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Accumulation of apoE-enriched triglyceride-rich lipoproteins in patients with coronary artery disease

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Abstract

Triglycerides (TGs) are vehicled by multiple particles with different abilities to promote atherosclerosis. Among plasma TG-rich lipoproteins (TRLs), subspecies may or may not contain apolipoprotein E (apoE) molecules: in this study, we evaluated the relative contribution of apoE-rich and apoE-poor TRLs to coronary atherosclerosis. We selected a group of males with premature coronary artery disease (CAD) without any of the classical nonlipid risk factors and/or high plasma lipid levels and evaluated the plasma concentration of TRL subspecies in comparison with healthy controls. Patients with CAD and controls had total cholesterol and TG levels within the normal range (despite slightly, even if significantly, higher TG levels in patients with CAD) and low-density lipoprotein cholesterol levels near optimal values. Nevertheless, patients with CAD had significantly lower high-density lipoprotein cholesterol, smaller low-density lipoprotein peak particle size, and a reduced HDL2b subfraction than controls. In addition, we observed higher concentrations of total TRL in patients with CAD together with a selective increase in apoE-rich particles. All these data were confirmed after correction for TG levels. We also investigated which parameters were associated with the spread of coronary atherosclerosis. Subjects with a single-vessel disease had selectively lower levels of apoE-rich fractions than patients with a multivessel disease. This was confirmed by multivariate analysis. Patients with a premature CAD free of nonlipid conventional risk factors, despite not having elevated lipid levels, show several lipoprotein abnormalities. Besides known atherogenic alterations, the accumulation of apoE-rich TRL subfractions may represent an additive factor that can potentially promote and initiate the atherosclerotic process.

1. Introduction

A number of risk factors have been commonly associated with atherosclerosis [1]. Although classical risk factors account for most of the risk of coronary artery disease (CAD) worldwide in both sexes, many patients show a lack of any of the conventional risk factors [2,3]. This finding implies that other factors might play a significant role in CAD and leads to a considerable interest in nontraditional risk factors and genetic determinants of CAD. Among lipoprotein abnormalities, total and low-density lipoprotein cholesterol (LDL-C) are the major predictors of atherosclerosis, but many other "hidden" lipoprotein alterations may have a potential role in the pathogenesis of atherosclerosis [4,5]. Triglycerides (TGs), which are not usually considered as an independent risk factor for CAD, are vehicled by multiple particles in plasma [6-8]. Increasing experimental and clinical evidence suggests that the ability of different TG-rich lipoproteins (TRLs) to promote atherosclerosis is not the same [5,9,10]. This is mainly reflected by the different incidence of cardiovascular disease in genetic forms of hypertriglyceridemia [11].

Apolipoprotein E (apoE) is a 299AA glycoprotein that has a pivotal role in TRL metabolism [12]. Among plasma TRLs, subspecies may or may not contain apoE molecules:

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the metabolic pathways and functions of these fractions are not completely known, and only a few data relate these particles to atherosclerosis [5,13-16].

To evaluate the relative contribution of apoE-rich or apoE-poor TRLs on coronary atherosclerosis, we selected a group of male patients with a premature CAD without any of the classical nonlipid risk factors and/or high plasma lipids and evaluated the plasma concentration of these TRL particles in comparison with non-CAD controls. Based upon the strong relationship between high-density lipoprotein (HDL) and coronary atherosclerosis [17], we also further analyzed HDL subfractions in these subjects.

2. Materials and methods

2.1. Patients

During a 1-year follow-up of subjects undergoing an angiography at the Intensive Care Unit of Villa Maria Eleonora Hospital (Palermo, Italy), we selected a group of 29 males who had a coronary stenosis of 75% or greater without any of the classical nonlipid cardiovascular risk factors and who gave informed consent. Inclusion criteria into the study were age less than 55 years, absence of hypertension (systolic and diastolic blood pressure lower than 140 and 90 mm Hg, respectively, and no pharmacological therapy with antihypertensive drugs), diabetes mellitus or impaired glucose tolerance (fasting glucose plasma concentrations <110 mg/dL and no pharmacological therapy with antidiabetic drugs or insulin), and cigarette smoking. Height and weight were recorded, and body mass index (BMI) was expressed as kilograms per meter squared. Participants were also included if BMI was lower than 30 kg/m^2. In addition, all patients who had total cholesterol (CHO) levels less than 200 mg/dL and TG levels less than 170 mg/dL were also included. Exclusion criteria were presence of renal, endocrine, or hepatic disease, or other conditions that can modify plasma lipoproteins, and use of drugs affecting lipid metabolism (hypolipidemic drugs, steroids, diuretics, β-blockers, etc). In 10 of them, angiogram was performed after an acute myocardial infarction (MI), in 13 because of unstable angina, and in 5 for stable angina; 1 patient had a peripheral artery disease. Overall, 17 subjects had a recent or previous MI, 17 had the involvement of only 1 vessel, and 12 had a 2- or 3-vessel disease or a damage of the left main coronary artery (see Table 1). As controls, we selected a group of healthy male subjects, matched for age and body weight, and had the same inclusion and exclusion criteria with the exception of the presence of CAD. Because there was no clinical indication, they did not undergo an angiogram. Procedures followed were in accordance with the Helsinki Declaration of 1975, as revised in 1983. The study was approved by the ethics committee of the University of Palermo. Clinical characteristics of patients with CAD and controls are presented in Table 1.

Table 1
Clinical characteristics of normolipidemic male patients with CAD free of conventional nonlipid risk factors and controls

	Controls	Patients with CAD	*P*
n	29	29	–
Age, y	42.0 ± 7.4	42.9 ± 7.6	NS
BMI, kg/m^2	25.4 ± 3.3	24.9 ± 4.7	NS
2/3, n	1	3	
3/3, n	26	25	
3/4, n	2	1	
Insulin, mU/L	47.4 ± 37.0	49.0 ± 33.6	NS
Obesity[a], n (%)	0 (0.0%)	0 (0.0%)	–
Diabetes, n (%)	0 (0.0%)	0 (0.0%)	–
Hypertension, n (%)	0 (0.0%)	0 (0.0%)	–
Smokers, n (%)	0 (0.0%)	0 (0.0%)	–
Genetic dyslipidemia, n (%)	0 (0.0%)	0 (0.0%)	–
Familial history of premature CHD, %	0 (0.0%)	5 (17.2%)	–
Myocardial infarction, n (%)	0 (0.0%)	17 (58.6%)	–
Vessels with stenosis ≥7 5, n (%)			
One	–	17 (58.6%)	–
Two	–	7 (24.1%)	–
Three or LMCA disease	–	5 (17.2%)	–
PTCA, n (%)	0 (0.0%)	6 (20.7%)	–
Stroke, n (%)	0 (0.0%)	2 (6.9%)	–
Peripheral artery disease, n (%)	0 (0.0%)	1 (3.4%)	–

LMCA indicates left main coronary artery; PTCA, percutaneous transluminal coronary angioplasty.
[a] BMI ≥ 30 kg/m^2.

2.2. Laboratory procedures

Blood samples were collected in sodium-EDTA tubes after an overnight fast before the angiographic procedure. Analyses of CHO and TGs were performed by standard enzymatic procedures (Boehringer, Mannheim, Germany), HDL-C after phosphotungstic acid/magnesium chloride precipitation and enzymatic determination of cholesterol, and LDL-C by the Friedewald formula (LDL-C = CHO − TG/5 − HDL-C); non–HDL-C was calculated by the difference; protein content was measured by a commercial protein assay (BCA, Pierce Biotechnology, Rockford, Ill) [18]. All mentioned procedures were performed on a COBAS MIRA PLUS auto-analyzer (Roche, Basel, Switzerland), with a low sample absorbance threshold to avoid possible interferences due to sample turbidity. Lipoprotein (a) [Lp(a)] was measured using a commercial latex immunonephelometric method (Dade Behring, Newark, Del). Insulin levels were determined by radioimmunoassay. The apoE genotypes were determined by using the polymerase chain reaction method with primers and condition as described by Hixson and Vernier [19].

2.3. Nondenaturing polyacrylamide gradient gel electrophoresis

Nondenaturing polyacrylamide gradient gel electrophoresis of whole plasma was performed in 2% to 16%

Table 2
Plasma lipids and lipoproteins of normolipidemic male patients with CAD free of conventional nonlipid risk factors and controls

	Controls	Patients with CAD	*P*
CHO, mg/dL	173.0 ± 18.0	165.8 ± 19.5	NS
TG, mg/dL	91.6 ± 39.2	115.5 ± 36.0	<.05
HDL-C, mg/dL	43.7 ± 11.0	31.4 ± 9.2	<.0001
LDL-C, mg/dL	111.1 ± 17.0	110.9 ± 15.9	NS
Non–HDL-C, mg/dL	129.4 ± 18.6	134.2 ± 18.3	NS
LDL peak particle size, Å	271.7 ± 5.0	262.7 ± 5.0	<.0001
Lp(a)[a]	13.6 ± 20.0	28.9 ± 15.1	<.0001
HDL2b, %	33.4 ± 9.2	27.5 ± 10.1	<.05
HDL2a, %	25.7 ± 3.1	24.6 ± 4.8	NS
HDL3a, %	21.0 ± 4.1	23.0 ± 3.7	NS
HDL3b, %	11.1 ± 4.2	13.5 ± 6.1	NS
HDL3c, %	9.1 ± 5.8	11.7 ± 5.8	NS

[a] After log transformation.

polyacrylamide gradient gels as previously described [20], using commercially available gels (Alamo Gels, San Antonio, Tex) and standards of previously determined particle size (kindly provided by RM Krauss and PJ Blanche from the Lawrence Berkeley National Laboratory, University of California, Berkeley, Calif). A group of randomized samples were also tested in a reference laboratory (Lawrence Berkeley National Laboratory), using gels made by established procedures, without significant differences in the results.

The analysis of HDL subfraction was performed by one of the authors (MR) at the Lawrence Berkeley National Laboratory with the help of Dr RM Krauss by gradient gel electrophoresis as described by Nichols et al [21] using 3% to 31% home-made gradient gels prepared by the method of Rainwater et al [22].

2.4. Isolation of TRLs

Triglyceride-rich lipoproteins were isolated from plasma by preparative ultracentrifugation at a density of less than 1.019 g/mL. Sodium bromide was used to adjust the density, and the samples were centrifuged at 40 000 rpm for 24 hours at 10°C in a Beckman 40 Ti fixed angle rotor. Under these standard conditions, both very low density lipoprotein and intermediate-density lipoprotein migrated together at the top of the tube.

2.5. Affinity chromatography of TRL

Apolipoprotein E–rich (bound [BND]) TRLs were separated from apoE-poor (unbound [UNB]) TRLs by heparin affinity chromatography. A 5-mL Hi Trap column (Amersham-Biosciences, Uppsala, Sweden) was used on a fast protein liquid chromatography system. We performed a slight modification of a previously described method [23]. Briefly, the whole TRL fraction, separated as above described, was dialyzed over a 24- to 48-hour period against the equilibrating buffer of the column (Trizma base–HCl 10 mmol/L, pH 8). After column equilibration with 2 to 3 beds of equilibrating buffer at a flux of 0.5 mL/min, the sample was loaded in an equilibrating buffer. The apoE-poor fraction was eluted in the same buffer at low ionic strength (0 mol/L NaCl). To release the apoE-rich fraction, the ionic strength was increased to 0.8 mol/L NaCl stepwise. Both fractions were monitored at 280 nm of wavelength, collected, dialyzed, and assayed for cholesterol, TG, and protein content.

2.6. Sodium dodecyl sulfate–polyacrylamide gradient gel electrophoresis

Three percent to 15% sodium dodecyl sulfate–polyacrylamide gradient gel electrophoresis (SDS-PAGE) was used to assess apolipoprotein distribution in the 2 TRL subfractions as separated by heparin-sepharose affinity chromatography in a subset of subjects (n = 12). The samples were preliminarily solubilized in buffer containing 2% of SDS and incubated for 10 minutes at 90°C. Then the samples were loaded at 50 μg per lane immediately after incubation in 3% to 15% acrylamide home-made gradient gels (pH 8.8). As previously described [24], after electrophoresis at constant voltage, the gels were fixed with an isopropanol–acetic acid solution, stained with Coomassie Brillant Blue R250, and destained with acetic acid. The relative amounts of apolipoproteins in the different fractions were determined by densitometry.

2.7. Statistical analysis

Statistical analysis was performed using the software SPSS 10.1 for Windows (SPSS, Chicago, Ill). Mean values and standard deviations were calculated, and the differences between groups were compared using 2-tailed Student *t* test for unpaired data. Because Lp(a) levels were found not normally distributed by the Kolgomorov-Smirnov test, statistical analysis for this variable was performed after log transformation of the data. Statistical significance was considered when differences between groups had *P* values less than .05. Multivariate analysis was performed using a multiple logistic regression model with the number of vessels involved (one or more vessels) as the dependent

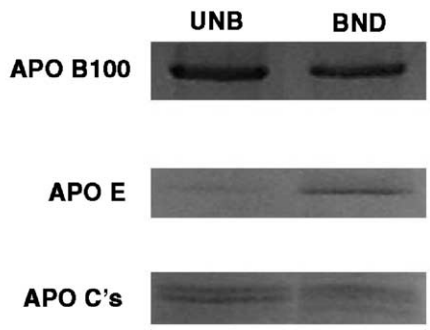

Fig. 1. Three percent to 15% SDS-PAGE of apoE-rich (BND) and apoE-poor (UNB) fractions, separated by heparin affinity chromatography as described in the Materials and methods section.

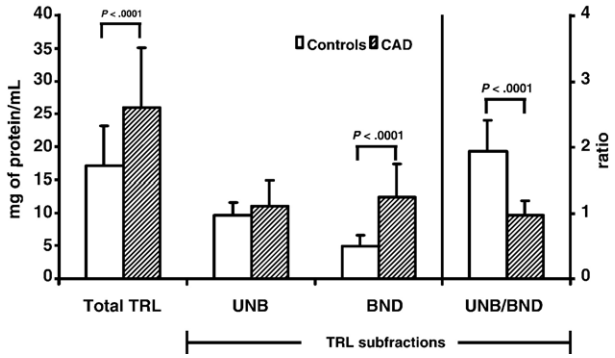

Fig. 2. Total TRLs and apoE-poor (UNB) and apoE-rich (BND) particle TRL in normolipidemic male patients with CAD free of conventional nonlipid risk factors and in controls. Mean ± SD.

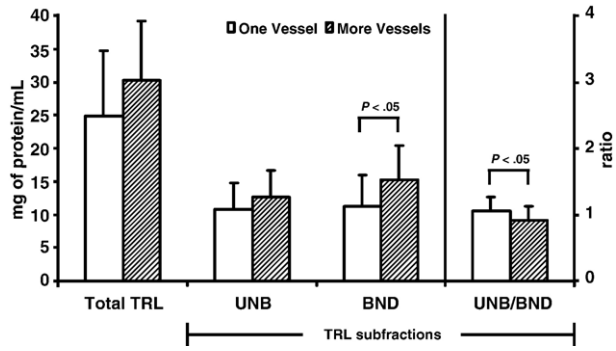

Fig. 3. Total TRLs and apoE-poor (UNB) and apoE-rich (BND) particle TRL in normolipidemic male CAD patients free of conventional nonlipid risk factors divided according to the number of stenotic vessels as demonstrated by angiography. One vessel: patients with a single-vessel disease; more vessels: patients with a multivessel disease or a damage of the main left coronary artery. Mean ± SD.

variable, and age, BMI, insulin, and all lipoprotein parameters as predictors.

3. Results

Patients with CAD and controls had similar mean age, BMI, and insulin levels. No subject was homozygote for 2 or 4 alleles, and all showed at least one 3 allele (Table 1). Plasma lipids and lipoproteins are shown in Table 2. Patients with CAD had significantly higher TG and Lp(a), lower HDL-C, a smaller LDL peak particle size, and reduced HDL2b subfractions than controls. To define the characteristics of BND or UNB fraction, we analyze the composition of these fractions as well as the distribution of apoE by 3% to 15% SDS-PAGE. Our findings indicated that BND represents a cholesterol-enriched particle in comparison with UNB (cholesterol/protein and TG/protein ratios, respectively, 1.8 and 6.0 in BND vs 0.2 and 6.5 in UNB) and that apoE was almost completely in the BND fraction, whereas UNB contained only apoE traces (see Fig. 1). When we compared the TRLs between the 2 groups, we observed higher concentrations of the total fraction in patients with CAD together with a selective increase in BND particles; UNB/BND ratio was significantly lower in subjects with CAD (Fig. 2). All differences were confirmed after correction of data for TG levels (data not shown). We also investigated the association of studied parameters with the spread of coronary atherosclerosis. Among patients with CAD, we compared those with the involvement of a single-vessel disease with those with a multiple-vessel disease or with the involvement of the left main coronary artery. We were not able to find any difference in age, BMI, insulin levels, plasma lipids, lipoproteins, LDL peak particle size, or HDL subfractions (Table 3). However, subjects with a single-vessel disease had selectively lower levels of BND fractions with a higher UNB/BND ratio than the other patients (Fig. 3). Even the logistic analysis performed with the number of vessels involved (one or more vessels) as dependent variable and all clinical and lipoprotein parameters as predictors demonstrated that the only parameter significantly associated with the spread of the disease was BND (β = .172, SE = 0.088, χ^2 = 3.80, P < .05, data not shown). We also analyzed the possible difference between patients with CAD based on a previous MI, but we were not able to find any difference (data not shown).

Table 3
Plasma lipids and lipoproteins in patients divided according to the number of stenotic vessels as demonstrated by angiography

	One vessel	More vessels	*P*
n	17	12	
Age, y	45.6 ± 4.8	46.9 ± 7.0	NS
BMI, kg/m^2	26.0 ± 2.3	26.8 ± 2.3	NS
Insulin, mU/L	53.1 ± 35.8	43.7 ± 31.0	NS
CHO, mg/dL	165.9 ± 21.8	165.3 ± 15.9	NS
TG, mg/dL	117.1 ± 40.7	113.3 ± 29.5	NS
HDL-C, mg/dL	32.1 ± 10.2	30.5 ± 7.8	NS
LDL-C, mg/dL	110.0 ± 17.2	112.1 ± 14.6	NS
Non–HDL-C, mg/dL	133.8 ± 20.7	134.8 ± 14.9	NS
LDL peak particle size, Å	263.0 ± 4.0	262.2 ± 6.2	NS
Lp(a)[a]	25.5 ± 15.8	33.4 ± 13.5	NS
HDL2b, %	27.4 ± 10.6	27.5 ± 9.8	NS
HDL2a, %	24.5 ± 4.8	24.8 ± 5.0	NS
HDL3a, %	22.5 ± 3.9	23.6 ± 3.5	NS
HDL3b, %	13.7 ± 6.1	13.2 ± 6.3	NS
HDL3c, %	12.0 ± 6.1	11.3 ± 5.7	NS

One vessel indicates patients with a single-vessel disease; more vessels, patients with a multivessel disease or a damage of the LMCA.
[a] After log transformation.

4. Discussion

Atherosclerosis is a multifactorial disorder, but plasma lipids represent the major predictors of vascular damage and clinical events [1,4-6,9,17]. The success of cardiovascular prevention by cholesterol-lowering drugs is well documented, and all guidelines define optimal lipid targets

according to the individual risk level [25,26]. However, a subset of patients with coronary heart disease (CHD) do not show any evident lipid abnormality or established risk factors [2,3]. A genetic susceptibility or new "emerging" conditions (eg, hyperhomocysteinemia, inflammatory, or infective factors) may contribute to the development of atherosclerosis in these patients even if the role of plasma lipoproteins may not be excluded at all [5,27,28].

Despite the usual failure of epidemiological studies to identify TG levels as an independent risk factor, patients with CHD often show a lipid profile characterized by high TG/low HDL-C levels [6,29-31]. However, TRLs comprise largely heterogeneous and discrete subspecies differing in composition, metabolic pathways, and atherogenic potentiality [7-9]. A major role in TRL metabolism is carried out by apoE, a protein that mediates the binding and clearance of TRL remnants, a highly atherogenic TRL subfraction; it may also influence lipoprotein lipase–mediated hydrolysis of large TRLs [12]. Apolipoprotein E is a polymorphic protein: 3 codominant alleles (2, 3, 4) encode for 3 different isoforms (E2, E3, E4), determining 6 different genotypes. Apolipoprotein E isoforms have a different receptor affinity, higher for apoE4 and lower for apoE2, and for this reason apoE polymorphisms may influence many lipoprotein features including remnant levels [32,33]. The relevance of apoE in remnant metabolism has been further confirmed by the accumulation of remnants in subjects with dysbetalipoproteinemia, which is commonly associated with apoE2 homozygosis or apoE variant [34,35]. Triglyceride-rich lipoproteins may have a different apoE content, and either apoE-rich or apoE-poor TRLs have been demonstrated in human plasma: based on their characteristics, it is not known which of these fractions better represents TRL remnants [5,13,16,24,36]. Accumulation of these particles is often associated with other metabolic conditions with a high cardiovascular risk, such as hyperlipidemia or diabetes, and therefore it is difficult to evaluate their own atherogenic potentiality [37,38]. In transgenic rabbits, we documented that overexpression of apoE mediated a selective removal of large TRL with accumulation of small-sized TRLs; these animals showed a larger, even if thin, atherosclerotic involvement of the proximal aorta than control animals, suggesting that their predominant lipoprotein particles may facilitate the initiation of the atherosclerotic process [39,40]. Fewer data are available in humans, and we designed this study to evaluate the relative contribution of the plasma concentration of these TRL subspecies to coronary atherosclerosis. For this reason, we accurately selected, during a long follow-up, a group of subjects free of all classical nonlipid risk factors for atherosclerosis, who had a premature, severe CAD demonstrated by angiography. To avoid the possible influence of sex and hyperlipidemias, we recruited only males and excluded subjects with high plasma lipids. Because it is known that in such patients HDL may have an important role in the development of coronary damage, we also measured HDL subfractions [17]. In this way, we were able to select a group of subjects who had CHO and TG levels within the normal range (despite slightly, even if significantly, higher TG levels in patients) and LDL-C levels near optimal values (in the range at which according to their risk profile, guidelines suggest therapeutic lifestyle changes and only optional drug therapy) [25]. Nevertheless, our findings show that patients with CAD still have an atherogenic profile with lower HDL-C levels than controls, mainly because of the largest particle, and a smaller LDL peak particle size. Large HDL particles are known to represent the protective subfractions, whereas subjects with a predominance of small, dense LDL (LDL phenotype B) particles have a 3- or 4-fold increased risk of CHD [41,42]. In agreement with previous data, Lp(a) levels were also higher in patients with CAD [30]. In addition, these patients showed increased levels of TRL particles with a selective accumulation of the apoE-rich lipoproteins; the differences were confirmed after correction of data by TG concentrations. These particles resulted in higher relative cholesterol content, supporting a greater atherogenic potentiality. Because apoE genotypes may represent a factor contributing to modulate plasma concentrations of apoE-rich lipoproteins [33], we analyzed apoE isoforms in our patients but we could not demonstrate any difference in or influence on apoE-rich particle levels. When we evaluated the relationship between lipoprotein particles with the number of coronary vessels involved in the disease, we found that the apoE-rich fraction was the only parameter associated with a wider spread of coronary damage at both univariate and multivariate analyses, whereas no association was demonstrated with the incidence of MI. In animal models, apoE-rich particles seem to be able to begin the atherosclerotic process and to widen lesion areas but not to thicken them [40]. Our data exactly express these findings in humans: accumulation of cholesterol-enriched, apoE-rich particles, despite no additional risk factor, led to premature atherosclerosis and was related to the number of atherosclerotic vessels, indicating that these particles are highly atherogenic per se. On the contrary, because cardiovascular clinical events are usually determined by thrombotic factors working on unstable plaques [43], it is not surprising that there is lack of association between lipoprotein parameters and history of a previous MI. In this study, we have no direct measurements of remnants (eg, by the determination of remnant-like particle cholesterol) [38] and therefore we were not able to relate TRL subclasses with these atherogenic fractions. However, non–HDL-cholesterol levels, which reflect the content of remnant particles and represent a good predictor of cardiovascular disease in the general population [25,44], were not different between patients and controls, suggesting that apoE-rich TRLs are not likely to be the expression only of remnant particles. These alterations overlap, in part, with those of a particular disorder, defined as *atherogenic lipoprotein phenotype*, characterized by an accelerated vascular damage [45]. Atherogenic lipoprotein phenotype may be genetically

determined, but it is also a typical feature of the metabolic syndrome, a high-risk condition determined by hyperinsulinemia and visceral obesity. In this study, we excluded obese subjects; our patients did not show any difference in body weight in comparison with controls, and also insulin levels were similar. These data argue against an increased prevalence of the metabolic syndrome as an explanation for the increased levels of atherogenic particles.

There is no published evidence of the benefit of drug intervention on apoE-rich TRLs. For this reason, in the attempt to evaluate the effects of statin treatment, we studied a subgroup of patients with CAD (n = 11) after 3 months of therapy with simvastatin 20 mg/d. In this period, despite a significant decrease in LDL-C levels, we were not able to find any significant difference in apoE-rich TRLs (14.7 ± 3.6 milligram of proteins per milliliter before simvastatin vs 12.6 ± 2.6 milligram of proteins per milliliter after simvastatin; data not shown), and this suggests that the removal pathway of these fractions is probably not through the LDL receptor. Thus the prevention of cardiovascular clinical events documented also in this kind of patients during statin treatment must be attributed to other mechanisms [46,47].

In conclusion, our data suggest that young patients with CAD without classical nonlipid risk factors, regardless of apoE genotype distribution, accumulate apoE-rich TRLs together with a complex of other lipoprotein abnormalities that may potentially promote and initiate the atherosclerotic process. Even if underlying causes remain unclear, some alternative putative mechanisms may be suggested to explain the accumulation of apoE-rich particles in these patients: (*a*) the in vivo clearance of these particles is dependent on receptors whose function may be impaired in patients with CAD; (*b*) other characteristics of apoE-rich TRLs in patients with CAD (eg, increased content of apoCIII) may result in an impairment of their metabolism or clearance; (*c*) the increased production of such particles may saturate normal pathways for their catabolism or clearance.

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