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### Research Article

Apo A-IMilano is a mutant form of apo A-I in which cysteine is substituted for arginine at amino acid 173. Subjects with apo A-IMilano are characterized by having low levels of plasma HDL cholesterol and apo A-I. To determine the kinetic etiology of the decreased plasma levels of the apo A-I in these individuals, normal and mutant apo A-I were isolated, radiolabeled with either <sup>125</sup>I or <sup>131</sup>I, and both types of apo A-I were simultaneously injected into two normal control subjects and two subjects heterozygous for apo A-IMilano. In the normal subjects, apo A-IMilano was catabolized more rapidly than the normal apo A-I (mean residence times of 5.11 d for normal apo A-I vs. 3.91 d for apo A-IMilano), clearly establishing that apo A-IMilano is kinetically abnormal and that it has a shortened residence time in plasma. In the two apo A-IMilano subjects, both types of apo A-I were catabolized more rapidly than normal (residence times ranging from 2.63 to 3.70 d) with normal total apo A-I production rates (mean of 10.3 vs. 10.4 mg/kg per d in the normal subjects). Therefore, in the subjects with apo A-IMilano, the decreased apo A-I levels are caused by rapid catabolism of apo A-I and not to a decreased production rate, and the abnormal apo A-IMilano leads to the rapid catabolism of both [...]

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# In Vivo Metabolism of a Mutant Form of Apolipoprotein A-I, apo A-I<sub>Milano</sub>, Associated with Familial Hypoalphalipoproteinemia

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## Abstract

Apo A-I<sub>Milano</sub> is a mutant form of apo A-I in which cysteine is substituted for arginine at amino acid 173. Subjects with apo A-I<sub>Milano</sub> are characterized by having low levels of plasma HDL cholesterol and apo A-I. To determine the kinetic etiology of the decreased plasma levels of the apo A-I in these individuals, normal and mutant apo A-I were isolated, radiolabeled with either <sup>125</sup>I or <sup>131</sup>I, and both types of apo A-I were simultaneously injected into two normal control subjects and two subjects heterozygous for apo A-I<sub>Milano</sub>. In the normal subjects, apo A-I<sub>Milano</sub> was catabolized more rapidly than the normal apo A-I (mean residence times of 5.11 d for normal apo A-I vs. 3.91 d for apo A-I<sub>Milano</sub>), clearly establishing that apo A-I<sub>Milano</sub> is kinetically abnormal and that it has a shortened residence time in plasma. In the two apo A-I<sub>Milano</sub> subjects, both types of apo A-I were catabolized more rapidly than normal (residence times ranging from 2.63 to 3.70 d) with normal total apo A-I production rates (mean of 10.3 vs. 10.4 mg/kg per d in the normal subjects). Therefore, in the subjects with apo A-I<sub>Milano</sub>, the decreased apo A-I levels are caused by rapid catabolism of apo A-I and not to a decreased production rate, and the abnormal apo A-I<sub>Milano</sub> leads to the rapid catabolism of both the normal and mutant forms of apo A-I in the affected subjects. (*J. Clin. Invest.* 1993. 91:1445-1452.) Key words: kinetics • lipoproteins • genetic defects • hypoalphalipoproteinemia • apolipoprotein A-I • lipoproteins • HDL

## Introduction

HDLs are macromolecular complexes composed of proteins, free and esterified cholesterol, phospholipids, and triglycerides. Apo A-I, the major apolipoprotein of HDL, is synthesized in both the liver and the intestine as a preproprotein (1); this is cotranslationally cleaved to the proprotein, which is secreted into plasma. Within the vascular compartment, proapo A-I is converted to the mature form, a 243-amino acid long polypeptide, by the action of a calcium-dependent protease (2, 3). Apo A-I has both a structural and a functional role in lipoprotein metabolism: it is the major structural protein of HDL, it is an activator of the enzyme lecithin:cholesterol acyltransferase

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(LCAT)<sup>1</sup> (4) and there is evidence that it has a role as a specific ligand for the HDL receptor (5-7).

HDL cholesterol and apo A-I levels are important negative risk factors for the development of coronary artery disease in humans. Epidemiological and family studies have demonstrated a strong inverse correlation for both HDL cholesterol and apo A-I levels and the incidence of coronary artery disease (8-12), whereas longevity has been reported in individuals with hyperalphalipoproteinemia (13). A mutant form of apo A-I, apo A-I<sub>Milano</sub>, has been reported by Franceschini et al. (14), in which a cysteine is substituted for an arginine at amino acid position 173 (15). All the individuals with this mutation detected so far are heterozygous for the mutant allele (16) and have low to extremely low levels of HDL associated with an increased incidence of hypertriglyceridemia (17). Interestingly, in spite of their hypoalphalipoproteinemia, an increased incidence of cardiovascular disease has not been reported for individuals with apo A-I<sub>Milano</sub> (16).

Because of the presence of a cysteine residue, apo A-I<sub>Milano</sub> can form disulfide-linked dimers, and it is present on plasma lipoproteins as a monomer, a heterodimer with apo A-II, and a homodimer with itself (18). In addition, with the loss of the positively charged arginine at position 173, apo A-I<sub>Milano</sub> has one less net-positive charge than normal apo A-I. This allows visualization of both normal and mutant apo A-I with isoelectric focusing gel electrophoresis of plasma (15). Even though HDL cholesterol and apo A-I levels are low in individuals with apo A-I<sub>Milano</sub>, a high degree of interindividual variability has been observed (19). It is also of interest that the amount of the mutant form of apo A-I is always greater than that of the normal form of the protein, even though the total mass of apo A-I is decreased (14).

Very little is known about the kinetic etiology of the decreased HDL cholesterol and apo A-I levels in subjects with apo A-I<sub>Milano</sub> and the kinetic mechanism behind the variable ratio of mutant to normal apo A-I in these subjects. We, therefore, performed studies on the in vivo kinetics of both normal apo A-I and apo A-I<sub>Milano</sub> in normal and apo A-I<sub>Milano</sub> subjects to investigate the metabolism of the mutant protein and to obtain insights into the mechanism(s) that determine(s) the absolute and relative amounts of the two forms of apo A-I in plasma.

## Methods

**Patients and controls.** The two study subjects with apo A-I<sub>Milano</sub> were identified during the screening of asymptomatic family members of the proband for apo A-I<sub>Milano</sub>. Patient 1, a 23-yr-old male, has a history of mild hypertension but has never been treated with medications. Patient

1. Abbreviations used in this paper: LCAT, lecithin:cholesterol acetyltransferase.

2, a 33-yr-old male, has a history of duodenal ulcer with bleeding, which occurred at the age of 31. The patient was started on H2 receptor blockers, and at the time of the study was taking 20 mg famotidine/d. The control subjects, two females, 20 and 21 yr old, were normolipidemic volunteers without evidence of acute or chronic illness. The study protocol was approved by the Investigational Review Board, National Heart, Lung, and Blood Institute, and written informed consent was obtained from each subject. The patients and controls were on an ad libitum diet before admission to the metabolic ward of the Clinical Center at the National Institutes of Health. 1 wk before the beginning of the study, they were put on an isocaloric diet consisting of 16% protein, 42% fat, 42% carbohydrate, 200 mg cholesterol/1,000 kcal, and a polyunsaturated to saturated fat ratio of 0.2. Starting 1 d before the injection of the tracer and continuing throughout the experiment the subjects were given 300 mg potassium iodide three times per day.

**Compositional analysis of plasma lipoproteins.** Fasting plasma lipoproteins were isolated by sequential ultracentrifugation at 4°C in a 40.3 rotor (Beckman Instruments, Inc., Palo Alto, CA) at the densities of 1.063, 1.125 (HDL<sub>2</sub>), and 1.210 g/ml (HDL<sub>3</sub>) (20). Protein content was determined by the method of Lowry et al. (21), using BSA as a standard. Enzymic assays were used for the quantitation of total cholesterol (cholesterol CII, cat. 996-54001; Wako), free cholesterol (free cholesterol C, cat. 274-47109; Wako), triglycerides (triglyceride G, cat. 997-69801; Wako), and phospholipids (phospholipids, cat. 996-54001; Wako Bioproducts, Richmond, VA).

**Preparation of tracer apolipoproteins and lipoproteins.** Normal apo A-I was isolated as previously described (22) and iodinated with <sup>131</sup>I (NEZ 035H, New England Nuclear/DuPont, Boston, MA) in the presence of 6 M guanidine hydrochloride, 1 M glycine, pH 8.5, as previously reported (3). Less than 0.5 mol of iodine were covalently bound to 1 mol of apo A-I. Apo A-I<sub>Milano</sub> was isolated by affinity chromatography on thiopropyl Sepharose 6B (Pharmacia Fine Chemicals, Piscataway, NJ), as previously described (15) and stored as a monomer in a reducing buffer containing 4 M guanidine hydrochloride, 0.1 M Tris HCl, pH 7.4, 0.04% EDTA, 0.01% sodium azide, and 20 mM DTT. Before iodination apo A-I<sub>Milano</sub> was desalted on a Sephadex G-100 column (Pharmacia Fine Chemicals) eluting with 0.01 M NH<sub>4</sub>HCO<sub>3</sub> buffer, pH 7.0. The iodination was performed as for normal apo A-I, using <sup>125</sup>I (NEZ 033L; New England Nuclear/DuPont). After iodination apo A-I<sub>Milano</sub> was reduced by the addition of DTT in 6 M guanidine hydrochloride, 1 M glycine, pH 8.5, to a final DTT concentration of 50 mM. Iodinated apolipoproteins were added at a 1:1,000 dilution (vol/vol) to fresh plasma (anticoagulated with 1 mg/ml EDTA) from either a control subject or an apo A-I<sub>Milano</sub> patient and incubated for 15 min at 37°C. Plasma was adjusted to a density of 1.21 g/ml with solid KBr, ultracentrifuged at 59,000 rpm for 24 h at 4°C in a 60 Ti rotor with an ultracentrifuge (L8-M; Beckman Instruments Inc.) and the plasma lipoproteins were collected by tube slicing. The labeled lipoproteins were extensively dialyzed against PBS and human serum albumin was added to the lipoproteins at a final concentration of 10 mg/ml. The lipoproteins were sterile filtered and tested for pyrogenicity and sterility (3). Doses of 10 μCi of normal <sup>131</sup>I-apo A-I and 18–23 μCi of <sup>125</sup>I-apo A-I<sub>Milano</sub> were injected into each study subject. The mass of

labeled apo A-I injected per subject was < 25 μg for each form of apo A-I.

**Experimental protocol.** Control subjects received labeled normal apo A-I and apo A-I<sub>Milano</sub> reassociated with control lipoproteins whereas the apo A-I<sub>Milano</sub> subjects received both forms of labeled apo A-I reassociated with apo A-I<sub>Milano</sub> lipoproteins. After the injection of labeled proteins, blood samples were drawn into tubes containing EDTA (1 mg/ml) and placed immediately on ice. Samples were obtained after the injections at 5 min, at 1, 2, 3, 4, 6, 9, 12, 18, 24, and 36 h, and on days 2, 3, 4, 5, 7, 9, 11, and 12. Plasma was obtained by centrifugation at 1,500 g for 30 min at 4°C. Sodium azide (0.05%) and aprotinin (200 Kallikrein inhibitor units/ml, Boehringer Mannheim GmbH, Mannheim, Germany) were added to the plasma. Quantitation of radioactivity was obtained by counting 5-ml aliquots of plasma in an Autogamma Counter (model 5260; Packard Instruments Co., Inc., Downers Grove, IL). Total plasma (150 μl) from samples from the first portion of the study (5 min to 36 h) were subjected to nonreducing 15% NaDodSO<sub>4</sub> PAGE (23). The gels were stained with Coomassie blue and the apo A-I-containing bands were sliced and counted.

**Analysis of kinetic data.** Multiexponential functions were fit to the plasma radioactivity decay curves using the SAAM 29 program (24) on a VAX-11/780 computer (Digital Equipment Corp., Maynard, MA). The residence times (RT) were determined from the area under the plasma decay curves. The production rates (PR) were calculated from the following formula:

$$PR = \frac{\text{apo A-I (mg/dl)} \times \text{plasma vol (dl)}}{RT \text{ (d)} \times \text{body wt (kg)}}$$

with the plasma volume being determined by dividing the total amount of radioactivity injected into each subject by the amount of radioactivity in 1 dl of plasma from the first time point that plasma was obtained (5 min). The plasma volumes were determined from both tracers injected, and the average of these two determinations were used for all further calculations. In all cases, the differences between the two determinations were ≤ 2% of the mean value.

**Other methods.** Cholesterol and triglycerides were determined in plasma using the VP Super System (Abbott Laboratories, Diagnostic Division, Irving, TX). Total HDL cholesterol was quantitated after dextran SO<sub>4</sub>-MgCl<sub>2</sub> precipitation of apo B-containing lipoproteins in plasma (25). A-I and A-II apolipoproteins were quantitated in total plasma with a radial immunodiffusion assay (26). NaDodSO<sub>4</sub> PAGE of plasma and two-dimensional gel electrophoresis of reduced total plasma were performed as described (27).

## Results

The characteristics of the study subjects are reported in Table I. The plasma cholesterol of the patients was in the normal range. HDL cholesterol was below the fifth percentile of age- and sex-matched controls (21 mg/dl in patient 1 and 12 mg/dl in patient 2). Patient 1 had low levels of apo A-I (86 mg/dl) and

Table I. Characteristics of Patients and Control Subjects

Subject	Age	Weight	Body mass index*	Plasma				
				Cholesterol	Triglycerides	HDL <sup>‡</sup>	ApoA-I	ApoA-II
	yr	kg	kg/m <sup>2</sup>			mg/dl		
Patient 1	23	77	22.5	199	181	21	86	29
Patient 2	33	77	22.4	169	166	12	51	14
Control 1	20	48	18.2	160	45	43	119	34
Control 2	21	64	24.2	130	43	47	120	43

\* Body mass index = wt/ht<sup>2</sup>; <sup>‡</sup> HDL, cholesterol.

normal levels of apo A-II (29 mg/dl). Patient 2 had markedly reduced levels of both apolipoproteins (51 and 14 mg/dl for apo A-I and apo A-II, respectively). The ratios of apo A-I versus apo A-II in the plasma (3.0 and 3.6, respectively) were comparable to those obtained for the control subjects (3.6 and 2.8, respectively). The plasma triglyceride levels were high normal to modestly elevated in both subjects.

Both patients, as all other individuals with apo A-I<sub>Milano</sub> so far identified, are heterozygous for the mutant form of apo A-I. Fig. 1 illustrates the results of the two-dimensional gel electrophoresis performed on reduced total plasma. Similar to normal apo A-I, apo A-I<sub>Milano</sub> has pro, mature, and deamidated isoforms of apo A-I, but with one additional positive charge compared with normal apo A-I. The relative increase in the mass of the mutant apo A-I compared with the normal apo A-I and the interindividual difference in the ratio of the mutant to normal apo A-I in these two patients are consistent with the previously reported relative mass differences and the interindividual variability in the ratio of mutant to normal apo A-I (19).

Composition analysis was performed on lipoproteins isolated at the densities of 1.063–1.125 g/ml (HDL<sub>2</sub>) and 1.125–1.210 g/ml (HDL<sub>3</sub>). The plasma concentration and the relative percentage of the lipoprotein components are reported in Table II. Both HDL<sub>2</sub> and HDL<sub>3</sub> in the two patients were relatively enriched in triglycerides and poor in esterified cholesterol. Triglyceride content (6% in HDL<sub>2</sub>, 4 and 3% in HDL<sub>3</sub>) was two to three times higher than in control lipoproteins. Cholesterol esters were 9 and 3% in HDL<sub>2</sub> and 5 and 2% in HDL<sub>3</sub>. Control values were 16 and 15% in HDL<sub>2</sub>, 9 and 12% in HDL<sub>3</sub>. In addition, the free cholesterol to cholesterol ester ratios were elevated for both patients in both HDL subfractions (ranging

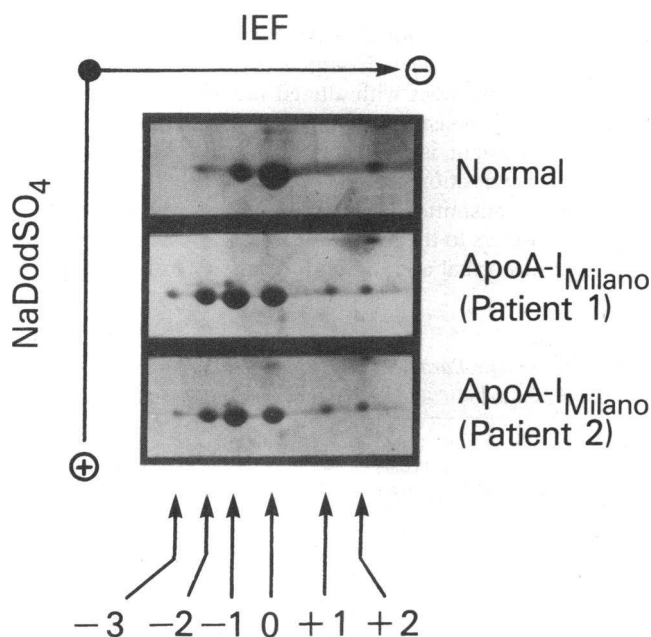


Figure 1. Comparison of the isoforms of normal apo A-I and apo A-I<sub>Milano</sub> by two-dimensional gel electrophoresis. Isoelectric focusing of 3  $\mu$ l of reduced plasma was performed in the first dimension, followed by NaDodSO<sub>4</sub> polyacrylamide gel electrophoresis in the second dimension. Proteins were detected by silver stain. Only the portions of the gels containing the apo A-I isoforms are shown. Panels are, from the top, apo A-I from a normal subject and from patients 1 and 2, respectively.

from 0.32 to 1.71 for the patients compared with 0.20 to 0.27 for the controls) with the elevations being greater in patient 2. When one compared the ratios of free cholesterol with total core components (cholesterol esters plus triglycerides), patient 1 had ratios comparable to the controls (0.24 vs. 0.22 and 0.22 for HDL<sub>2</sub>, and 0.19 vs. 0.22 and 0.18 for HDL<sub>3</sub>) whereas patient 2 still had elevated ratios (0.55 for HDL<sub>2</sub> and 0.50 for HDL<sub>3</sub>). Protein represented a slightly higher than normal percentage of HDL mass. The data in Table II also indicate that the low HDL concentration is mainly due to a decrease in the concentration of HDL<sub>2</sub> in patient 1 and to a decrease in the concentration of both HDL subfractions in patient 2.

After incubation in plasma at 37°C and isolation of the lipoprotein fraction at the density of 1.210 g/ml, the iodinated apo A-I<sub>Milano</sub> was examined by nonreducing NaDodSO<sub>4</sub> gel electrophoresis. A comparison of an autoradiogram of the gel with the Coomassie blue stain (Fig. 2) demonstrated that the iodinated apo A-I<sub>Milano</sub> was present in three bands, at the molecular weights of 28,000 (apo A-I<sub>Milano</sub> monomer), 35,000 (apo A-I<sub>Milano</sub>/apo A-II dimer), and 55,000 (apo A-I<sub>Milano</sub> dimer), corresponding to the protein bands for the native apo A-I<sub>Milano</sub> with the stained gels. In addition, the relative intensities of the autoradiogram bands are very similar to the intensities of the stained bands, indicating that the different forms of apo A-I<sub>Milano</sub> labeled in proportion to their relative mass. Therefore, the kinetic behavior of the radiolabeled apo A-I<sub>Milano</sub> should reflect the metabolism of its three native circulating forms.

The results of the kinetic studies are illustrated in Figs. 3–5 and in Table III. Fig. 3 depicts the plasma decay curves of apo A-I in one normal control and one apo A-I<sub>Milano</sub> subject. Apo A-I<sub>Milano</sub> was catabolized at a faster rate than normal apo A-I with a residence time of 3.9 versus 5.1 d in the normal control; virtually identical results were obtained from the other normal control with residence times also of 5.1 and 3.9 d for normal apo A-I and apo A-I<sub>Milano</sub>, respectively. In the two patients, both apo A-I<sub>Milano</sub> and normal apo A-I were catabolized at a faster rate than in the normal subjects. The residence time of apo A-I<sub>Milano</sub> was shorter than that of normal apo A-I in patient 1 (3.1 and 3.7 d, respectively) (Fig. 3, Table III) and virtually identical in patient 2 (2.6 and 2.7 d, respectively) (Table III). The plasma decay curves of normal apo A-I in a normal control and of apo A-I<sub>Milano</sub> in the two apo A-I<sub>Milano</sub> subjects are directly compared in Fig. 4 with patient 2 having the fastest catabolic rate. The apo A-I production rates were normal in both patients (10.9 and 9.7 mg/kg per d for patients 1 and 2, respectively; Table III).

A further insight into the metabolism of apo A-I<sub>Milano</sub> was obtained by following the radioactivity associated with its monomer and dimer bands after nonreducing NaDodSO<sub>4</sub> gel electrophoresis of total plasma (Fig. 5). Apo A-I<sub>Milano</sub> was catabolized much more rapidly as a monomer than as a dimer: 50% of the activity initially present in the monomeric form was metabolized within the first 6 h; at 36 h counts had further decreased to 30% in the normal control and to 20% in the patient. Conversely, after an initial raise, 80% of the activity associated with the dimers was still present in the plasma of the control subject at 36 h; at the same time 50% of the initial dimer activity was found in the plasma isolated from the patient (Fig. 5).

The amount of labeled apo A-I that was lipoprotein free (i.e. in the 1.21 g/ml bottom fraction) after ultracentrifugation was determined at the 1-h time point with the following results:

Table II. Mass and Percent Composition of HDL<sub>2</sub> and HDL<sub>3</sub>

	HDL <sub>2</sub>				
	Protein	CE	FC	TG	PL
	mg/dl (%)				
Patient 1	0.379 (56)*	0.062 (9)	0.024 (4)	0.039 (6)	0.176 (26)
Patient 2	0.164 (61)	0.007 (3)	0.012 (4)	0.015 (6)	0.071 (27)
Control 1	0.649 (51)	0.201 (16)	0.053 (4)	0.040 (3)	0.342 (27)
Control 2	0.718 (56)	0.198 (15)	0.048 (4)	0.022 (2)	0.306 (24)
	HDL <sub>3</sub>				
Patient 1	0.552 (72)	0.041 (5)	0.013 (2)	0.027 (4)	0.134 (17)
Patient 2	0.386 (77)	0.009 (2)	0.013 (3)	0.017 (3)	0.079 (16)
Control 1	0.610 (70)	0.074 (9)	0.020 (2)	0.016 (2)	0.150 (17)
Control 2	0.622 (65)	0.117 (12)	0.023 (2)	0.012 (1)	0.178 (19)

CE, cholesterol esters; FC, free cholesterol; TG, triglycerides; PL, phospholipids. \* The numbers in parentheses are the percent composition of that component in that density range.

for patient 1, apo A-I<sub>Milano</sub> in the 1.21 g/ml bottom fraction, 10.1%; normal apo A-I, 10.7%; for patient 2, apo A-I<sub>Milano</sub> 13.4%; normal apo A-I, 20.7%; for control 1, apo A-I<sub>Milano</sub> 9.8%; normal apo A-I, 6.8%; for control 2, apo A-I<sub>Milano</sub> 9.0%; for normal apo A-I, 6.1%. The percentage of free normal apo

A-I at each time point remained relatively constant throughout the course of the study for all four subjects. The percentage of free apo A-I<sub>Milano</sub> at each time point decreased by ~ 35–40% by 12 h in both patients and controls and then remained relatively constant for the rest of the study.

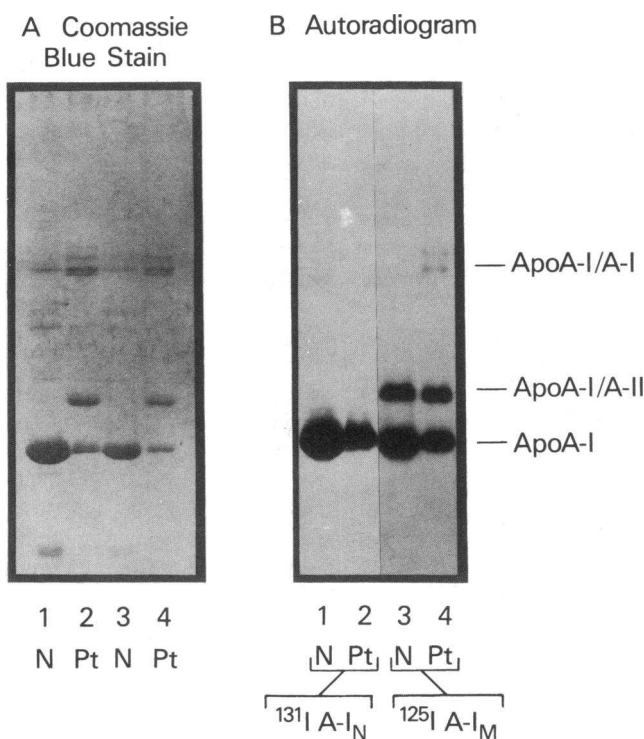


Figure 2. Coomassie blue stain (A) and autoradiogram (B) of a 12% nonreducing NaDodSO<sub>4</sub> gel of the 1.21 g/ml density top fraction isolated after incubation for 15 min at 37°C of lane 1, <sup>131</sup>I normal apo A-I with normal plasma; lane 2, <sup>131</sup>I normal apo A-I with A-I<sub>Milano</sub> plasma; lane 3, <sup>125</sup>I apo A-I<sub>Milano</sub> with normal plasma; and lane 4, <sup>125</sup>I apo A-I<sub>Milano</sub> with A-I<sub>Milano</sub> plasma.

## Discussion

Several variant forms of apolipoproteins that are caused by point mutations, deletions, or rearrangements in the corresponding genes have been described. A number of these mutations result in peptides with altered functions and abnormal lipoprotein metabolism. Apo A-I<sub>Milano</sub>, the first described mutant apolipoprotein, is characterized by a cysteine for arginine substitution at position 173 in the amino acid sequence and the mutation is transmitted as an autosomal dominant trait. The mutation confers to the protein a more acidic isoelectric point than that of normal apo A-I and the ability to form disulfide

Table III. Kinetic Parameters of Apo A-I Metabolism in Apo A-I<sub>Milano</sub> Patients and in Control Subjects

Subject	Apo A-I mg/dl	Plasma volume dl	Apo A-I residence time		Production rate mg/kg per d
			Normal	Mutant	
Patient 1	86	33.1	3.70±0.06*	3.13±0.05	10.9
Patient 2	51	38.7	2.65±0.02	2.63±0.02	9.7
Control 1	119	22.5	5.09±0.06	3.94±0.23	11.0
Control 2	120	26.6	5.12±0.31	3.88±0.37	9.7
Historic Controls (n = 7)	126±17		4.91±0.43	—	10.3±1.2

\* ±SD

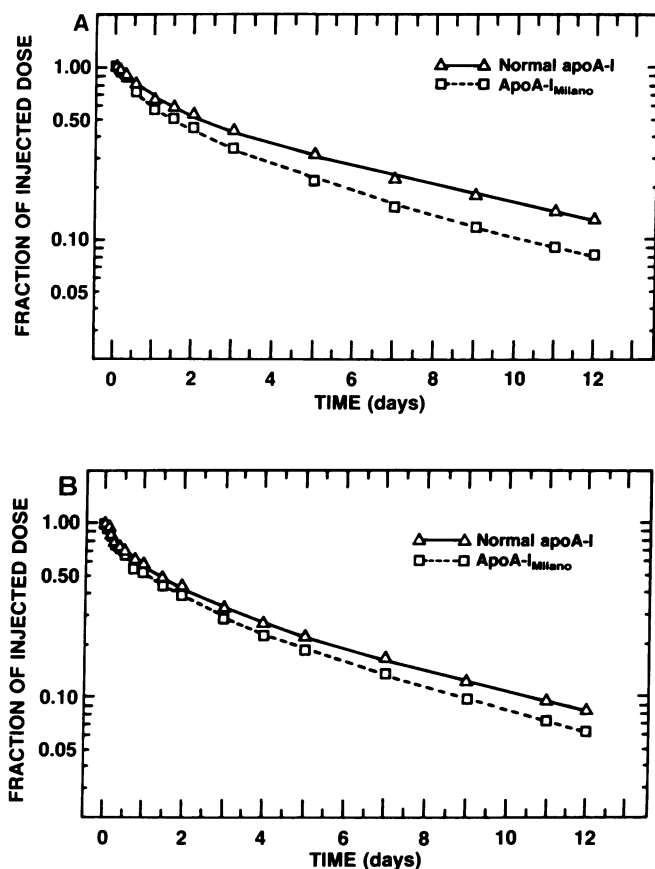


Figure 3. (A) Plasma radioactivity decay curves of normal apo A-I ( $\Delta$ ) and apo A-I<sub>Milano</sub> ( $\square$ ) in a normal subject. (B) Plasma radioactivity decay curves of normal apo A-I ( $\Delta$ ) and apo A-I<sub>Milano</sub> ( $\square$ ) in an apo A-I<sub>Milano</sub> subject.

bonds, principally as homodimers and as heterodimers with apo A-II. Since the description of the first case (14, 15), a number of additional affected individuals have been found as a result of screening in the geographic area from which the proband originated. Although to various degrees, the plasma lipoprotein profile is altered in all of these individuals. Apo A-I<sub>Milano</sub> is associated with very low levels of both HDL cholesterol and apo A-I with an increased incidence of hypertriglyceridemia (17), which in the index case was resistant to drug therapy (14). In spite of the altered plasma lipid and lipoprotein profile, no increased incidence of clinical manifestations of atherosclerotic vascular disease was observed among apo A-I<sub>Milano</sub> subjects (17, 19). In vitro studies on the interaction of apo A-I<sub>Milano</sub> with lipids (28) and with HDL particles isolated from plasma of apo A-I<sub>Milano</sub> subjects (29–32) have been performed. The arginine to cysteine substitution in apo A-I<sub>Milano</sub> results in an accelerated rate of association of apo A-I<sub>Milano</sub> with lipids and reduced stability of the lipid-apolipoprotein complexes (28). Preferential distribution of apo A-I<sub>Milano</sub> monomers and dimers with HDL<sub>3</sub> was also observed in HDL particles separated by gel filtration chromatography (29). In addition, the occurrence of apo A-I<sub>Milano</sub> dimers did not significantly affect the relative distribution of HDL particles with and without apo A-II isolated by immunoaffinity chromatography (30) but did affect the in vitro metabolic activity of these particles (31).

Also of interest, individuals with apo A-I<sub>Milano</sub> have been demonstrated to have reduced levels of LCAT protein and LCAT activity (32).

The two apo A-I<sub>Milano</sub> patients taking part in this study were healthy young males with the characteristics of previously reported subjects. They were modestly hypertriglyceridemic, had normal levels of plasma cholesterol, and significantly decreased levels of HDL cholesterol. Their plasma apo A-I levels were decreased, although not to as great an extent as their HDL cholesterol levels would predict. Studies on plasma lipoproteins demonstrated an abnormal composition of both HDL subclasses, with triglycerides being proportionally increased and cholesterol esters decreased. The change in composition was particularly evident in patient 2, who had the lower HDL level. In addition, on an absolute mass basis, HDL<sub>2</sub> levels were more decreased than HDL<sub>3</sub> in these two subjects, a common finding among individuals with hypoalphalipoproteinemia caused by a number of different etiologies.

The decrease in cholesterol esters in HDL, and particularly the increase in the free cholesterol to cholesterol esters and the free cholesterol to cholesterol esters plus triglycerides ratios in patient 2, is consistent with the previously described decrease in LCAT activity in subjects with apo A-I<sub>Milano</sub> (32). The reasons for the substantial differences in lipoprotein composition and apo A-I kinetics between these two subjects is unknown, but these types of differences have been previously described (19, 29, 32). It is interesting to speculate that there may be a second genetic defect in some apo A-I<sub>Milano</sub> individuals, such as a partial LCAT deficiency or a primary over production of triglycerides, resulting in these compositional and metabolic differences; although other factors such as diet or level of physical activity or the hormonal status of women may secondarily result in these differences. The current study does not and was not designed to provide any direct evidence to support any of these hypotheses.

Apo A-I<sub>Milano</sub> is present in native plasma principally in three forms, as a monomer, a homodimer, and a heterodimer. To determine the kinetics of their metabolism, it was important to have all three forms labeled with the radioactive tracer. Our results with NaDodSO<sub>4</sub> PAGE of the labeled apo A-I<sub>Milano</sub>, which was added to plasma in a reduced state after iodination, indicated that during the incubation with plasma at 37°C the

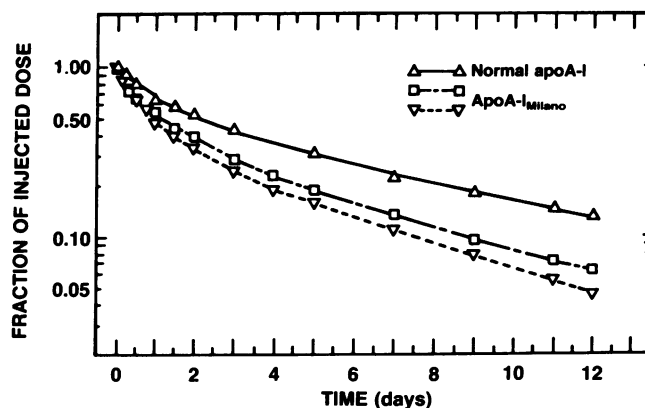


Figure 4. Plasma radioactivity decay curves of normal apo A-I in a normal subject ( $\Delta$ ) and of apo A-I<sub>Milano</sub> in the two apo A-I<sub>Milano</sub> subjects ( $\square$ ,  $\nabla$ ).

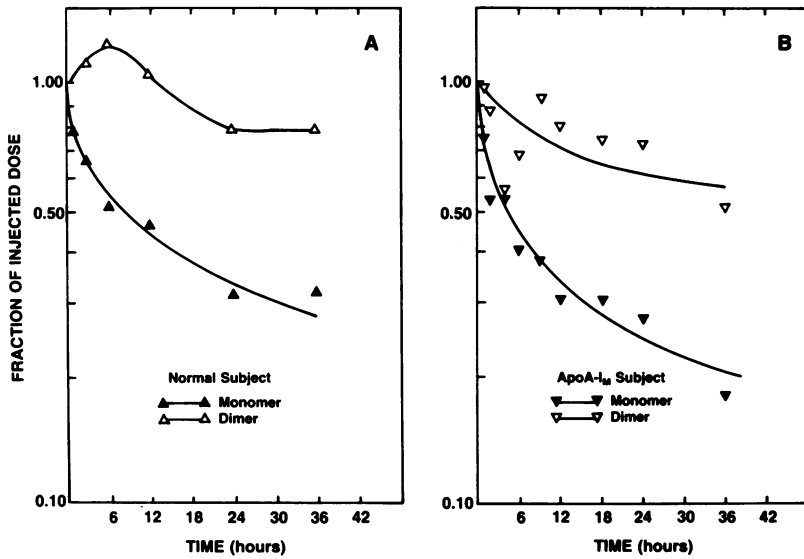


Figure 5. Plasma radioactivity decay curves of apo A-I monomer ( $\blacktriangle$ ,  $\blacktriangledown$ ) and dimers ( $\triangle$ ,  $\triangledown$ ) in normal subject 1 (A) and in apo A-I<sub>Milano</sub> subject 2 (B), as determined from NaDodSO<sub>4</sub> gel electrophoresis of nonreduced total plasma. Counts at each time point are expressed as a percentage of radioactivity of the respective bands at 5 min after the injection of labeled apo A-I<sub>Milano</sub>.

protein was able to form homodimers and heterodimers with apo A-II in approximately the same proportion as they exist in native plasma. Thus, we were able to appropriately radiolabel all three forms of apo A-I<sub>Milano</sub> for the kinetic study.

The results obtained from this study clearly indicate that apo A-I<sub>Milano</sub> is metabolically abnormal; this is most directly demonstrated by the significantly more rapid catabolism of apo A-I<sub>Milano</sub> compared with normal apo A-I in the normal control subjects. The kinetics of metabolism of apo A-I were more complex in the two subjects with apo A-I<sub>Milano</sub> in that both normal apo A-I and apo A-I<sub>Milano</sub> were catabolized more rapidly than either type of apo A-I in normal subjects. Since there are two to four molecules of apo A-I per HDL particle, when tracer amounts of apo A-I<sub>Milano</sub> are injected in normal subjects, there will be only one mutant apo A-I per particle, whereas virtually all of the normal apo A-I will be on particles containing only normal apo A-I. Therefore, one will be determining the metabolism of normal apo A-I on a normal particle or the metabolism of apo A-I<sub>Milano</sub> on a particle that contains only one copy of the abnormal apo A-I. In the apo A-I<sub>Milano</sub> subjects, both the normal and mutant radiolabeled forms of apo A-I will be on particles that contain unlabeled forms of both normal apo A-I and apo A-I<sub>Milano</sub>. Our studies suggest that at least some HDL particles in the apo A-I<sub>Milano</sub> subjects are catabolized as a whole and that the presence of a rapidly catabo-

lized, unlabeled, abnormal apo A-I<sub>Milano</sub> in the affected patients resulted in the rapid catabolism of both radiolabeled normal and mutant apo A-I.

When the catabolic rates of the monomer and dimer forms of apo A-I<sub>Milano</sub> were evaluated, it was found that the monomer form, in addition to being catabolized more rapidly than normal apo A-I, was catabolized much more rapidly than the dimer forms of apo A-I<sub>Milano</sub>. In contrast, the dimer forms were catabolized somewhat more slowly than normal apo A-I in these subjects. These findings are important in the understanding of the kinetics of apo A-I metabolism that lead to rapid catabolism of both normal and mutant apo A-I. In addition, they can also explain the paradoxical findings that in subjects with apo A-I<sub>Milano</sub>, even though the mutant apo A-I is catabolized more rapidly than apo A-I in a normal subject, the apo A-I<sub>Milano</sub> is present in plasma at a higher concentration than is the normal form of apo A-I and that the subjects with the lowest levels of apo A-I and HDL cholesterol have the highest amounts of apo A-I<sub>Milano</sub> relative to normal apo A-I.

The results of the determination of the amount of free apo A-I in the study are consistent with previous findings that apo A-I<sub>Milano</sub> destabilizes lipid-apolipoprotein complexes (28) in that both of the study subjects had a larger percentage of both forms of free apo A-I in plasma. In addition, the relative decrease in the percentage of free apo A-I<sub>Milano</sub> over time parallels the rapid catabolism of apo A-I<sub>Milano</sub> monomer, suggesting that the dimer binds more tightly to lipoprotein particles than does the monomer. One can not determine from this study if the apo A-I<sub>Milano</sub> is catabolized primarily as free apo A-I or as part of a particle. Apo A-I<sub>Milano</sub> may be recognized by a cell surface receptor that leads to internalization and degradation of the whole particle. This is consistent with the rapid catabolism of both normal and mutant apo A-I in the apo A-I<sub>Milano</sub> patients. In addition, the site of uptake would be more likely to be in the liver in that these subjects do not have xanthomas or accelerated atherosclerosis, which would be more consistent with a peripheral uptake of whole HDL particles. However, there is evidence that apo A-I<sub>Milano</sub> destabilizes the whole HDL particle, resulting in increased amounts of both normal and mutant free apo A-I, and one can not rule out that this results in the rapid

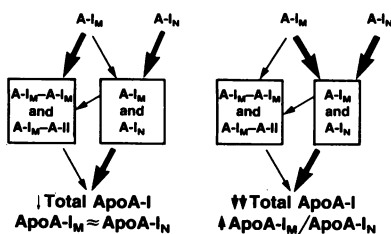


Figure 6. Schematic representation of the metabolism of apo A-I in apo A-I<sub>Milano</sub> subjects. *Left*, the situation in which plasma levels of HDL cholesterol and apo A-I are modestly decreased and the concentration of mutant and normal apo A-I are

similar. *Right*, the situation in which plasma levels of HDL cholesterol and apo A-I are markedly decreased and the ratio of mutant vs. normal apo A-I is relatively increased. See text for further details.

catabolism of both normal and mutant apo A-I. The authors currently favor the first hypothesis for rapid catabolism of apo A-I in apo A-I<sub>Milano</sub> subjects, although there is no convincing evidence favoring one mechanism over the other.

Our proposed model for the metabolism of apo A-I in subjects with apo A-I<sub>Milano</sub> is schematically depicted in Fig. 6. The left panel depicts the situation in apo A-I<sub>Milano</sub> subjects with modest decreases in plasma apo A-I and HDL cholesterol levels, similar to patient 1, whereas the right panel represents the metabolism of apo A-I in apo A-I<sub>Milano</sub> subjects with more marked decreases in plasma apo A-I and HDL, similar to patient 2. After synthesis, apo A-I<sub>Milano</sub> can enter two metabolically different pools, the rapidly catabolized monomer pool or the slowly catabolized dimer pool. If the newly formed apo A-I preferentially enters the dimer pool, the majority of apo A-I<sub>Milano</sub> will be catabolized somewhat more slowly than normal apo A-I. Since monomeric apo A-I<sub>Milano</sub> is a minor proportion of the total plasma apo A-I, only a minor amount of mutant and normal apo A-I will be rapidly catabolized, resulting in a modest decrease in the plasma levels of mutant and normal apo A-I and of HDL cholesterol. In addition, since apo A-I<sub>Milano</sub> dimers are catabolized only slightly slower than normal apo A-I, there will not be a substantial decrease of normal apo A-I relative to apo A-I<sub>Milano</sub>.

In contrast, if the newly formed apo A-I<sub>Milano</sub> preferentially enters the monomer pool, a larger amount of the mutant apo A-I will be rapidly catabolized. This will also result in a much greater amount of normal apo A-I being rapidly catabolized, with a substantial depletion of both normal and mutant forms of apo A-I from plasma. On the other hand, the apo A-I<sub>Milano</sub> dimers will not be as rapidly catabolized, and although decreased in concentration compared with those in the left-hand panel, they will be increased in concentration relative to normal apo A-I. The end result of this is a marked decrease in plasma apo A-I and HDL cholesterol levels with normal apo A-I being decreased more than apo A-I<sub>Milano</sub>.

These investigations in subjects with apo A-I<sub>Milano</sub> have resulted in important information on the regulation of the metabolism of apo A-I in this disease and have led to the first insights into the metabolic etiology of the paradoxical relative increase in the levels of mutant apo A-I compared with normal apo A-I. It remains for future investigations to determine where the dimerization of apo A-I<sub>Milano</sub> occurs and what determines the relative proportion of the mutant apo A-I that goes into the dimer versus the monomer pool. Thus apo A-I<sub>Milano</sub>, the first mutant apolipoprotein discovered, continues to provide important insights into the function and metabolic control of apo A-I in humans.

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