Cardiovascular Status of Carriers of the Apolipoprotein A-I Milano Mutant
The Limone sul Garda Study

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Background—Carriers of the apolipoprotein A-I Milano (apoA-I M ) mutant present with very low plasma HDL cholesterol and moderate hypertriglyceridemia, apparently not leading to premature coronary heart disease. The objective of this study was to establish whether this high-risk lipid/lipoprotein profile is associated with structural changes in the carotid arteries and heart, indicative of preclinical atherosclerosis.

Methods and Results—Twenty-one A-I M carriers were compared with age- and sex-matched control subjects from the same kindred and with 2 series of matched subjects with primary hypoalphalipoproteinemia (HA). Structural changes in the carotid arteries were defined as the intima-media thickness (IMT) measured by B-mode ultrasound. HA subjects, both recruited among patients attending our Lipid Clinic and blood donors, showed significant thickening of the carotids (average IMT, 0.86±0.25 and 0.88±0.29 mm, respectively) compared with control subjects (average IMT, 0.64±0.12 mm); the apoA-I M carriers instead showed normal arterial thickness (average IMT, 0.63±0.10 mm). Moreover, a significantly higher prevalence of atherosclerotic plaques was found in patients and blood donors with HA (both 57%) compared with apoA-I M carriers (33%) and control subjects (21%). Echocardiographic findings and maximal treadmill ECG did not differ significantly between apoA-I M carriers and control subjects, apart from a slight increase in left ventricular end-diastolic dimension in the carriers.

Conclusions—Despite severe HA, carriers of the apoA-I M mutant do not show structural changes in the arteries and heart, in contrast to HA subjects, who are characterized by a marked increase in carotid IMT and increased prevalence of atherosclerotic plaques. (Circulation. 2001;103:1949-1954.)

Key Words: lipoproteins ■ apolipoproteins ■ carotid arteries ■ intima-media thickening ■ heart diseases

The apolipoprotein A-I Milano (apoA-I M ) mutation was described in 1980 in a family originating from Limone sul Garda in northern Italy. This apoA-I variant shows a single amino acid substitution, arginine 173 to cysteine, that leads to the formation of homodimers (A-I M/A-I M ) and heterodimers with apoA-II (A-I M/A-II). All carriers are heterozygous for the mutation and share a lipoprotein disorder characterized by very low plasma levels of HDL cholesterol with moderate hypertriglyceridemia, a condition that has been associated with a high risk of premature coronary heart disease in numerous epidemiological studies.

The clinical status of the apoA-I M carriers has been generally associated with a reduced cardiovascular risk. This conclusion was essentially based on historical data pertaining to the whole Limone sul Garda population and, in particular, to a prior study investigating the clinical conditions of the apoA-I M carriers. This early study, conducted on all citizens >10 years of age, was clearly suggestive of a relatively low global incidence of cardiovascular disease in the population and, in the carriers, of an apparent lack of clinically evident disease despite the “atherogenic” lipoprotein phenotype. Twenty years later, we wanted to carry out more detailed cardiovascular investigations, making use of the most advanced noninvasive technologies for the evaluation of the cardiovascular status of the apoA-I M carriers. In the present study, adult carriers were compared with a double number of control subjects from the same kindred and, in view of the lipoprotein abnormality typically found in the carriers, with dyslipidemic patients and healthy blood donors, both characterized by a low HDL phenotype.
Methods

Subjects
All apoA-I-M carriers living in Limone sul Garda who were between 20 and 70 years of age were asked to attend the Centro di Semeiotica Medica, University of Brescia, for a biochemical and cardiovascular check-up. Of the 25 eligible subjects, 21 agreed to participate in the study. A double number of control subjects of the same age and sex was selected among the close relatives who were not carriers of the mutation.3

Two series of subjects with hypoalphalipoproteinemia (HA), defined on the basis of a plasma HDL cholesterol level below the 10th percentile for age- and sex-matched Italian subjects,7 were recruited from the databases of the Lipid Clinic of the Center E. Grossi Paoletti (LC) and of blood donors attending the Servizio Immunomematologico Trasfusionale of the Niguarda Hospital in Milano (BD). One hundred fifty-one HA subjects were found in the LC database. Of these, 50 subjects were excluded because of a personal history of cardiovascular or cerebrovascular disease. Among the remaining 101 HA subjects, the 21 subjects who matched for sex and were the closest in age to an apoA-IM carrier were recruited for the study (HA-LC).

We found 789 HA subjects in the BD database; 81 were occasional blood donors or their addresses were not known. Among the 708 remaining subjects, 121 could be matched for sex and age to 1 of the apoA-IM carriers, but after HDL cholesterol testing in our laboratory, only 103 subjects proved to have HA. They were asked to take part in the study if they did not have a personal history of cardiovascular disease. The first 21 HA subjects corresponding to each match who agreed to participate were selected for comparison (HA-BD).

None of the selected subjects was taking drugs known to affect plasma lipid/lipoprotein levels. A detailed medical history was collected from all subjects, with particular emphasis on metabolic diseases, smoking habits, and drug treatments. All subjects gave informed consent, and the procedures were approved by the Internal Review Board. The Centro di Semeiotica Medica, University of Brescia, carried out the carotid intima-media thickness (IMT) evaluation, cardiovascular and carotid stress tests. The Center E. Grossi Paoletti, University of Milano, was responsible for the biochemical evaluations and data handling.

Biochemical Methods
Fasting blood samples were collected from all subjects and patients, and plasma (Na2-EDTA, 1 mg/mL) was prepared by low-speed centrifugation. Plasma total cholesterol, triglycerides, and glucose content of lipoprotein fractions was measured by enzymatic techniques. The apoE phenotype was determined by isoelectric focusing.10

Cardio Ultrasonography
High-resolution B-mode carotid ultrasonography was performed with a Hewlett-Packard Sonos 1500 echocardiographic unit equipped with a 7.5-MHz imaging transducer according to a standard protocol.11 Arterial walls of the carotid arteries were investigated by 2 trained sonographers. Videotape recordings were subsequently examined by 2 independent readers using morphometric software that allowed direct evaluation of the IMT2,12 at different sites. Both sonographers and readers were blinded to the subject’s identity. Several measurements were taken on the far wall of the common carotid artery (CCA), bifurcation, and internal carotid artery (ICA) according to previously described protocols.14,15 The ICA was analyzed 10 mm distal from the flow divider that separates the external carotid artery and ICA. Mean values of all measurements on each carotid segment were then calculated. Average IMT was calculated as the average of the 6 carotid segments examined; maximum IMT was defined as the largest IMT among the 6 examined segments. A lesion was considered a plaque in the presence of an average IMT ≥1.3 mm or a maximum IMT ≥1.5 mm.16

Cardiac Echography
Echocardiographic data were obtained according to an established protocol17 by use of a Sonos 1500 echocardiographic unit equipped with a 2.5-MHz transducer. The echocardiographic studies were performed in the morning with the subject in supine left lateral decubitus after 30 minutes of rest. Only 2 physicians were responsible for recording the echocardiograms. Echocardiographic tracings were recorded on light-sensitive paper at a paper speed of 50 mm/s. In all analyzed subjects, left ventricular (LV) mass (g); LV mass index (LVMi, g/m2); intraventricular septal, posterior wall, and relative wall thicknesses (all mm); LV end-diastolic dimension (LVEDD, mm); LV end-systolic dimension (mm); meridional end-systolic stress (dyne/cm2); ejection fraction (%); midwall fractional shortening (%); and cardiac output (L/min) were calculated.

Treadmill Exercise ECG
ApoA-I-M carriers and appropriate control subjects exercised on a bicycle against progressive resistance (30 W every 3 minutes) until the maximum resistance according to age was reached. A 12-lead ECG was recorded during the final 30 seconds of each 2-minute stage and every 2 minutes for ≥6 minutes of recovery. Exercise ECG was interpreted according to the Minnesota Code.18 Heart rate (bpm) was monitored at baseline and up to maximal exercise. The rate-pressure product (107 mm Hg/min) was calculated at baseline and after maximal exercise.

Statistical Analyses
Results are expressed as mean±SD. Differences in means or proportions of the different biochemical and cardiovascular variables were analyzed by ANOVA and χ2 statistics, respectively. A value of P<0.05 was considered significant.

Results
Clinical Characteristics of Participating Subjects
Twenty-one adult carriers of the apoA-I-M mutant and 42 age- and sex-matched control subjects from the large apoA-I-M kindred1 were recruited for the study. Two groups of 21 HA subjects without symptomatic cardiovascular disease were selected among those attending the E. Grossi Paoletti Lipid Clinic (HA-LC) and from a large database of blood donors (HA-BD), again with the same age and sex distribution as control subjects and apoA-I-M carriers. These last 2 groups of subjects underwent only biochemical evaluations and IMT measurements.

Clinical evaluation of the 4 groups showed minimal differences in body mass index, maximal in the HA-BD subjects and lowest in the control subjects (Table 1). The average fasting blood glucose level was higher in the HA-LC subjects than in control subjects, apoA-I-M carriers, and HA-BD subjects, who displayed similar values; none of the recruited subjects had impaired fasting glucose or diabetes mellitus. Blood pressure, both SBP and DBP, did not differ significantly in the 4 groups, with the highest average SBP found in the apoA-I-M carriers and the highest DBP in the HA-LC patients. The distribution of hypertension did not differ in the 4 groups, and duration ranged between 2 and 30 years; all
hypertensive patients, except for 1 apoA-I<sub>M</sub> carrier, were currently under treatment, mostly with ACE inhibitors and calcium antagonists. Similarly, there was no difference in the percentage of smokers among the 4 groups.

As expected and as per protocol, apoA-I<sub>M</sub> carriers and HA subjects showed marked reductions in HDL cholesterol (Table 1). In the apoA-I<sub>M</sub> carriers, HDL cholesterol concentrations were on average less than half of that of control subjects from the same kindred. Otherwise, cholesterol distribution did not differ significantly among the 4 groups, except for a higher average VLDL cholesterol in the apoA-I<sub>M</sub> carriers but were significantly higher than both apoA-I<sub>M</sub> carriers and control subjects (Table 1). There was no significant difference in the total and LDL cholesterol levels among the 4 groups, except for a higher average VLDL cholesterol in the HA-LC patients (Table 1). There was no significant difference in the LDL/HDL cholesterol ratios and apoA-I/B ratios in control subjects were both in the normal range (3.47 ± 1.73 and 1.60 ± 0.64, respectively). ApoA-I<sub>M</sub> carriers had the highest LDL/HDL cholesterol ratios of the 4 groups (8.10 ± 4.07 versus 5.24 ± 1.22 in HA-LC and 5.20 ± 1.63 in HA-BD subjects), and their apoA-I/B ratios were similar to those of the HA subjects (0.83 ± 0.35 versus 0.84 ± 0.20 in HA-LC and 1.03 ± 0.22 in HA-BD).

**Intima-Media Thickness**

The average thicknesses of the 3 examined carotid segments were remarkably similar in apoA-I<sub>M</sub> carriers and control subjects (Table 2). These thicknesses are somewhat lower than those recently reported for a geographically neighboring population (Vobarno Study, which had slightly older participants, i.e., ~57 years).<sup>11</sup> In contrast, the HA subjects, regardless of whether they were selected among the LC patients or among BD subjects without symptomatic cardiovascular disease, had markedly increased average thicknesses at the CCA, ICA, and bifurcation (Table 2). The average IMT was

| Table 1: Demographic, Hemodynamic, Clinical, and Lipid/Lipoprotein Data in Control Subjects, ApoA-I<sub>M</sub> Carriers, and HA Subjects |
|---------------------------------|---------------|-------------|-------------|-------------|
| n | 42 | 21 | 21 | 21 |
| M/F | 22/20 | 11/10 | 11/10 | 11/10 |
| Age, y | 42.3 ± 16.7 | 42.2 ± 17.5 | 43.0 ± 16.9 | 43.6 ± 16.6 |
| BMI, kg/m<sup>2</sup> | 24.1 ± 3.4 | 25.1 ± 2.7 | 24.7 ± 3.9 | 25.6 ± 2.7 |
| Glucose, mg/dL | 86.8 ± 11.8 | 84.1 ± 8.9 | 94.9 ± 10.0* | 83.9 ± 8.5 |
| SBP, mm Hg | 126.9 ± 18.2 | 135.2 ± 24.5 | 134.5 ± 9.6 | 124.7 ± 11.5 |
| DBP, mm Hg | 79.3 ± 7.6 | 84.2 ± 11.2 | 86.4 ± 7.9 | 77.5 ± 7.5 |
| Smokers, n | 17 | 7 | 6 | 6 |

| Table 2: IMT in the CCA, Bifurcation, and ICA Segments in Control Subjects, ApoA-I<sub>M</sub> Carriers, and HA Subjects |
|---------------------------------|---------------|-------------|-------------|-------------|
| CCA | 0.63 ± 0.14 | 0.61 ± 0.15 | 0.78 ± 0.36* | 0.82 ± 0.30* |
| Bifurcation | 0.70 ± 0.14 | 0.74 ± 0.18 | 1.18 ± 0.46* | 1.11 ± 0.46* |
| ICA | 0.60 ± 0.13 | 0.60 ± 0.11 | 0.76 ± 0.35 | 0.77 ± 0.34* |
| Average | 0.64 ± 0.12 | 0.63 ± 0.10 | 0.86 ± 0.25* | 0.88 ± 0.29* |
| Maximum | 0.83 ± 0.12 | 0.89 ± 0.36 | 1.86 ± 0.68* | 1.73 ± 0.78* |

Data are reported as mean ± SD. *Significantly different vs control subjects and ApoA-I<sub>M</sub> carriers.
TABLE 3. Echocardiographic Findings in Control Subjects and ApoA-IM Carriers

<table>
<thead>
<tr>
<th></th>
<th>Control Subjects</th>
<th>ApoA-IM Carriers</th>
</tr>
</thead>
<tbody>
<tr>
<td>LV mass, g</td>
<td>166.8±50.5</td>
<td>169.3±39.1</td>
</tr>
<tr>
<td>LVMI, g/m²</td>
<td>61.8±21.2</td>
<td>57.6±12.8</td>
</tr>
<tr>
<td>IVS thickness, mm</td>
<td>8.4±1.2</td>
<td>8.2±1.2</td>
</tr>
<tr>
<td>PW thickness, mm</td>
<td>7.7±1.1</td>
<td>7.4±1.1</td>
</tr>
<tr>
<td>Relative wall thickness, mm</td>
<td>0.36±0.04</td>
<td>0.33±0.05</td>
</tr>
<tr>
<td>LVESD, mm</td>
<td>50.9±4.0</td>
<td>52.9±3.8*</td>
</tr>
<tr>
<td>LVESD, mm</td>
<td>33.4±3.3</td>
<td>33.5±4.9</td>
</tr>
<tr>
<td>mESS, dyne²/cm²</td>
<td>76.0±10.9</td>
<td>79.4±25.9</td>
</tr>
<tr>
<td>EF, %</td>
<td>62.7±5.3</td>
<td>65.7±8.9</td>
</tr>
<tr>
<td>MWFS, %</td>
<td>16.1±2.3</td>
<td>17.6±3.0</td>
</tr>
<tr>
<td>Cardiac output, L/min</td>
<td>5.16±0.99</td>
<td>5.97±1.28*</td>
</tr>
</tbody>
</table>

IVS indicates intraventricular septal; PW, posterior wall; LVESD, LV end-systolic dimension; mESS, meridional end-systolic stress; EF, ejection fraction; and MWFS, midwall fractional shortening. Data are reported as mean±SD.

*Significantly different vs control subjects.

~44% and 40% higher in the HA-LC and HA-BD subjects than in the other 2 groups, and the maximum IMT, close to 2 mm, was about twice as large as that found in the apoA-IM carriers and control subjects (Table 2). A significantly higher prevalence of atherosclerotic plaques was observed in HA subjects (plaques/subjects, 12/21 in both HA-LC and HA-BD) than in control subjects (plaques/subjects, 9/42); a slightly higher, not significantly different prevalence of plaques was observed in the apoA-IM carriers (plaques/subjects, 7/21) than in control subjects.

Echocardiography and Maximal Treadmill Exercise ECG

Echocardiographic findings and maximal treadmill exercise ECGs were essentially within normal limits and did not show any significant difference between apoA-IM carriers and appropriate control subjects, except for a slight but significant increase in LVESD in the former (Table 3). Interestingly, apoA-IM carriers also had an ~17% increase in cardiac output compared with control subjects from the same kindred. These findings, possibly suggesting that apoA-IM carriers may be somewhat more fit compared with control subjects from the same families, were associated with a nonsignificant ~25% increase in the workload at maximal exercise (125±98 versus 101±55 W).

Discussion

Genetic, biochemical, and general clinical information on the carriers of the apoA-IM mutation has been provided in detail.3,4 In contrast to other conditions associated with apoA-I/HDL abnormalities, eg, fish-eye disease19 or Tangier disease,20 there does not appear to be any clear association between the apolipoprotein abnormality and any pathological condition.3 In the present investigation, quantitative data on the cardiovascular condition are provided to investigate in detail comparable series of apoA-IM carriers and control subjects from the same kindred. In addition, because the apoA-IM carriers are characterized primarily by dramatic reductions in HDL cholesterol levels, 2 matching series of HA individuals without symptomatic cardiovascular disease were recruited from the LC patients and among the 13 000 BD individuals attending our hospital service.

The biochemical evaluation of apoA-IM carriers and HA subjects clearly reflected what had been the background information and purpose of the present investigation. ApoA-IM carriers are typically characterized by reduced HDL levels, elevated triglyceride levels, and LDL cholesterol levels in the normal range. The LDL/HDL cholesterol ratios are therefore in a very-high-risk range. According to Grover et al,21 an LDL/HDL cholesterol ratio >4.9 defines high risk, and according to the model proposed by these authors, there should be an ~4.3-fold difference in cardiovascular risk between apoA-IM carriers and control subjects. One of the carriers (53 years of age) actually underwent an episode of sudden cardiovascular death 6 months after the study. He had been a heavy smoker throughout his life and had uncontrolled hypertension. A similar episode had occurred earlier in this century in an apoA-IM obligate carrier (49 years of age), the ultimate cause of death being cerebral hemorrhage. It is therefore apparent that the severely atherogenic lipoprotein phenotype of HA in apoA-IM carriers is not associated with an increased cardiovascular risk, confirming the initial clinical observations in the earlier study in Limone sul Garda.3

A major finding in the present study was the remarkable difference between IMT at the carotid artery level among the 4 groups of investigated subjects. In fact, control subjects and apoA-IM carriers did not differ in IMT, whereas HA individuals, both HA-LC patients and HA-BD individuals, had markedly increased IMT, particularly at the level of the CCA and bifurcation. When data from the HA individuals are expressed as the maximum IMT, in both cases they reach values close to double those of apoA-IM carriers and appropriate control subjects. IMTs in HA individuals are extremely close to those recently reported in men with low HDL cholesterol levels but without LDL elevations who were participating in the secondary-prevention Veterans Affairs’ HDL Intervention Trial.22 In these men, in fact, who had a mean age of 64.3 years, mean IMTs for the CCA, bifurcation, and ICA were 1.16, 1.72, and 1.40 mm, respectively, with a mean maximum IMT... of 1.41 mm and a single maximum IMT of 2.58 mm. According to a comparative evaluation carried out by these same authors, it appears that these findings fall in the same range as those reported in studies in patients of a similar age who were characterized by LDL cholesterol elevations.23 In a recent report on hypercholesterolemic patients, a significant negative correlation was found between IMT, HDL cholesterol, and the HDL/LDL ratio.24 A similar increase in the coronary atherosclerosis burden, negatively correlated with the HDL/LDL ratio, had been previously demonstrated in asymptomatic patients with marked elevations of plasma cholesterol and triglyceride levels through the use of intravascular ultrasound methodology.25 The apoA-IM carriers, despite a dramatic reduction in HDL cholesterol levels, thus do not differ to any extent in atheromatous burden, as assessed by a highly sensitive, noninvasive method, from close relatives living in the same environment.
with HDL levels in the normal range. Of particular interest is the observation that asymptomatic blood donors with low HDL levels and generally normal triglyceride levels had IMT values that were essentially identical to those of the HA-LC patients, most of whom were hypertriglyceridemic and had elevated plasma apoB levels. These results suggest that isolated low HDL may contribute to increased carotid IMT even in the absence of elevated triglycerides or LDL cholesterol.

ApoA-I M carriers and appropriate control subjects also underwent cardiac echocardiographic evaluation and dynamic studies to detect eventual contractile alterations, changes in cardiac output, and/or impaired cardiac performance during exercise.26 There were no observations of segmental contractile alterations in either group. Both had normal ventricular mass, and all dynamic echocardiographic data were within normal limits; the apoA-I M carriers showed only a statistically significant increase in cardiac output associated with moderate increase of LVEDD, possibly suggestive of improved ventricular diastensibility.

Considerable effort was expended to understand more clearly the mechanism whereby the apoA-I M mutation might be linked to the apparent cardiovascular protection.3 Studies in human carriers and transgenic mice expressing the apoA-I M mutant disclosed a high capacity of serum to extract cholesterol from peripheral cells,27 consequent to the peculiar structural and functional properties of the mutant. Indeed, earlier studies have shown that monomeric apoA-I M has a higher affinity for lipids28 and a faster catabolism than the wild-type apoA-I.29 More recent data demonstrate that the apoA-I M dimer is more likely the “protective” component, characterized by a very slow turnover in both the carriers and normal volunteers29 and by a high efficiency in promoting cell cholesterol efflux.30 A recombinant version of apoA-I M/ apoA-I,31 administered as a phospholipid complex, has been shown to significantly reduce vascular stenosis after balloon angioplasty32 or periarterial manipulation33 in cholesterol-fed rabbits, to prevent or reduce atheroma formation in apoE-deficient mice,34 and to delay thrombus formation in rats.35

In conclusion, a detailed series of cardiovascular studies investigating a group of carriers of the apoA-I M mutant characterized by extreme reductions of HDL cholesterol indicates that despite an atherogenic lipoprotein profile, they do not show any clear evidence of vascular disease at the preclinical level. Comparison with a double number of appropriate control subjects from the same kindred who all have HDL cholesterol levels in the normal range shows that the apoA-I M carriers have remarkably normal IMT at the CCA level and a somewhat improved cardiac performance. In contrast, individuals characterized by reduced HDL cholesterolemia, selected among HA-LC patients and asymptomatic HA-BD subjects, were characterized by a marked increase in CCA IMT, which corresponds well with previous observations in patients with this same biochemical abnormality.22 These data confirm the hypothesis that the apoA-I M carrier status, even in a heterozygous expression, may exert some cardiovascular protective effect. Present knowledge of the potential therapeutic properties of the recombinant apoA-I M dimer36 suggests that the presence of this abnormal form of apoA-I in the circulation may be partly or totally responsible for the arterial protection observed in the carriers.

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References

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