# High density lipoprotein particle size restriction in apolipoprotein A-I$_{Milano}$ transgenic mice

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**Abstract** Human carriers of apolipoprotein A-I$_{Milano}$ ($Arg_{173}$ → Cys substitution in apolipoprotein A-I) are characterized by an HDL deficiency in which small, dense HDL accumulate in plasma. Because affected individuals are heterozygous for this mutation, the full impact of apolipoprotein A-I$_{Milano}$ (apoA-I$_{Milano}$) on HDL-cholesterol metabolism is unknown. In this study, apoA-I$_{Milano}$ transgenic mice were used to evaluate the extent of apoA-I$_{Milano}$ dimerization and HDL particle size restriction in the absence of wild-type apoA-I. Murine apoA-I knockout mice were utilized to express apoA-I$_{Milano}$ and human apoA-II in the presence of wild-type, human apoA-I (apoA-I*Milano*/A-I*wt*/A-II) and in its absence (apoA-I*Milano*/A-II). Plasma HDL-cholesterol concentrations were similar (30 mg/dl) in both lines of apoA-I$_{Milano}$ transgenic mice. In the apoA-I*Milano*/A-I*wt*/A-II phenotype, 14% of the apoA-I$_{Milano}$ formed homodimers and 33% formed heterodimers with apoA-II. ApoA-I$_{Milano}$ homodimers increased by 71% in the apoA-I*Milano*/A-II transgenics and was associated with an abundance of small, 7.6-nm $HDL_3$-sized particles compared to the 9.5, 8.3, and 7.6-nm-sized particles in apoA-I*Milano*/A-I*wt*/A-II mice. The unesterified cholesterol/cholesteryl ester mole ratio of HDL was elevated by 45% in apoA-I*Milano*/A-I*wt*/A-II mice and by 90% in apoA-I*Milano*/A-II transgenics compared to wild-type (human apoA-I/A-II). Both apoA-I$_{Milano}$ transgenics possessed normal levels of plasma LCAT activity, but endogenous cholesterol esterification rates were reduced by 50% compared to controls. Thus, HDL particle size restriction was not the result of impaired LCAT activation; rather, dimerization of apoA-I$_{Milano}$ limited the esterification of cholesterol on endogenous HDL. In the absence of wild-type apoA-I, the more extensive dimerization of apoA-I$_{Milano}$ severely limited cholesteryl ester accumulation on plasma HDL accounting for the abundance of small, 7.6-nm $HDL_3$ particles in apoA-I*Milano*/A-II mice.—**Bielicki, J. K., T. M. Forte, M. R. McCall, L. J. Stoltzfus, G. Chiesa, C. R. Sirtori, G. Franceschini, and E. M. Rubin.** High density lipoprotein particle size restriction in apolipoprotein A-I$_{Milano}$ transgenic mice. *J. Lipid Res.* 1997. **38:** 2314–2321.

**Supplementary key words** apolipoprotein A-I$_{Milano}$ • HDL size restriction • LCAT activity • transgenic mice

Plasma concentrations of high density lipoproteins (HDL) are inversely correlated with the development of atherosclerosis (1–3). The protective effects of HDL are due, in part, to its role in reverse cholesterol transport in which HDL mediates cholesterol efflux from peripheral cells and transports cholesterol to the liver for catabolism (4, 5). Apolipoprotein A-I (apoA-I) is the major protein of HDL and the physiological activator of lecithin:cholesterol acyltransferase (LCAT). The esterification of cholesterol in plasma catalyzed by LCAT is responsible for HDL maturation from small, cholesteryl ester-poor $HDL_3$ particles to larger, cholesteryl ester-rich $HDL_2$ subpopulations (6).

Mutations in apoA-I primary sequence often manifest HDL deficiencies, but only some of these mutations predispose to atherosclerosis (7–11). Individuals who inherit the apoA-I$_{Milano}$ mutation are heterozygous for an $Arg_{173}$ → Cys substitution in apoA-I primary sequence. Despite severe reductions in plasma HDL-cholesterol and apoA-I concentrations, affected subjects do not develop coronary artery disease (10, 11).

The $Arg_{173}$ → Cys substitution enables the dimerization of apoA-I$_{Milano}$ via a disulfide bridge and, in human carriers, apoA-I$_{Milano}$ homodimers and apoA-I$_{Milano}$/apoA-II heterodimers are abundant (12). As a consequence of apoA-I$_{Milano}$ dimerization, HDL-cholesterol metabolism is altered in affected carriers. Most noted is a skewing of the HDL particle size distribution where the $HDL_3$ subpopulations predominate over a nearly depleted $HDL_2$ (13). This restriction in HDL particle size may be related to a partial LCAT deficiency. Indeed, LCAT mass is reduced by about 50% in apoA-I$_{Milano}$ carriers (14). An alternative explanation for the accumulation of small $HDL_3$ particles is that the disulfide bridge prevents particle expansion thus limiting

Abbreviations: HDL, high density lipoproteins; apoA-I, apolipoprotein A-I; LCAT, lecithin:cholesterol acyltransferase; apoA-I$_{Milano}$, apolipoprotein A-I$_{Milano}$; apoA-II, apolipoprotein A-II; KO, knockout.

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the accumulation of cholesteryl esters in the particle core. This explanation is supported by the observations by Franceschini et al. (15) that conversion of $HDL_3$ to $HDL_2$ in the plasma of apoA-$I_{Milano}$ subjects can be accomplished by restoring apoA-$I_{Milano}$ to the monomeric form by chemical reduction of the disulfide bridge.

Because human carriers are heterozygous for the apoA-$I_{Milano}$ mutation, the full impact of apoA-$I_{Milano}$ dimerization on HDL particle size restriction cannot be assessed in vivo. Moreover, the question of whether wild-type apoA-I exerts an influence over apoA-$I_{Milano}$ dimerization is not possible to explore in humans but can be examined using transgenic mice.

In the present study, transgenic mice were created that expressed apoA-$I_{Milano}$ in the presence and absence of wild-type, human apoA-I. Comparisons between these mice revealed that *1*) regardless of the presence of wild-type apoA-I, apoA-$I_{Milano}$ formed an abundance of heterodimers with human apoA-II; *2*) the presence of wild-type apoA-I did, however, limit the extent of apoA-$I_{Milano}$ homodimerization; *3*) dimerization of apoA-$I_{Milano}$ restricted the HDL particle size distribution to small 7.6-nm particles; the accumulation in plasma of 7.6-nm particles was maximum in the absence of wild-type apoA-I; and *4*) wild-type apoA-I is not required for normal transport of murine LCAT on apoA-$I_{Milano}$ HDL. These new findings, together with our previously published work demonstrating that apoA-$I_{Milano}$ activates LCAT normally (16), suggest that dimerization of apoA-$I_{Milano}$ limits cholesterol esterification, in vivo, by imposing a steric constraint that limits HDL particle size expansion.

## MATERIALS AND METHODS

### Creation of apoA-$I_{Milano}$ transgenic mice

The apoA-$I_{Milano}$ mutation was prepared by site directed mutagenesis (Muta-Gene kit, Bio-Rad) using a 2.2-kb Pst1 genomic DNA fragment of the human apoA-I gene; DNA sequencing verified that no other sporadic mutations had occurred. Transgenic mice expressing the following combinations of human transgenes were used in the present study: apoA-I*Milano*/A-I*wt*/A-II and apoA-I*Milano*/A-II. DNA constructs were coinjected into fertilized eggs from the FvB stain of mice. Mice expressing human transgenes were crossed into C57BL/6 mice that lacked murine apoA-I (apoA-I KO) due to gene targeting (kindly provided by Dr. N. Maeda). Mice that had been bred for six to eight generations into the C57BL/6 strain were used in this study. Transgenic mice expressing wild-type, human apoA-I (A-I*wt*) and apoA-II (A-II) have been described (17).

### Plasma apolipoprotein, lipid, and lipoprotein concentrations in transgenic mice

Two- to eight-month-old mice were studied. After an overnight fast, blood was collected from a tail vein into tubes containing EDTA; plasma was obtained following low speed centrifugation (500 *g*) to remove blood cells; gentamicin sulfate (50 μg/ml) was added to prevent bacterial contamination; and plasma samples were stored at −20°C for 1 to 2 weeks before analyses. Plasma concentrations of apoA-I and A-II were determined by radial immunodiffusion as previously described (18). Triglycerides were quantified using a commercially available kit (Sigma diagnostics); plasma "free" glycerol concentrations were subtracted from each sample. Total cholesterol was measured using a reagent kit (Boehringer-Mannheim), and HDL cholesterol was determined in supernatants after dextran-sulfate precipitation of plasma (19). Unless otherwise stated, data from male and female mice were combined to calculate means.

### Physical/chemical properties of HDL from apoA-$I_{Milano}$ transgenic mice

High density lipoproteins (d 1.063–1.21 g/ml) were isolated from plasma (0.1 ml) of individual mice by preparative ultracentrifugation (20) using a type 42.2 ti rotor. After isolations, HDL was dialyzed into 0.15 mM NaCl containing 2.7 mM EDTA. Protein was measured by the method of Markwell et al. (21), phospholipid by the method of Chen, Toribara and Warner (22), and cholesterol as described by Sale et al. (23). SDS-PAGE was performed (24) to evaluate the distribution of apoA-$I_{Milano}$ homodimers, heterodimers, and monomers; gels were stained with Coomassie R-250 and the apolipoprotein distribution was determined by densitometric scanning. HDL particle size distribution was determined by nondenaturing gradient gel electrophoresis as described by Nichols, Krauss, and Musliner (25).

### Measurements of plasma LCAT activity

Two methods were used to measure plasma LCAT activity in transgenic mice. The first method utilized an exogenous proteoliposome substrate for quantification of total plasma LCAT activity as described by Chen and Albers (26). This method is not affected by changes in endogenous substrates and cofactors; thus, the absolute levels of plasma LCAT activity were evaluated. The proteolipsome substrate was composed of human apoA-I: egg-yolk phosphatidylcholine:unesterified cholesterol (0.8:250:12.5 mole ratios) containing trace amounts of [$^{14}$C]cholesterol (57.1 mCi/mmol). Reaction mixtures (0.25 ml final volume) contained saturating substrate concentrations of [$^{14}$C]cholesterol proteoliposomes ($4.4 \times 10^5$ dpm/ml), 20 mM Tris-HCl (pH 8.0), 0.15

TABLE 1. Plasma concentrations of human apolipoprotein A-I and A-II, lipids, and HDL-cholesterol in apoA-$I_{Milano}$ transgenic mice

| Phenotype | n | ApoA-I | ApoA-II | TC | HDL-Chol | TG |
|---|---|---|---|---|---|---|
| | | | | *mg/dl* | | |
| A-I*Milano*/A-I*wt*/A-II | 15 | 43 ± 12 | 35 ± 13 | 50 ± 9 | 28 ± 9 | 46 ± 18 |
| A-I*Milano*/A-II | 16 | 45 ± 13 | 31 ± 8 | 54 ± 9 | 31 ± 6 | 46 ± 22 |
| Murine A-I KO | 15 | np | np | 57 ± 17 | 38 ± 11 | 15 ± 8[a] |

Values are given as means ± SD; np, none present.
[a] $P < 0.001$ compared to apoA-I*Milano*/A-I*wt*/A-II and apoA-I*Milano*/A-II transgenic mice (Student's unpaired *t*-test).

mM NaCl, 0.27 mM EDTA, 0.5% human serum albumin, 2 mM β-mercaptoethanol, and 7.5 μl of mouse plasma. Samples were incubated at 37°C for 1 h. Reactions were stopped by cooling samples to 4° C and by adding ethanol (0.25 ml). Lipids were extracted with hexane. Cholesterol and cholesteryl esters were separated by thin-layer chromatography using toluene as the mobile carrier phase. Radioactivity was quantified by liquid scintillation counting, and results were expressed as initial rates (i.e., the percentage of [$^{14}$C]cholesterol at t = 0 that was converted to [$^{14}$C]cholesteryl esters per 1 h).

The second method used to measure plasma LCAT activity involved the direct labeling of mouse plasma with [$^{14}$C]cholesterol. Unlike the exogenous proteoliposome substrate procedure described above, this method is sensitive to changes in endogenous substrates and cofactor. For these measurements, pools of plasma were prepared from 3–5 mice in each group. Pooled plasma samples (0.1 ml final volume) were labeled at 4°C with 8.88 × $10^4$ dpm of [$^{14}$C]cholesterol added in ethanol. Samples were brought to 37°C; at specified times, aliquots (25 μl) were removed and lipids were extracted with hexane. Results were expressed as a percentage of the initial [$^{14}$C]cholesterol that was esterified. Three experiments were performed with different batches of pooled plasma obtained from different individual mice.

### Statistics

Student's unpaired *t*-test was used to evaluate significance.

## RESULTS

### Plasma lipid and apolipoprotein concentrations

**Table 1** shows the plasma concentrations of lipid and apolipoproteins in apoA-I*Milano*/A-I*wt*/A-II and apoA-I*Milano*/A-II transgenic mice; for comparison, data for murine apoA-I knockout (KO) mice are provided. Both apoA-$I_{Milano}$ transgenic mouse lines exhibited similar plasma concentrations of human apoA-I and apoA-II. No differences were observed in the concentration of total cholesterol and HDL-cholesterol between the apoA-$I_{Milano}$ transgenics and apoA-I KO mice. However, mice expressing apoA-$I_{Milano}$ showed a 3-fold increase in plasma triglycerides compared to apoA-I KO mice. The large variability in plasma triglyceride concentrations observed in apoA-$I_{Milano}$ transgenic mice is not unlike the wide range of plasma triglycerides seen in human apoA-$I_{Milano}$ carriers (13).

### Distribution of monomers, dimers, and heterodimers of apoA-$I_{Milano}$ in transgenic mouse plasma

SDS-PAGE (**Fig. 1**) of HDL (d 1.063–1.21 g/ml) isolated from apoA-$I_{Milano}$ transgenic mice revealed high molecular weight forms reflecting the formation of apoA-$I_{Milano}$ homodimers (56 kD) and apoA-$I_{Milano}$/A-II heterodimers (37 kD) in addition to the monomeric form of apoA-I (28 kD). Upon reduction with β-mercaptoethanol (lanes 6 and 7), apoA-$I_{Milano}$ migrated like that of wild-type apoA-I while human apoA-II migrated at 8.7 kD commensurate with monomeric apoA-II. Figure 1 also shows the major HDL-apolipoproteins found in murine apoA-I KO mice; proteins with molecular weights corresponding to murine apoE and apoA-II were consistently observed. Note that the expression of the human apolipoproteins dramatically reduces the amount of murine apoA-II associated with HDL.

The relative distributions of apoA-$I_{Milano}$ monomeric and dimeric forms, determined by densitometric scans of Coomassie-stained SDS-PAGE gels, is shown in **Table 2.** In the presence of wild-type apoA-I (apoA-I*Milano*/A-I*wt*/A-II mice), 14% of the protein was present as homodimers and 33% as apoA-$I_{Milano}$/A-II heterodimers. In the absence of wild-type apoA-I (apoA-I*Milano*/A-II mice), however, there was a 71% increase in the formation of apoA-$I_{Milano}$ homodimers. These results demonstrate that although apoA-$I_{Milano}$ readily forms heterodimers with human apoA-II, the presence of wild-type apoA-I limits the extent of apoA-$I_{Milano}$ homodimerization.

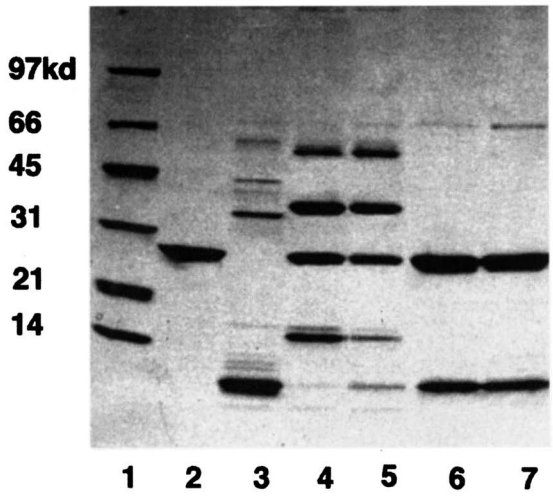


**Fig. 1.** Evaluation of HDL protein composition and apoA-$I_{Milano}$ dimerization by SDS-PAGE electrophoresis. HDL (d 1.063–1.21 g/ml) were isolated from 100 µl of plasma obtained from individual male mice by density gradient ultracentrifugation. A Coomassie R-250-stained gel is shown. Lane 1 shows molecular weight standards; lane 2 contains purified human apoA-I (MW = 28 kD). Lane 3 shows HDL from murine apoA-I KO mice; note the abundance of murine apoA-II (naturally occurring monomer). Lane 4 shows nonreduced HDL apolipoproteins from apoA-I*Milano*/A-I*wt*/A-II transgenic mice, and lane 5 nonreduced apolipoproteins from apoA-I*Milano*/A-II mice; lanes 6 and 7 show the reduced (5 mM β-mercaptoethanol) forms of apoA-$I_{Milano}$ and apoA-II in these samples, respectively. Under reducing conditions, molecular weight forms corresponding to monomeric human apoA-I and murine apoA-II were observed.

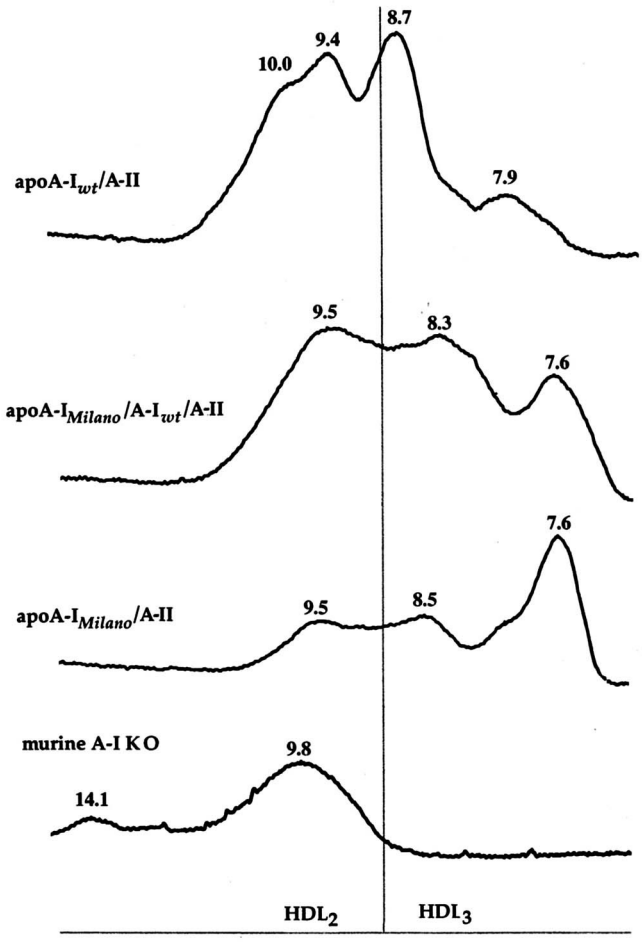


**Fig. 2.** HDL particle size distribution determined by nondenaturing gradient gel electrophoresis. HDL (d 1.063–1.21) was isolated from plasma pools (3–5 male mice/group) by density gradient ultracentrifugation. Shown are densitometric scans of a Coomassies G-250-stained gel. Numbers above the peaks are particle diameters in nanometers.

### Effect of apoA-$I_{Milano}$ on HDL size distribution

To determine whether expression of apoA-$I_{Milano}$ in mice restricts HDL particle size to the $HDL_3$-sized interval as in human apoA-$I_{Milano}$ carriers, HDL (d 1.063–1.21 g/ml) from apoA-I*Milano*/A-I*wt*/A-II and apoA-I*Milano*/A-II transgenic mice were evaluated by nondenaturing gradient gel electrophoresis. For comparison, the HDL particle size distribution of mice expressing wild-type, human apoA-I and A-II (apoA-I*wt*/A-II) are provided as these mice exhibit an HDL profile similar to that of normal humans (18). As shown in **Fig. 2,** HDL from apoA-I*wt*/A-II mice possessed several HDL subpopulations mostly in the $HDL_2$ size range (12.9–8.8 nm). ApoA-I*Milano*/A-I*wt*/A-II mice, on the other hand, exhibited a skewing of the particle size distribution so that approximately half (55%) of the particles were within the $HDL_3$ size interval (7.2–8.8 nm in diameter). The most pronounced change in HDL particle size distribution was noted in apoA-I*Milano*/A-II mice

TABLE 2. Relative distribution of apoA-$I_{Milano}$ homodimers, heterodimers, and monomers associated with plasma HDL from transgenic mice

| Phenotype | n | A-I/A-I Homodimer | A-I/A-II Heterodimer | A-I Monomer | A-II |
|---|---|---|---|---|---|
| | | | *% distribution* | | |
| A-I*Milano*/A-I*wt*/A-II | 7 | 14 ± 3 | 33 ± 7 | 33 ± 7 | 20 ± 2 |
| A-I*Milano*/A-II | 6 | 24 ± 5[a] | 38 ± 3 | 25 ± 5[b] | 13 ± 3[a] |

Values were obtained from densitometric scans of Coomassie R-250-stained gels and represent the relative proportion of each of the major human apolipoproteins shown in Fig. 1. Values are means ± SD.
[a]$P < 0.001$ and [b]$P < 0.05$ compared to apoA-I*Milano*/A-I*wt*/A-II (Student's unpaired *t*-test).

TABLE 3. Chemical composition of plasma HDL (d 1.063–1.21 g/ml) isolated from apoA-$I_{Milano}$ transgenic mice

| Phenotype | n | Protein | PL | UC | CE | TG | UC/CE Mole Ratio |
|---|---|---|---|---|---|---|---|
| | | | | % *weight* | | | |
| ApoA-I*Milano*/A-I*wt*/A-II | 13 | 56 ± 2[a] | 29 ± 3 | 3.4 ± 0.3 | 11 ± 1[b] | 1.6 ± 0.2 | 0.54 ± 0.10[c,d] |
| ApoA-I*Milano*/A-II | 10 | 52 ± 7[a] | 32 ± 9 | 3.5 ± 1.0 | 10 ± 2[b] | 2.5 ± 2.0 | 0.69 ± 1.19[c] |
| ApoA-I*wt*/A-II | 8 | 43 ± 8 | 37 ± 2 | 3.5 ± 0.6 | 16 ± 5 | 1.1 ± 0.3 | 0.37 ± 0.06 |
| Murine A-I KO | | 45 ± 4 | 36 ± 5 | 4.8 ± 2.1 | 15 ± 4 | nd | 0.49 ± 0.27 |

Values are means ± SD; nd, not determined.
[a] $P < 0.02$, [b] $P < 0.01$, and [c] $P < 0.05$ compared to HDL from apoA-I*wt*/A-II transgenic mice.
[d] $P < 0.05$ compared to apoA-I*Milano*/A-II (Student's unpaired *t*-test).

where a majority of HDL were small (7.6 nm) $HDL_3$ sized particles. Murine apoA-I KO mice showed a single homogenous peak of large 9.8 nm particles. Taken together, these observations indicate that apoA-$I_{Milano}$ restricts HDL particle size; in the absence of wild-type apoA-I, more extensive dimerization (as shown in Table 2) of apoA-$I_{Milano}$ coincides with a dramatic shift in HDL particle size distribution to mostly small, 7.6 nm particles.

### Impact of apoA-$I_{Milano}$ on plasma LCAT activity

The accumulation of small $HDL_3$ particles in apoA-$I_{Milano}$ transgenic mice suggests that the esterification of cholesterol catalyzed by LCAT may be impaired. Indeed, human carriers of apoA-$I_{Milano}$ exhibit an elevated unesterified cholesterol/cholesteryl ester molar ratio in plasma which is partly the result of a decrease in LCAT mass (14). We have found that HDL from apoA-$I_{Milano}$ trangenic mice were enriched in protein and depleted in cholesteryl esters compared to HDL isolated from apoA-I*wt*/A-II transgenic mice (**Table 3**). Reductions in HDL cholesteryl ester were accompanied by 45% and 90% increases in the UC/CE mole ratios of apoA-I*Milano*/A-I*wt*/A-II and apoA-I*Milano*/A-II mice, respectively. These observations are consistent with nondenaturing gradient gel electrophoretic profiles in which the absence of wild-type apoA-I (apoA-I*Milano*/A-II transgenics) produces an abundance of small (7.6 nm) cholesteryl ester-poor $HDL_3$ particles in plasma.

In order to determine whether the increased unesterified cholesterol/cholesteryl ester ratio in apoA-$I_{Milano}$ transgenic mice was attributed to murine LCAT deficiency, total plasma LCAT activity was assessed using an exogenous proteoliposome substrate (**Fig. 3**). This figure shows that there was no difference in plasma LCAT activity between the apoA-$I_{Milano}$ transgenic mouse lines and mice expressing high levels of wild-type, human apoA-I (238 mg/dl) and A-II (56 mg/dl). Moreover, no differences in LCAT activity were observed between apoA-I*Milano*/A-I*wt*/A-II and apoA-I*Milano*/A-II transgenics indicating that wild-type apoA-I is not required to sustain normal levels of plasma LCAT activity.

The experiments described above (Fig. 3) using an exogenous substrate addressed the question of whether there was a deficiency of plasma LCAT activity in apoA-$I_{Milano}$ transgenic mice. It did not address the question of whether apoA-$I_{Milano}$ impairs the rate of cholesteryl ester accumulation in HDL; to this end, endogenous LCAT activity was measured in both lines of apoA-$I_{Milano}$ transgenic mice. Pools of plasma from transgenic mice were labeled with [$^{14}$C]cholesterol and the time-course of cholesterol esterification was evaluated. Both the apoA-I*Milano*/A-I*wt*/A-II and apoA-I*Milano*/A-II transgenic mice exhibited marked reductions (>50%) in [$^{14}$C]cholesterol esterification compared to control apoA-I*wt*/A-II mice (**Fig. 4**) indicating that apoA-$I_{Milano}$

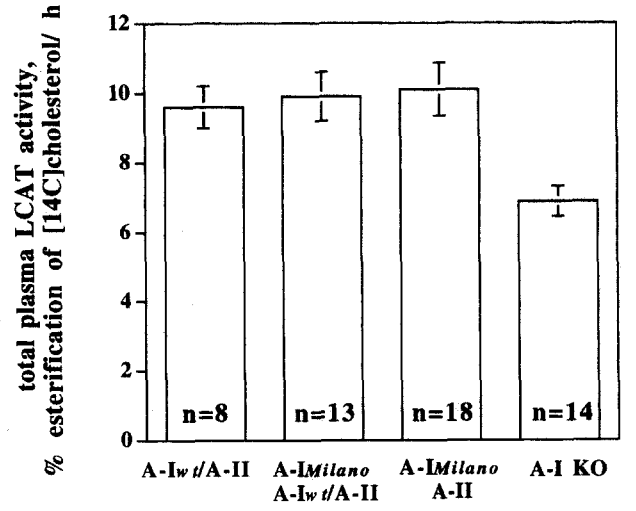


**Fig. 3.** Level of plasma LCAT activity in apoA-$I_{Milano}$ transgenic mice determined by an exogenous substrate procedure. Plasma was obtained from individual male and female mice, and LCAT activity was determined using an exogenous proteoliposome substrate as described in Methods. Initial rates of [$^{14}$C]cholesterol esterification are shown (i.e., % esterification of [$^{14}$C]cholesterol per 1 h). Values are means ± SD.

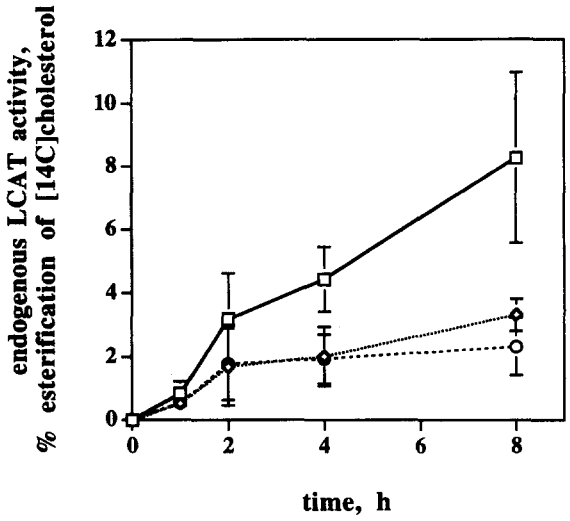


**Fig. 4.** Rates of endogenous cholesterol esterification in plasma of apoA-$I_{Milano}$ transgenic mice. Pools of plasma (3–5 male and female mice/group) were radiolabeled with [$^{14}$C]cholesterol and rates of [$^{14}$C]cholesterol esterification were evaluated. At specified times during the incubation (37°C), aliquots of plasma were removed and lipids were extracted with hexane; [$^{14}$C]cholesterol and [$^{14}$C]cholesteryl ester were separated by thin-layer chromatography as described in Methods. Results are expressed as a percentage of the initial [$^{14}$C]cholesterol that was esterified. Squares indicate apoA-I*wt*/A-II transgenic mice; diamonds and circles indicate apoA-I*Milano*/A-I*wt*/A-II and apoA-I*Milano*/A-II transgenic mice, respectively. Means ± SD, n = 3, are shown.

HDL had a limited capacity to support cholesterol esterification catalyzed by LCAT.

## DISCUSSION

In the present study, transgenic mice were created that exhibited an apoA-$I_{Milano}$ phenotype which does not exist in humans; namely, mice expressing apoA-$I_{Milano}$ in the absence of wild-type, human apoA-I. Comparisons between apoA-I*Milano*/A-II and apoA-I*Milano*/A-I*wt*/A-II mice revealed that expression of apoA-$I_{Milano}$ in the absence of wild-type apoA-I produces more extensive dimerization of apoA-$I_{Milano}$ that severely limits cholesteryl ester accumulation in HDL as judged by the presence of small (7.6 nm) $HDL_3$ sized particles in apoA-I*Milano*/A-II transgenics.

The high abundance of apoA-$I_{Milano}$ homodimers in mice lacking wild-type apoA-I (apoA-I*Milano*/A-II) could very well be related to the distribution of specific HDL subpopulations found in plasma. In human apoA-$I_{Milano}$ carriers, all of which exhibit a hypoalphalipoproteinemia, the distribution of apoA-I without apoA-II (LP-AI) versus apoA-I with apoA-II (LP-AI/AII) subpopulations is apparently normal (27); the LP-AI particles are composed of wild-type apoA-I, the monomeric form of apoA-$I_{Milano}$, and apoA-$I_{Milano}$ homodimers. As expected, apoA-$I_{Milano}$/apoA-II heterodimers are exclusively located on LP-AI/AII particles in human plasma. Our findings, in mice, that the formation of apoA-$I_{Milano}$ homodimers is more extensive in the absence of wild-type apoA-I is entirely consistent with the localization of apoA-$I_{Milano}$ homodimers on LP-AI particles. The presence of wild-type apoA-I on these particles is likely to "dilute-out" the apoA-$I_{Milano}$ monomer pool thereby lowering the frequency of apoA-$I_{Milano}$ monomer/monomer interaction; as a result, less apoA-$I_{Milano}$ homodimers are formed. Moreover, the observation that apoA-$I_{Milano}$/apoA-II heterodimerization is unaffected by wild-type, human apoA-I suggests that apoA-II resides on a metabolically distinct HDL subpopulation of LP-AI/AII particles.

In the present study, we also found that changes in HDL particle size distribution were correlated with the degree of apoA-$I_{Milano}$ forming homodimers. In apoA-I*Milano*/A-I*wt*/A-II mice, approximately 50% of the particles were restricted to the $HDL_3$ size interval (7.2–8.8 nm in diameter); whereas, most of the HDL from apoA-I*Milano*/A-II mice were small, 7.6 nm sized particles. The latter was associated with extensive dimerization of apoA-$I_{Milano}$ on such particles. Consistent with the relative proportion of small $HDL_3$ particles in apoA-I*Milano*/A-I*wt*/A-II and apoA-I*Milano*/A-II mice, the UC/CE mole ratios of HDL were elevated by 45% and 90% in these mice, respectively, compared to HDL obtained from apoA-I*wt*/A-II transgenic mice. Thus, by modulating the degree of apoA-$I_{Milano}$ forming homodimers, wild-type apoA-I enables some cholesteryl ester core expansion within the $HDL_3$-sized interval.

Despite the increased UC/CE mole ratio of HDL from apoA-$I_{Milano}$ transgenic mice, the level of plasma LCAT activity, as measured using an exogenous proteoliposome substrate, was normal compared to apoA-I*wt*/A-II mice. These observations indicate that the murine LCAT enzyme is functionally normal and can be activated in mouse plasma by an exogenous substrate. Humans carriers of apoA-$I_{Milano}$ are characterized by a deficiency of functional LCAT enzyme (14). Unlike humans, however, we have found that expression of apoA-$I_{Milano}$ in mice does not produce murine LCAT deficiency. The reason for this difference is not known at this time. The concentration of LCAT mass is much lower (5- to 8-fold) in mice (28) compared to humans (29). Thus, despite the markedly reduced plasma HDL-cholesterol concentrations in apoA-$I_{Milano}$ mice, a sufficient quantity of HDL may circulate to sustain the normal level of murine LCAT activity. In support of this ascertion, Parks et al. (30) found, in murine apoA-I knockout mice, that mice expressing half the gene dos-

age of apoA-I exhibit normal plasma LCAT activity and that homozygous apoA-I knockouts possessed a substantial amount of LCAT enzyme when assayed using an exogenous substrate. These observations, together with our findings in apoA-$I_{Milano}$ transgenic mice, indicate that HDL deficiencies do not appreciably alter the levels of murine LCAT enzyme.

Although the level of plasma LCAT activity in apoA-$I_{Milano}$ transgenic mice was normal, the endogenous esterification of cholesterol catalyzed by LCAT was greatly reduced compared to apoA-I*wt*/A-II transgenic mice. Impaired cholesterol esterification catalyzed by LCAT is probably not the result of reduced ability of apoA-$I_{Milano}$ to activate LCAT. In support of this hypothesis, transgenic mice that lack wild-type apoA-I (apoA-I*Milano*/A-II) exhibit similar rates of endogenous esterification compared to apoA-I*Milano*/A-I*wt*/A-II mice. Moreover, we have found in a previous study, using Chinese hamster ovary cells transfected with the genes for wild-type apoA-I and apoA-$I_{Milano}$, that nascent HDL with apoA-$I_{Milano}$ were just as effective as wild-type apoA-I lipid complexes at supporting LCAT activity, indicating that apoA-$I_{Milano}$ activated LCAT normally (16). A more likely explanation for reduced endogenous cholesterol esterification rate is that dimerization of apoA-$I_{Milano}$ imposes a steric constraint that limits HDL core expansion. This could account for the accumulation of small $HDL_3$-sized particles in both humans, apoA-$I_{Milano}$ subjects, and apoA-$I_{Milano}$ transgenic mice.

The unique feature of the present study was the ability to use transgenic mice to express apoA-$I_{Milano}$ in the absence of wild-type apoA-I; this enabled us to evaluate the full impact of apoA-$I_{Milano}$ dimerization on HDL particle size distribution. These transgenic mice revealed that wild-type apoA-I exerts an influence over apoA-$I_{Milano}$ dimerization and, hence, HDL particle size restriction. As HDL from apoA-I*Milano*/A-II mice exhibited an even greater UC/CE mole ratio compared to HDL from apoA-I*Milano*/A-I*wt*/A-II mice, expression of apoA-$I_{Milano}$ in the homozygous state, if ever occurring in humans, may severely limit HDL-cholesterol transport. The mechanisms by which apoA-$I_{Milano}$ exerts it putative antiatherogenic effects are currently unknown.

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## REFERENCES

1. Gordon, T., W. P. Castelli, M. C. Hjortland, W. B. Kannel, and T. R. Dawber. 1977. High density lipoprotein as a protective factor against coronary heart disease. *Am. J. Med.* **62:** 707–714.
2. Rifkind, B. M. 1990. High-density lipoprotein cholesterol and coronary artery disease: survey of the evidence. *Am. J. Cardiol.* **66:** 3A–6A.
3. Wilson, P. W. F. 1990. High-density lipoprotein, low-density lipoprotein and coronary artery disease. *Am. J. Cardiol.* **66:** 7A–10A.
4. Glomset, J. A. 1968. The plasma lecithin:cholesterol acyltransferase reaction. *J. Lipid Res.* **9:** 155–167.
5. Rothblat, G. H., and M. C. Phillips. 1991. Cholesterol efflux from arterial wall cells. *Curr. Opin. Lipidol.* **2:** 288–294.
6. Tall, A. R. 1990. Plasma high density lipoproteins: metabolism and relationship to atherogenesis. *J. Clin. Invest.* **86:** 379–384.
7. Assmann, G., A. von Eckardstein, and H. Funke. 1993. High density lipoproteins, reverse transport of cholesterol, and coronary artery disease: insights from mutations. *Circulation.* **87[suppl III]:** 28–34.
8. Matsunaga, T., Y. Hiasa, H. Yanagi, T. Maeda, N. Hattori, K. Yamakawa, Y. Yamanouchi, I. Tanaka, T. Obara, and H. Hamaguchi. Apolipoprotein A-I deficiency due to a codon 84 nonsense mutation of the apolipoprotein A-I gene. *Proc. Natl. Acad. Sci. USA.* **88:** 2793–2797.
9. Funke, H., A. von Eckardstein, P. H. Pritchard, M. Karas, J. J. Albers, and G. Assmann. 1991. A frameshift mutation in the human apolipoprotein A-I gene causes high density lipoprotein deficiency, partial lecithin:cholesterol acyltransferase deficiency, and corneal opacities. *J. Clin. Invest.* **87:** 371–376.
10. Weisgraber, K. H., T. P. Bersot, R. W. Mahley, G. Franceschini, and C. R. Sirtori. 1980. Isolation and characterization of a cysteine-containing variant of the A-I apoprotein from human high density lipoproteins. *J. Clin. Invest.* **66:** 901–907.
11. Franceschini, G., C. R. Sirtori, A. Capurso, K. H. Weisgraber, and R. W. Mahley. 1980. Decreased high density lipoprotein cholesterol levels with significant lipoprotein modifications and without clinical atherosclerosis in an Italian family. *J. Clin. Invest.* **66:** 892–899.
12. Franceschini, G., T. G. Frosi, C. Manzoni, G. Gianfranceschi, and C. R. Sirtori. 1982. High density lipoprotein-3 heterogeneity in subjects with the apoA-$I_{Milano}$ variant. *J. Biol. Chem.* **257:** 9926–9930.
13. Franceschini, G., L. Calabresi, C. Tosi, C. R. Sirtori, C. Fragiacomo, G. Noseda, E. Gong, P. Blanche, and A. V. Nichols. Apolipoprotein A-$I_{Milano}$: correlation between high density lipoprotein subclass distribution and triglyceridemia. *Arteriosclerosis.* **7:** 426–435.
14. Franceschini, G., M. Baio, L. Calabresi, C. R. Sirtori, and M. Cheung. 1990. Apolipoprotein A-$I_{Milano}$: partial lecithin:cholesterol acyltransferase deficiency due to low levels of a functional enzyme. *Biochim. Biophys. Acta.* **1043:** 1–6.
15. Franceschini, G., L. Calabresi, C. Tosi, G. Gianfranceschi, C. R. Sirtori, and A. V. Nichols. Apolipoprotein A-$I_{Milano}$:

disulfide-linked dimers increase high density lipoprotein stability and hinder particle interconversion in carrier plasma. *J. Biol. Chem.* **265:** 12224–12231.
16. Bielicki, J. K., M. R. McCall, L. J. Stoltzfus, A. Ravandi, A. Kuksis, E. M. Rubin, and T. M. Forte. 1997. Evidence that apolipoprotein A-$I_{Milano}$ has reduced capacity, compared to wild-type apolipoprotein A-I, to recruit membrane cholesterol. *Arterioscler. Thromb. Vasc. Biol.* In press.
17. Schultz, J. R., J. G. Verstuyft, E. L. Gong, A. V. Nichols, and E. M. Rubin. 1993. Protein composition determines anti-atherogenic properties of HDL in transgenic mice. *Nature.* **365:** 762–764.
18. Rubin, E. M., B. Y. Ishida, S. M. Clift, and R. M. Krauss. 1991. Expression of human apolipoprotein A-I in transgenic mice results in reduced plasma levels of murine apolipoprotein A-I and the appearance of two new high density lipoprotein size subclasses. *Proc. Natl. Acad. Sci. USA.* **88:** 434–438.
19. Steele, B. W., D. E. Koehler, and M. M. Azar. 1976. Enzymatic determination of cholesterol in high-density lipoprotein fractions prepared by precipitation techniques. *Clin. Chem.* **22:** 98–191.
20. Lindgren, F. T., L. C. Jensen, and F. T. Hatch. 1972. Blood Lipids and Lipoproteins: Quantitation, Composition, and Metabolism. Wiley-Interscience, NY. 181–274.
21. Markwell, M. K., S. M. Haas, L. L. Bieber, and N. E. Tolbert. 1978. A modification of the Lowry protein procedure to simplify protein determination in membrane and lipoprotein samples. *Anal. Biochem.* **87:** 206–210.
22. Chen, P. S., T. Y. Toribara, and H. Warner. 1956. Microdetermination of phosphorus. *Anal. Chem.* **28:** 1756–1759.
23. Sale, F. O., S. Marchesini, P. H. Fishman, and B. Berra. 1984. A sensitive enzymatic assay for determination of cholesterol in lipid extracts. *Anal. Biochem.* **142:** 347–350.
24. Laemmli, U. K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature.* **227:** 680–685.
25. Nichols, A. V., R. M. Krauss, and T. A. Musliner. 1986. Nondenaturing polyacrylamide gradient gel electrophoresis. *Methods Enzymol.* **128:** 417–431.
26. Chen, C., and J. J. Albers. 1982. Characterization of proteoliposomes containing apoprotein A-I: a new substrate for the measurement of lecithin:cholesterol acyltransferase activity. *J. Lipid Res.* **23:** 680–691.
27. Cheung, M. C., A. V. Nichols, P. Blanche, E. L. Gong, G. Franceschini, and C. R. Sirtori. 1988. Characterization of apoA-I -containing lipoproteins in subjects with apoA-$I_{Milano}$ variant. *Biochim. Biophys. Acta.* **960:** 73–82.
28. Vaisman, B. L., H. Klein, M. Rouis, A. M. Berard, M. R. Kindt, G. D. Talley, S. M. Meyn, R. F. Hoyt, S. M. Marcovina, J. J. Albers, J. M. Hoeg, H. B. Brewer, and S. Fojo. 1995. Overexpression of human lecithin:cholesterol acyltransferase leads to hyperalphalipoproteinemia in transgenic mice. *J. Biol. Chem.* **270:** 12269–12275.
29. Albers, J. J., R. O. Bergelin, J. L. Adolphson, and P. W. Wahl. 1982. Population-based reference values for lecithin:cholesterol acyltransferase (LCAT). *Atherosclerosis.* **43:** 369–379.
30. Parks, J. S., H. Li, A. K. Gebre, T. L. Smith, and N. Maeda. 1995. Effect of apolipoprotein A-I deficiency on lecithin:cholesterol acyltransferase activation in mouse plasma. *J. Lipid Res.* **36:** 349–355.