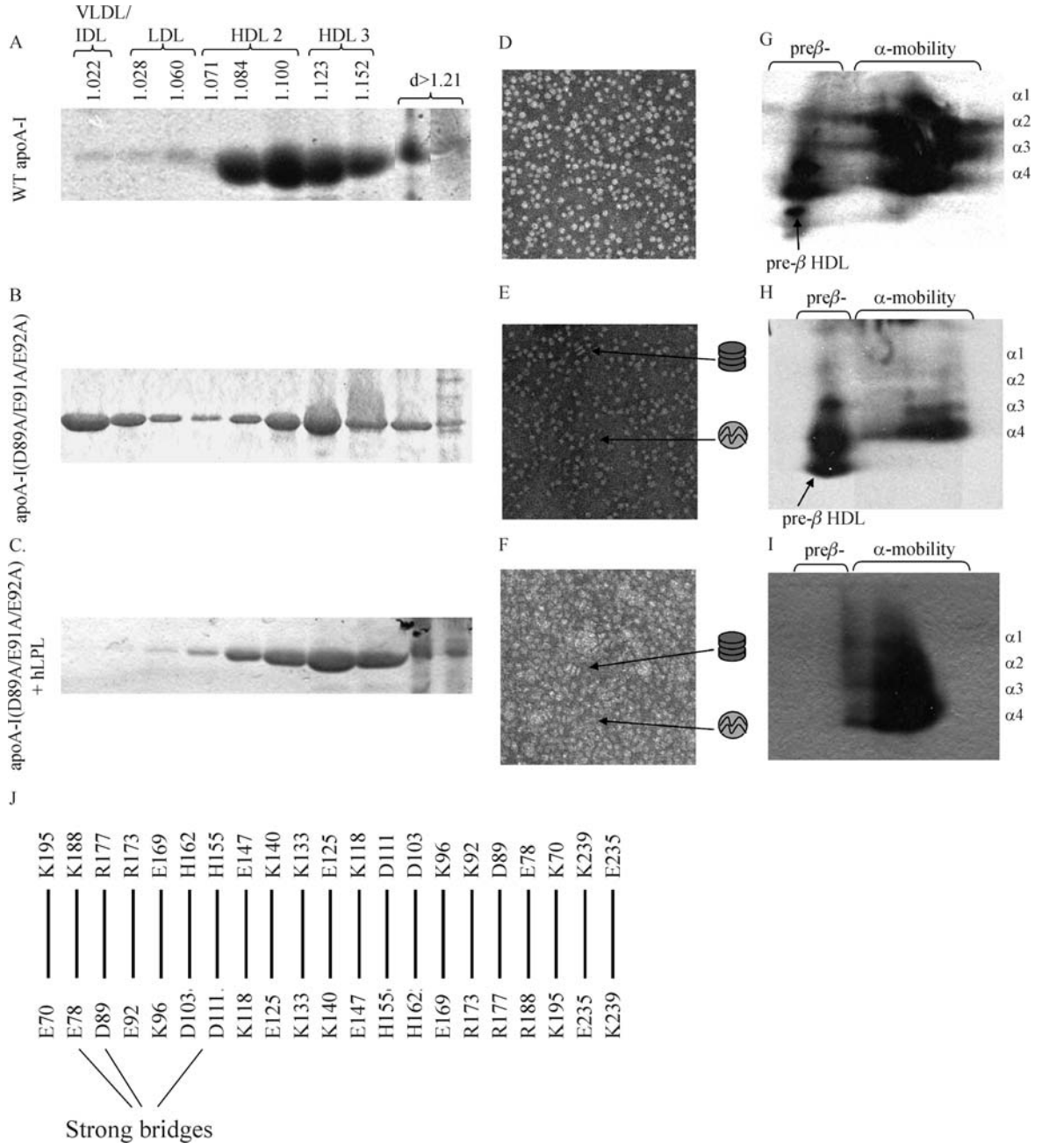

Fig. 3 **Analyses of the phenotype of the apoA-I[D89A/E91A/E92A] mutation that induces hypertriglyceridemia and the potential role of solvent inaccessible salt bridges in the induction of hypertriglyceridemia.** Analysis of plasma of *apoA-I*$^{-/-}$ mice infected with adenoviruses expressing the WT apoA-I or the apoA-I[D89A/E91A/E92A] mutant alone or in combination with human LPL, as indicated. Density gradient ultracentrifugation and SDS-PAGE of the resulting fractions (A-C). EM analysis of HDL fractions 6-7 following density gradient ultracentrifugation of plasma as indicated (D-F). Two dimensional gel electrophoresis of plasma, as indicated (G-I). Schematic representation of the solvent inaccessible interhelical charged interactions of apoA-I dimers arranged in an antiparallel orientation in the belt model of discoidal HDL (rHDL) (J).

rich lipoprotein particles is further supported by binding studies of these mutants to TG-rich emulsion particles[78–79].

A common feature of all these three mutants is that they caused accumulation of apoA-I in the VLDL/IDL region. It was shown previously that apoA-I containing TG-rich lipoprotein fractions inhibited their *in vitro* lipolysis by exogenous lipoprotein lipase[76–77]. All three apoA-I mutants studied have lost negative charged residues that are present in the WT sequence. The E78, D89 and E111 residues that are located in helices 2, 3, and 4 of the WT apoA-I have the ability to form solvent inaccessible salt bridges with the positively charged residues R188, R177 and H155 present in antiparallel

helices 8, 7, and 6 respectively on the surface of a discoidal HDL particle[37] (***Fig. 3J***). It is conceivable that loosening of the structure of apoA-I around the D89 or E92 area due to the substitution of the original residues by alanines may provide new surfaces for interaction of HDL with other proteins or lipoprotein particles such as VLDL in ways that inhibit TG hydrolysis.

The preceding analyses described in ***Fig. 2*** and ***3*** demonstrate that expression of mutant apoA-I forms in different mouse models disrupted specific steps along the pathway of the biogenesis of HDL and generated discrete lipid and HDL phenotypes[80]. ***Fig. 4A*** shows the location of the apoA-I mutations in the secondary structure of apoA-I. The phenotypes generated by these mutations include inhibition of the formation of HDL[3,48]; generation of unstable intermediates[75]; inhibition of the activation of LCAT[67]; increase in plasma cholesterol or increase in both plasma cholesterol and TG[76–78] (***Fig. 4B***).

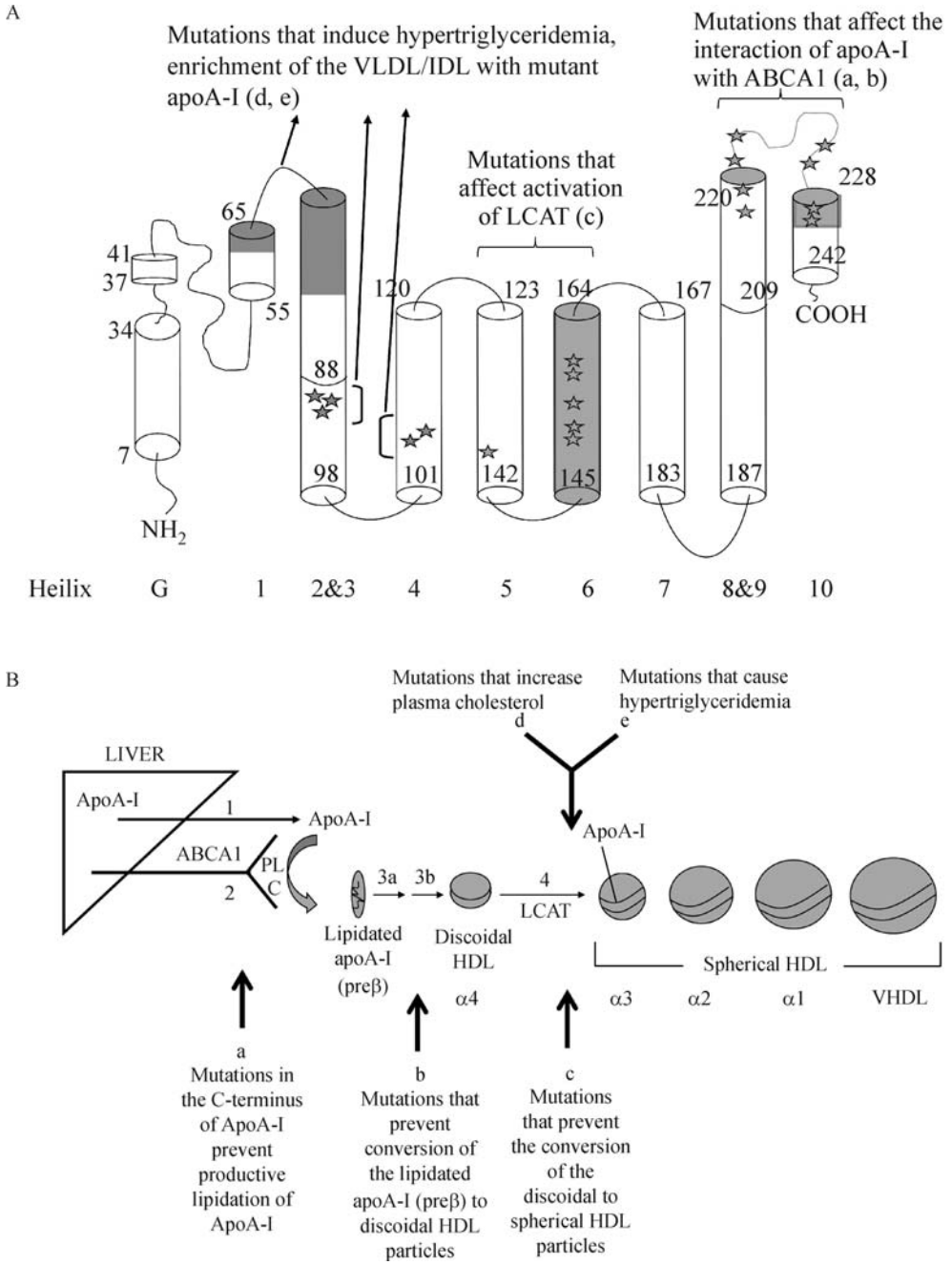

Fig. 4 **Schematic representation of the secondary structure of apoA-I[33,81–82].** Superimposed on the model are the different categories of mutations that are described in panel B (A). The pathway of biogenesis of HDL showing how different categories of apoA-I mutations disrupt distinct steps in the biogenesis of HDL or cause dyslipidemia, VHDL indicates very large HDL particles (B).

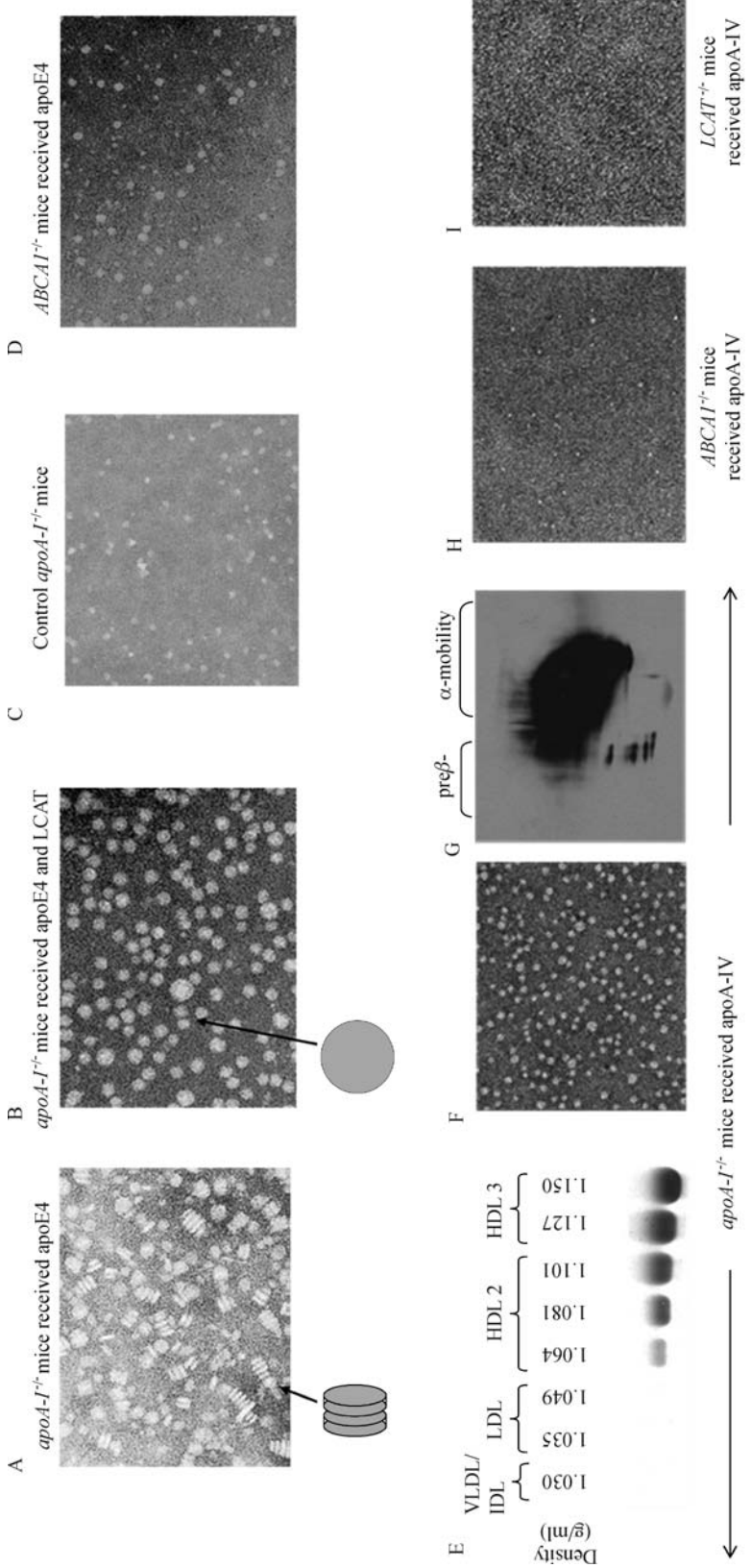

Fig. 5 **Participation of apoE and apoA-IV in the biogenesis of apoE- and apoA-IV containing HDL particles.** EM analysis of *apoA-I* $^{-/-}$ mice following infection with adenovirus expressing human apoE4 alone or in combination with human LCAT as indicated (A, B). EM analysis of *ABCA1* $^{-/-}$ mice following infection with adenovirus expressing human apoE4 (D) and of control *apoA-I* $^{-/-}$ mice (C). Analysis of plasma by density gradient ultracentrifugation and SDS-PAGE of *apoA-I* $^{-/-}$ mice infected with adenoviruses expressing human apoA-IV (E). EM analysis of HDL fractions 6-7 obtained from *apoA-I* $^{-/-}$ mice expressing human apoA-IV (F). The HDL fractions were obtained by density gradient ultracentrifugation of plasma. Two dimensional gel electrophoresis of plasma of *apoA-I* $^{-/-}$ mice infected with adenoviruses expressing human apoA-IV (G). EM analysis of HDL fractions 6-7 obtained from *ABCA1* $^{-/-}$ mice and *LCAT* $^{-/-}$ mice, as indicated (H, I).

ApoE and apoA-IV participate in the biogenesis of HDL particles containing the corresponding apolipoproteins

In vitro studies have shown that lipid-free apoE and apoA-IV promoted ABCA1-mediated cholesterol efflux, with the same efficiency as apoA-I[83–84]. Other studies have indicated that apoE may participate in the early stage of apoB lipidation in the ER[85] but no role has been assigned to apoE for the biogenesis of HDL.

Our in vivo studies have shown that apoE, regardless of its phenotype, participates in the biogenesis of apoE-containing HDL particles (HDL-E) using a similar pathway that is used for the biogenesis of apoA-I containing HDL particles[86]. Initially, gene transfer of anapoE4-expressing adenovirus in *apoA-I* $^{-/-}$ mice, which cannot synthesize HDL, increased both the HDL and the TG-rich VLDL/IDL/LDL fraction, generated discoidal HDL-E particles (***Fig. 5A***), and induced hypertriglyceridemia. The essential role of LCAT for the maturation of HDL-E was established by co-infection of the *apoA-I* $^{-/-}$ mice with a mixture of adenoviruses expressing both apoE4 and human LCAT. This treatment converted the discoidal to spherical HDL-E particles (***Fig. 5B***). It also cleared the TG-rich lipoproteins found in the VLDL/IDL/LDL region and increased the HDL cholesterol peak as determined by FPLC[86]. Control *apoA-I* $^{-/-}$ mice did not form HDL size particles (***Fig. 5C***). The involvement of ABCA1 in the biogenesis of HDL-E was established by gene transfer of apoE4 in *ABCA1* $^{-/-}$ mice. HDL was not present in the plasma of these mice after treatment with the apoE4 expressing adenovirus (***Fig. 5D***), indicating that apoE4 could not promote formation of HDL-E particles in the absence of ABCA1.

Overall, our findings combined with previous knowledge of the functions of apoE indicate that apoE has a dual functionality. In addition to its documented role in the clearance of TG-rich lipoproteins, it participates in the biogenesis of HDL-E in a process that is similar to that of apoA-I.

HDL-E thus formed may have antioxidant[13–14] and anti-inflammatory[16,18–20] and other functions similar to those described for apoA-I-containing HDL[9], which may account for its atheroprotective properties[87]. In addition, apoE-containing HDL may also have important biologic functions in the brain[88].

A similar set of gene transfer experiments established that similar to apoE, apoA-IV also participates in the biogenesis of apoA-IV containing HDL (HDL-AIV) and requires for this purpose the activity of ABCA1 and LCAT[84].

Thus, gene transfer of apoA-IV in *apoA-I* $^{-/-}$ mice, that express the endogenous mouse apoE gene, did not change plasma lipid levels and resulted in the appearance of the apoA-IV in HDL2/HDL3 region (***Fig. 5E***). This treatment also generated spherical particles (***Fig. 5F***) and α- and few preβ-like HDL subpopulations (***Fig. 5G***). Spherical HDL particles were not detectable following gene transfer of apoA-IV in *ABCA1* $^{-/-}$ or *LCAT* $^{-/-}$ mice (***Fig. 5H, I***). These findings indicate that both ABCA1 and LCAT are required for the biogenesis of HDL-AIV[84]. The ability of apoA-IV to promote biogenesis of HDL-AIV may explain its previously reported anti-inflammatory[89–90] and atheroprotective[90–92] properties.

In contrast to the experiments performed in *apoA-I* $^{-/-}$ mice, gene transfer of apoA-IV in the *apoA-I* $^{-/-}$ x *apoE* $^{-/-}$ mice, resulted in the distribution of 80% of apoA-IV in the VLDL/IDL region where apoB is also found and the mice developed hypertriglyceridemia (***Table 1***). This suggests that deficiency of both apoA-I and apoE may have increased the affinity of apoA-IV for apoB-containing lipoprotein particles and this might have triggered hypertriglyceridemia[84]. Other studies also showed that hepatic expression of apoA-IV in mice enhanced triglyceride secretion, reduced hepatic triglyceride content and increased the size of the VLDL particle without changing apoB secretion[93]. The opposite effect was obtained when SREBP-1a transgenic mice were crossed with *apoA-IV* $^{-/-}$ mice. In *SREBP-1a-Tg* x *apoA-IV* $^{-/-}$ mice, the hepatic triglyceride secretion rate and the size of the VLDL particles were decreased without change in apoB production[93]. Consistently with the in vivo data, earlier cell culture studies in McA-RH7777 rat hepatoma cells had shown that apoA-IV increased triglyceride and VLDL secretion[94]. In the light of these studies[93–94] a possible interpretation of our results could be that in the absence of apoA-I, the expression of apoA-IV in the liver promotes the formation of HDL-AIV. However, in the absence of both apoA-I and apoE, there appears to be an enhanced interaction of apoA-IV with apoB in the secretory pathway that increases VLDL secretion and causes hypertriglyceridemia. This hypothesis can be tested by measurement of VLDL triglyceride secretion rate in mice expressing apoA-IV in *apoA-I* $^{-/-}$ x *apoE* $^{-/-}$ mice.

Clinical relevance of the aberrant HDL phenotypes

The HDL phenotypes observed in human patients carrying the apoA-I[L141R]$_{Pisa}$ and apoA-I[L159R]$_{FIN}$ mutations resemble closely the phenotypes observed in *apoA-I* $^{-/-}$ mice expressing these mutants and indicates the validity of the gene transfer studies in mice to

establish defects in HDL biogenesis. It is possible that phenotypes generated by mutagenesis of apoA-I may exist in the human population and can be detected by one or more of the analyses described above. The correction of the aberrant HDL phenotypes by treatment with LCAT suggests a potential therapeutic intervention for HDL abnormalities that result from specific mutations in apoA-I or other conditions that result in low HDL levels[95].

The potential contribution of apoA-I mutations to hypertriglyceridemia in humans is interesting. Hypertriglyceridemia resulting from apoA-I mutations may be further aggravated by other genetic and environment factors such as diabetes and thyroid status. The contribution of apoA-I mutations to hypertriglyceridemia could be addressed in future studies in selected populations of patients with hypertriglyceridemia of unknown etiology.

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