Risk Factors, Biochemical Markers, and Genetic Polymorphisms in Early Coronary Artery Disease

Maria Cristina Izar, Francisco Antonio Helfenstein Fonseca, Silvia Saiuli Miki Ihara, Nelson Kasinski, Sang Won Han, Ieda Edite Lanzarini Lopes, Leonor do Espírito Santo de Almeida Pinto, Waldir Gabriel Miranda Relvas, Daisy Lourenço, Sérgio Tufik, Angelo Amato Vincenzo de Paola, Antonio Carlos Camargo Carvalho

São Paulo, SP - Brazil

Objective – To assess the risk factors, lipid and apolipoprotein profile, hemostasis variables, and polymorphisms of the apolipoprotein AI-CIII gene in early coronary artery disease (CAD).

Methods – Case-control study with 112 patients in each group controlled by sex and age. After clinical evaluation and nutritional instruction, blood samples were collected for biochemical assays and genetic study.

Results – Familial history of early CAD (64 vs 39%), arterial hypertension (69 vs 36%), diabetes mellitus (25 vs 3%), and previous smoking (71 vs 46%) were more prevalent in the case group (p<0.001). Hypertension and diabetes were independent risk factors. Early CAD was characterized by higher serum levels of total cholesterol (235 ± 6 vs 209 ± 4 mg/dL), of LDL-c (154 ± 5 vs 135 ± 4 mg/dL), triglycerides (205 ± 12 vs 143 ± 9 mg/dL), and apolipoprotein B (129 ± 3 vs 105 ± 3 mg/dL), and lower serum levels of HDL-c (40 ± 1 vs 46 ± 1 mg/dL) and apolipoprotein AI (132 ± 2 vs 146 ± 2 mg/dL) [p<0.01], in addition to an elevation in fibrinogen and D-dimer (p<0.02). The simultaneous presence of the rare alleles of the APO AI-CIII genes in early CAD are associated with hypertriglyceridemia (p=0.03).

Conclusion – Of the classical risk factors, hypertension and diabetes mellitus were independently associated with early CAD. In addition to an unfavorable lipid profile, an increase in the thrombotic risk was identified in this population. An additive effect of the APO AI-CIII genes was observed in triglyceride levels.

Keywords: Coronary artery disease, risk factors, genetic polymorphisms.

According to data from the Ministry of Health in Brazil, in 1998, cardiovascular disease accounted for more than 250,000 deaths, corresponding to 32% of all causes of death in our country. Of the cardiovascular causes, cerebral stroke was first, followed by myocardial ischemic disease. The contribution of traditional risk factors for coronary artery disease in the general population emerged from the studies in the city of Framingham. The recent guidelines of the National Cholesterol Education Program in the United States (Adult Treatment Panel III) recognize other markers of coronary risk, classified as risk factors related to lifestyle (obesity, physical inactivity, and atherogenic diet) and emerging risk factors [lipoprotein (a), homocysteine, markers of thrombosis and inflammation, altered fasting glycemia, and evidence of subclinical atherosclerosis]. The metabolic syndrome, whose substrate is insulin resistance, has been proposed to explain lipid, hemostatic, and inflammatory abnormalities, predisposing individuals to early coronary artery disease (CAD).

Apolipoprotein AI, the major protein component of HDL, is an in vivo activator of lecithin-cholesterol acyltransferase (LCAT) and plays a crucial role in reverse cholesterol transport. Apolipoprotein CII is a component of the particles rich in triglycerides and of HDL and influences the regulation of plasma concentrations of triglycerides. Apolipoprotein CIII was shown in vivo to inhibit the lipoprotein and hepatic lipases, reducing hydrolysis of triglycerides, making the recognition of the remnant particles by hepatic receptors difficult. The genes that regulate the expression of apolipoproteins AI and CII are very closely located in a gene complex in the long arm of human chromosome 11. Polymorphisms in nontranslated regions of the APO AI gene with substitutions G/A (-75 pb) and C/T (+83 pb) (M1 and M2 alleles) and in exon 4 of the APO CII gene (3238 C/G) have been reported in association with alterations in
The present study aimed at identifying cardiovascular risk factors and markers in a population with early CAD.

Methods

A case-control study was carried out in consecutive patients, 112 with early coronary artery disease (men < 45, women < 55 years) and 112 with no manifestation of atherosclerosis, controlled by sex and age. All patients were consecutively selected from the cardiology outpatient care clinic at the Federal University of São Paulo (UNIFESP). The criteria for CAD included a history of myocardial infarction, stable or unstable angina, and surgical or percutaneous revascularization.

The controls comprised the spouses, neighbors, and people from the same workplace of the patients, with the same sociocultural conditions, in whom the clinical history, the objective search for signals of CAD, and the electrocardiographic examination did not suggest the presence of that disease.

The following patients were excluded from the study: those with acute coronary syndromes, those who had undergone myocardial revascularization surgery, those who had undergone percutaneous intervention during the first 3 months of those events, and those with renal (serum creatinine > 2.0 mg/dL) or hepatic failure, uncontrolled hypothyroidism, or neoplasia.

The protocol was approved by the Committee on Ethics in Research of the UNIFESP. After obtaining informed consent, clinical and nutritional assessments were performed. The lipid-lowering drugs were withdrawn (statins for 4 weeks and fibrates for 8 weeks), and the patients were advised to follow the American Heart Association diet (AHA step I). After this period of time, blood samples were collected after a fasting period of 12 hours for general biochemistry and other specific assays.

The risk factors were identified according to the recommendations of the II Brazilian Guidelines on Dyslipidemias. In regard of tobacco consume, patients smoking any number of cigarettes regularly for a period longer than six months were considered previous smokers; those smoking any number of cigarettes within one month prior to the interview were considered current smokers; these guidelines were recently validated by the National Cholesterol Education Program of the USA (NCEP III).

A complete lipid profile was performed by an automated enzymatic method, and LDL–c was estimated by using the Friedewald formula. The apolipoproteins were determined with nephelometry [AI, B, and Lp (a), Beckman, and apolipoprotein E, Behring].

Fibrinogen was estimated according to the Clauss method, and factor VII was estimated by the addition of a plasma deficient in factor VII and thromboplastin (Simplastin Excel), both using the photomechanical method (Thrombotimer). Type-1 plasminogen activator inhibitor, von Willebrand factor, and D-dimer were assessed with the immunoenzymatic assay technique (American Diagnostica). Because the patients in the case group were in secondary prevention of coronary atherosclerotic disease, the analyses were performed under antiplatelet therapy (acetyl salicylic acid in 103 patients); the patients receiving oral anticoagulants were excluded from coagulation studies. No patient in the control group was taking antiplatelet agents or anticoagulants.

Total genomic DNA was extracted from leukocytes with an extraction set (GFX™ Genomic Blood Purification Kit, Amersham Bioscience). Amplification of the genes of interest occurred with polymerase chain reaction (PCR), in a thermocycler (Peltier Effect Cycling, MJ Research) programmed for 5 minutes at 94°C, 30 cycles, with 1 minute at 94°C, 1.5 minutes at 60°C, and 1.5 minutes at 72°C, followed by a final extension of 10 minutes at 72°C.

The primers (Gibco) used were:

- APO AI: sense 5’-AGGGACAGAGCTGATCTTTGAACCTTTAAG-3’, anti-sense 5’-TTAGGGGACCTAGGCCCTCGAGAAGAGAGCA-3’.
- APO CIII: sense 5’-GCTGACCGATGGCTCAAGTTCAGTT-3’, anti-sense 5’-CAGAACGTGGGATAGAGCGCT-3’.

The purified products of PCR were digested with Msp I (for APO AI) and Sst I (for APO CIII) restriction endonucleases and the appropriate buffers (Invitrogen) for 3 hours at 37°C. The digestion products were separated by 1.5% agarose gel electrophoresis (45 minutes, 5 V/cm), stained with ethidium bromide, and visualized under ultraviolet light. The Msp I restriction endonuclease cleaves DNA in the 5’-CCG3’ sequence. In the APO AI gene (433 pb), it causes the cleavage of a normally existing site, determining 2 alleles, M1 (177 bp) and M2 (255 bp). It also determines the polymorphic sites with the following bands: 177 (M1-), 177, 110, 67 (M1 +) and 110, 67 pb (M1++) for the M1 allele; and 255 (M2), 255, 207, 48 (M1++) and 207 and 48 pb (M2++) for the M2 allele.

The Sst I restriction endonuclease recognizes A in the 5’-GAGCT-C3’ sequence, causing cleavage of the APO CIII gene into 2 fragments with 265 and 163 pb bands.

The rare alleles are characterized by the absence (APO AI) and presence (APO CIII) of restriction sites of the enzymes.

The chi-square test was used to analyze the categorical variables, to test the deviations of the Hardy-Weinberg genotypic distribution, and also to compare the genotypic frequencies between cases and controls. The continuous variables were expressed as mean ± EPM. The means were tested with the nonpaired Student t test for equal or unequal variances as appropriate. The lipid distribution according to the genotypes, and the number of rare alleles were compared by the Student t test and analysis of variance (ANOVA), respectively. Multiple logistic regression was used to assess the associations among parameters and coronary artery disease. P values < 0.05 were considered significant.
Results

The baseline characteristics of the patients are shown in Table I. No differences were seen regarding sex and age distribution of patients among groups. One hundred and nine patients underwent coronary angiography and had a wide range of coronary obstructions. Two- or three-vessel involvement was the predominant distribution pattern of coronary lesions in the case group patients (tab I).

Familial history of early coronary artery disease (64% vs 39%; p=0.0002), arterial hypertension (69% vs 36%; p<0.0001), diabetes mellitus (25% vs 3%; p<0.0001), and previous smoking (71% vs 46%; p<0.0001) were highly prevalent in the group with early CAD as compared with those in the control group. Prevalence of current smoking, however, did not differ (26% vs 25%) between the groups. Body mass index (BMI) was similar in both groups (27.5 ± 4.8 vs 26.7 ± 4.6), and most patients were obese or overweight (65% vs 62%, p=0.05).

Total cholesterol (235 ± 6 vs 209 ± 4 mg/dL; p=0.0002), LDL-c (154 ± 5 vs 135 ± 4 mg/dL; p=0.0002), and triglycerides (205 ± 12 vs 143 ± 9 mg/dL; p=0.0001) were higher in patients with early CAD, whose levels of HDL-c (40 ± 1 vs 46 ± 1 mg/dL; p=0.0006) were lower. Lower levels of apolipoprotein AI (134 ± 2 vs 146 ± 2 mg/dL; p=0.0003) and higher levels of apolipoprotein B (129 ± 3 vs 105 ± 3 mg/dL; p<0.0001) were observed in early CAD. No difference was observed in the levels of apolipoprotein E (3.7 ± 0.4 vs 3.3 ± 0.3 mg/dL) and Lp(a) (43 ± 4 vs 33 ± 5 mg/dL). Figures 1 and 2 depict the levels of apolipoprotein E (3.7 ± 0.4 vs 3.3 ± 0.3 mg/dL) and diabetes mellitus (25% vs 3%; p<0.0001), and von Willebrand factor (81 ± 2 vs 79 ± 2%) did not differ. Figure 3 depicts the distribution of markers of hemostasis per quartiles.

DNA for APO AI genotyping was obtained in 104 patients in each group, and for APO CIII in 107 cases and 104 controls. The genotypic frequencies observed and expected for the APO AI and APO CIII genes were in Hardy-Weinberg equilibrium. Table II shows the distribution of APO AI (M1 and M2) and APO CIII genotypes in case and control groups, no differences being observed between them.

Apolipoprotein AI, HDL-c, and triglycerides did not differ between APO AI and APO CIII genotypes, even when the 2 groups were considered as a whole, or when each group was considered separately. When the effect of multiple rare alleles (M1 - /M2 - /S2) was considered, hypertriglyceridemia was observed in the presence of 2 rare alleles in the group with early CAD (TG with 2 alleles > TG with 0 allele, p=0.03, ANOVA) (tab III).

Multiple logistic regression showed that only arterial hypertension and diabetes mellitus were independently associated with early CAD (tab IV).

Discussion

The aim of this study was to identify in patients with early CAD the classical risk factors and some of the new risk markers, including apolipoproteins and hemostasis variables, in addition to polymorphisms of the APO AI-CIII genes. These genetic markers were chosen because they participate in lipoprotein metabolism, affecting the removal of remnant particles, and also reverse cholesterol transport. In addition, those markers are related to the metabolic syndrome and to a form of dyslipidemia commonly found in individuals surviving an early infarction (familial combined hyperlipidemia) 18.

As a case-control study, the choice of each group is critical to the correct interpretation of the data. Therefore, constitution of early CAD group was based on the presence of coronary atherosclerosis, while the control group was characterized by the complete absence of symptoms and coronary history, as well as normal electrocardiographic findings. Although subclinical atherosclerosis cannot be ruled out, the absence of manifest atherosclerosis, in a population controlled by age and sex, was considered appropriate, because autopsy studies have reported the gradual development of initial lesions throughout life 19,20. Therefore, sophisticated methods, such as ultrasonography, angioresonance, and ultrafast tomography have been questioned in regard to their capacity for foretelling coronary events 21,22.

The relevant findings in early CAD were as follows: a high prevalence of classic risk factors, with hypertension and diabetes being independently associated, and an un-
favorable lipid profile characterized by elevation in total cholesterol, LDL-c, and triglycerides, and reduction in HDL–c. Among the new risk markers, higher levels of apolipoprotein B and lower levels of apolipoprotein AI were observed in early CAD. In addition, higher levels of fibrinogen and D-dimer in a chronic and stable phase of coronary artery disease suggest an increased thrombotic risk, even under antiplatelet therapy. Although the genotypic distribution between the groups did not differ, an association between the number of rare alleles and high triglycerides was observed in early CAD.

In the PROCAM study, 48.4% of the men aged 45 to 65 years who developed CAD were hypertensive, while in the MRFIT study, in a 12-year follow-up, 49% of the deaths caused by CAD, in the same age group, were related to hypertension. Likewise, the prevalence of diabetes mellitus observed in early CAD (25%) was 8 times greater than that observed in controls, greater than that observed in the individuals who developed coronary artery disease in the PROCAM study, and much greater than its prevalence in the general Brazilian population, around 7.5%. A positive familial history of early CAD suggests a strong inherited or environmental component. In the PROCAM study, family history characterized a high-risk group, and, recently, NCEP III began to consider it as a major risk factor. The risk of CAD among smokers in the PROCAM study was more than twice that of nonsmokers, while the risk of an ex-smoker was only mildly increased as compared with that of nonsmokers. The high prevalence of smoking at the time of the initial manifestation of coronary artery disease contributed to the formation and growth of the atherosclerotic plaque in oxidative processes, and prothrombotic and proinflammatory phenomena.

The high prevalence of overweight and obesity in both groups suggests the presence of a metabolic component in this population. Exposure of the individuals to a similar BMI promoted great differences, both in the occurrence of diabetes mellitus and in dyslipidemia. Therefore, complete expression of the metabolic syndrome or of the coronary risk seems to depend on other conditions, possibly genetic,

Fig. 1—Lipid distribution according to quartiles. Box-plots contain the 1st and 3rd quartiles, the median, and the whiskers represent the lowest and the greatest values. *p<0.001 vs control (paired Student t-test).
to express the typical lipid phenotype. The aggregation of risk factors found in our study was another determinant of the early occurrence of the disease. The association of arterial hypertension and diabetes, an elevation in TG, and a reduction in HDL-c favored the hypothesis that metabolic syndrome participates in the development of early CAD.

Abnormal levels of serum lipids and apolipoproteins characterizes the patients with early CAD, who had a type of dyslipidemia with an increase in atherogenic lipoproteins and in apolipoprotein B, lower levels of HDL-c and apo AI. A study assessing cohorts of young individuals showed a strong association between serum cholesterol, coronary artery disease, and cardiovascular death, greater than that observed for middle-aged men with the same cholesterolemia. The HDL-c levels in early CAD were similar to those observed in the PROCAM study (40 vs 46 mg/dL) as well as those from the Brazilian Society of Cardiology. In our study, hypertriglyceridemia alone was not observed in the group with early CAD, but in association with low HDL-c or high LDL-c, or both, representing an additional risk to those patients.

Several studies have related the higher levels of apolipoprotein B and the lower levels of apolipoprotein AI to the early occurrence of CAD, to the presence of recurring events, and to thrombotic processes. Total apolipoprotein E reflects both the atherogenic particles containing Apo E and the Apo E of HDL, and their levels may not have differed among groups because the higher content of Apo E in Apo E in triglyceride-rich particles, and lower in HDL may have masked differences in patients with early CAD. The Lp(a) levels are genetically determined and are under ethnic influences. Both groups showed increased Lp(a) levels, which could be related to the ethnic heterogeneity in our community.

In vitro platelet aggregation was significantly attenuated in early CAD due to the chronic use of antiplatelet agents. However, other markers of hemostasis, such as fibrinogen and D-dimer, were elevated despite the use of those agents in a chronic and stable phase of the disease, suggesting increased thrombotic risk, which may reflect a continuous process of thrombosis and fibrinolysis, occurring with no clinical manifestation and under insufficient protection of platelet aggregation inhibitors. Several prospective and transversal studies related high levels of fibrinogen to
CAD, and a meta-analysis reassured these findings. Recently, other authors reported an independent association between CAD and fibrinogen, present even after correction for covariates. Fibrinogen influences platelet aggregation, interacts with a binding site of plasminogen, and participates in thrombus formation. Fibrinogen has a positive association with age, obesity, smoking, diabetes, and LDL-c levels, and an inverse association with HDL-c levels. Fibrinogen is an acute-phase protein, but it may also reflect the chronic process of atherosclerosis, because it is incorporated into the plaque, stimulating the proliferation of smooth muscle cells and contributing to the development of coronary artery disease. Our findings in
regard of fibrinogen elevation may be attributed to the lipid profile of these patients, to the increased BMI, and to diabetes mellitus, all increasing the thrombotic risk.

Elevated levels of D-dimer in the chronic phase of CAD were observed in our patients, and similar findings were reported by Salomaa et al.36 After a myocardial infarction, the increase in D-dimer was associated with the extension of atherosclerosis, with ventricular dysfunction, and with the presence of ventricular aneurysms, acting like a hemodynamic marker 13 and a marker of recurrent events 36. Because D-dimer levels increase with age, and considering the relative stability of the patients in our study, elevated D-dimer levels should not be expected and reflect the presence of fibrinolysis, even with no clinical evidence of thrombosis. Autopsy data from patients who died due to acute myocardial infarction showed rupture of plaques and silent thrombosis in unrelated arteries 39. These plaques are more frequent in diabetic and hypertensive patients. As a whole, our findings suggest a dynamic and silent process of thrombosis and fibrinolysis.

PAI-1 levels were elevated in both groups and did not identify early CAD. Those levels may be explained by the influence of smoking, increased body mass index, ethnic differences, and the high prevalence of metabolic syndrome.

Similar levels of factor VII and von Willebrand factor in our patients may be related to ethnic background the first case, and to the chronic and stable phase of the disease, in both 40.

Genotypic frequencies of M1 and M2 alleles did not differ between the case and control groups, and they were similar to those reported in a meta-analysis 40 of 14 studies including patients with CAD, healthy individuals, and mixed groups. No difference was observed in regard to the levels of HDL-c and apolipoprotein AI between the genotypes. However, a trend towards lower levels of Apo AI was observed in M1- carriers (p=0.07). In early CAD, Reguero et al.42 reported a high frequency of the M1- allele in unstable angina, with no difference in apolipoprotein AI levels. The M1- allele was also associated with combined familial hyperlipidemia 43. The same study showed lower HDL-c levels in M2-carriers in early CAD.

No difference was found in the genotypic frequencies of the APO CII gene among groups, as well as no association between genotypes and triglyceride levels, although these associations have been reported in another study 44. Our results regarding triglycerides may have been masked because these patients were under nutrition counselling, and different responses to diet modification are observed among genotypes. In addition, the analysis of triglyceride levels during a fasting period may not allow the assessment of the metabolic role of triglycerides for CAD.

On the other hand, hypertriglyceridemia was observed in the presence of 2 rare alleles only in patients with early CAD. This finding suggests an additive effect of the alleles of the APO AI and APO CII genes as a result of the effect of apolipoproteins AI and CII in the metabolism of the triglyceride-rich lipoproteins and in HDL. Although these polymorphisms are located in an untranslated region, they interfere with the efficiency of the transcription of the APO AI and APO CII genes, affecting the stability of mRNA.

In conclusion, patients with early coronary artery disease were characterized by the presence of classic risk factors, an unfavorable lipid profile, and an increase in thrombotic risk even in the chronic phase of the disease. Arterial hypertension and diabetes mellitus were indepen-
ently associated with early CAD. The rare alleles of the APO AI and APOCIII genes had an additive effect on triglycercide levels only in patients with early CAD, suggesting gene–gene and gene–environment interactions. Early identification of patients with metabolic syndrome seems crucial for preventing premature coronary artery disease.

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