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Systemic Markers of the Redox Balance and Apolipoprotein E Polymorphism in Atherosclerosis

The Relevance for an Integrated Study

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ABSTRACT

Prospective studies have demonstrated that an imbalance between oxidative damage and antioxidative protection can play a role in the development and progression of atherosclerosis. Also, genotypes with the apolipoprotein E $\varepsilon 4$ allele have been associated with an increase risk for this pathology. Based on this knowledge, the aim of this study was to evaluate indicators of the redox balance, trace elements, and apolipoprotein E allelic profile in subjects from the Lisbon population with clinically stable atherosclerosis, at risk for atherosclerotic events, and in healthy subjects for comparison. The activities of superoxide dismutase in erythrocytes and glutathione peroxidase in whole blood, plasma total thiols, and serum ceruloplasmin were kept unchanged among the three groups. Serum α-tocopherol was increased in atherosclerotic patients. Total malondialdehyde in serum and protein carbonyls in plasma, which are indicators of

lipid and protein oxidative damage, respectively, reached their highest values in risk subjects. The concentrations of potassium and calcium, in plasma and in blood cells, were slightly elevated in patients and might reflect an electrolytic imbalance. Regarding the apolipoprotein E polymorphism, atherosclerotic patients had an increased incidence of the high-risk genotypes for atherogenesis ($\varepsilon3/\varepsilon4$ and $\varepsilon4/\varepsilon4$). A multivariate model applied to the general population using most of the parameters clearly separated the three groups at study (i.e., the healthy group from the steady-state group of risk disease and from the atherosclerotic one). As shown by us, the usefulness of biochemical and complementary genetic markers is warranted for a better knowledge on atherosclerosis molecular basis.

Index Entries: Atherosclerosis; antioxidant defenses; oxidative damage; trace elements; apolipoprotein E.

INTRODUCTION

Although mortality from coronary heart disease has declined recently, atherosclerosis and related vascular diseases are still the major causes of death in Western countries *(1,2)*, including Portugal. Atherosclerosis is a chronic disease of large and medium-sized arteries, with hardening and loss of elasticity of the arterial walls and narrowing of the arterial lumen *(1)*. High blood pressure, diabetes, smoking, and a diet rich in lipids and cholesterol clearly increase the likelihood of premature atherosclerosis, although other factors, such as age and the individual genetic makeup (as apolipoprotein E), might play an additional role *(3–5)*. Apolipoprotein (apo) E gene expression affects the cholesterol metabolism *(6,7)* (more than any other gene identified so far), and the $\varepsilon4$ allele, in particular, has been associated with coronary artery and heart diseases, stroke, cognitive dysfunction, and Alzheimer's disease, as it promotes enhanced levels of both total cholesterol and low-density lipoprotein (LDL) cholesterol in plasma *(8,9)*.

The oxidation theory of atherosclerosis proposes that the formation of oxidized LDL in the subendothelial space of the artery wall represents a causative event for atherogenesis *(5,10,11)*, through their enhanced uptake by unregulated macrophage scavenger receptor, and their immunogenicity, and capacity for inducing chemotactic, cytotoxic, and growth factors *(12)*. Antioxidants are thought to be potential antiatherogenic compounds *(13)*. LDL is the primary carrier of cholesterol in the circulation, and its components, unsaturated fatty acids and proteins, are sensitive to oxidation. Endothelial cells, smooth muscle cells, and macrophages can release free radicals and other oxidant species leading to LDL modification. However, differences exist in the susceptibility of these lipoproteins to oxidative stress because small, dense LDL particles are more susceptible to oxidation than larger LDL subfractions and because LDL particles enriched with monounsaturated fatty acids are less prone to oxidation than those containing high levels of polyunsaturated fatty acids *(14)*.

Although a number of randomized trials have failed to show any benefit from antioxidant treatment, there remains a preponderance of basic and clinical evidence in supporting the role of oxidative processes in atherogenesis *(15–17)*. Fundamental research on redox status and oxidative damage mechanisms in atherosclerosis and associated pathologies (such as cardiovascular disease and stroke) becomes an important goal and might help one to select more appropriate study designs in terms of preventive measures in populations. The present study was developed under the scope of a project aiming to evaluate possible variations on redox balance markers, trace elements, and apo E polymorphism in subjects from the Lisbon population, from the healthy to clinically stable atherosclerotic condition, including the hazardous situation of hyperlipidemia and/or hypertension. This article reports data on blood activities of superoxide dismutase (SOD) and glutathione peroxidase (GPx), plasma concentration of total thiols, and α-tocopherol levels in serum. The protein ceruloplasmin (Cp), which is considered to have an antioxidant action and to be an inflammatory marker, was quantified in serum. Serum levels of total malondialdehyde (MDA) and plasma contents of protein carbonyls were evaluated as markers of oxidative modifications in lipids and proteins, respectively. The levels of essential elements—K (potassium), Ca (calcium), Fe (iron), Cu (copper), Zn (zinc), and Se (selenium)—playing a role in electrolytic mechanisms and oxidant/antioxidant pathways were determined in blood. The allelic profile of apo E was assessed for each subject and correlated with its clinical condition.

MATERIALS AND METHODS

Study Design

A total of 72 nonsmoker subjects from Lisbon, ranging in age from 40 to 78 yr, were included in the study and divided into 3 groups (Table 1). The pathological group was composed of ambulatory patients with a history of atherosclerotic disease: previous myocardial infarction, angina, and lower limb chronic arterial insufficiency, under therapy. Patients with acute or decompensated disorders (heart failure, respiratory disorders, and other metabolic abnormalities) were excluded from the study. The subjects from the risk group were hypertensive and/or hyperlipidemic. The reference group was composed by healthy subjects whose lipid and blood pressure values were within normal ranges. All subjects gave informed consent and filled out a clinical report stating information relevant to the study, namely baseline characteristics and lifestyles (e.g., smoking habits). Subjects under dietary supplements or drugs with a known antioxidant capacity were excluded. The study was approved by the Human Ethics Committee of the National Health Institute Dr. Ricardo Jorge and conformed to standard ethical guidelines for human research.

Blood Collection

The blood was collected by venipuncture after an overnight fast and aliquots were drawn into Z-Gel, lithium–heparin, and EDTA S-Monovette® tubes. Serum and plasma were removed after centrifugation at 1500*g* for 10 min at 4°C. Lipid parameters and the enzyme activities were evaluated within 24 h. Samples for DNA extraction were stored at –70°C. Aliquots for elemental analysis were frozen at –20°C and those for total thiols, proteins, protein carbonyls, and Cp quantification were frozen at –80°C. For the determination of α-tocopherol levels and lipid peroxidation index, aliquots were kept under liquid nitrogen. Serum, plasma, and blood samples from the three groups were analyzed in the same run.

Anthropometric Measurements

Body height and weight of the participants (without shoes and heavy clothing) were registered and body mass index (BMI = weight [in kg]/height^2 [in m^2]) was calculated as a measure of obesity. The World Health Organization reports the following categories for BMI: obese ($\geq$30 kg/m^2), overweight (25.0–29.9 kg/m^2), normal (18.5–24.9 kg/m^2), and underweight (<18.5 kg/m^2) *(18)*. Blood pressures were recorded to the nearest mm Hg and hypertension was defined according to the guidelines provided by the National Institutes of Health (systolic blood pressure >140 mm Hg and/or diastolic blood pressure >90 mm Hg or under antihypertensive therapy) *(19)*.

Lipid Profile Evaluation

High density lipoproteins (HDL) were separated by adding polyethylene glycol to fresh samples in order to precipitate other lipoproteins *(20)*. Total cholesterol, HDL cholesterol, and triglycerides concentrations in serum were estimated by using appropriate enzymatic kits (CHOD-PAP®, HDL-C Plus®, and GPO-PAP®, respectively; Boehringer, Manheim, Germany). LDL cholesterol concentration was estimated by the original Friedewald formula *(21)*. Based on cholesterol and/or triglycerides levels in serum, subjects were divided into normolipidemics (with total cholesterol and triglycerides levels below 200 mg/dL and 150 mg/dL, respectively) and hyperlipidemics, who had one or both parameters above these reference values *(22)*. At the laboratory, personnel were unaware of the status (patient, risk, or reference subject) of the specimens.

Determination of Enzyme Activities

Measurement of SOD activity in erythrocytes and GPx in whole blood were carried out with commercial kits (Ransod SD 125 and Ransel RS 506, respectively; Randox Laboratories, UK). For both enzyme activities, results were expressed per gram of hemoglobin, which was also determined by using a commercial kit (HG 980, Randox kit).

Determination of Total Thiols, α-Tocopherol, and Ceruloplasmin

Total thiols in plasma were measured spectrophotometrically using the Ellman's reagent *(23)*. Serum α-tocopherol was evaluated by a reverse-phase high-performance liquid chromatography (HPLC) method, according to Julianto et al. *(24)*. Cp in serum was determined by rate nephelometry using a commercial kit (CER Beckman-Array® System(s), Germany).

Determination of Total MDA and Protein Carbonyls

Malondialdehyde was measured in serum by liquid chromatography–mass spectrometry (LC/MS) according to a method derived from an initial ultraviolet (UV) version *(25)* with an electrospray ionization (ESI) positive detection mode and an internal standard (dideuterated tetra methoxypropane) for correction of MDA protein binding *(26)*. The carbonyl contents in plasma were evaluated according to Levine et al. *(27)* and expressed as nanomoles per milligram of protein, being the protein content determined based on the method of Lowry et al. *(28)*.

Elemental Analysis

Concentrations of Fe, Cu, Zn, and Se in plasma and blood cells were determined by the PIXE (particle-induced X-ray emission) multielemental technique. This technique also allowed one to evaluate the levels of K and Ca. The methodology applied is described elsewhere *(29)*. The analytical procedure was checked using Gent second-generation freeze-dried human serum reference material *(30)*, which was analyzed together in each sample batch. Differences in the certified value were below 5% for Fe and Se and below 10% for Cu, Zn, K, and Ca.

Apo E Genotyping

DNA extraction was achieved through the method of Miller et al. *(31)*. Apo E genotyping started with a polymerase chain reaction (PCR) to amplify a 267-basepair fragment from the exon 4 of the apo E gene. The amplification products were then submitted to digestion with the *Hha*I restriction endonuclease, as described elsewhere *(32)*. Each apo E allele yields a distinct pattern after electrophoresis on polyacrylamide gel *(32,33)*. Samples were analyzed in a random fashion and the laboratory technician carrying out the genotyping procedures was blinded for the cholesterol levels of the samples concerned.

Statistical Analysis

Statistical analysis was performed using STATISTICA 5.0 and SPSS 10.0 softwares. Data were expressed as mean ± standard deviation. The presence of outliers was verified by running box plots, and when the

parameters did not fit a Normal distribution, a logarithmic transformation was performed *(34)*. The values were analyzed using the Student's *t*-test for independent samples, with a significance level of $p < 0.05$. Correlation analyses using the Pearson's coefficient were also applied for assessing linear relationships between the evaluated parameters. A forward stepwise discriminant analysis, using blood pressure, BMI, lipids (total cholesterol, HDL cholesterol, and triglycerides), SOD and GPx activities, nonenzymatic parameters (total thiols, α-tocopherol, Cp), oxidative damage parameters (total MDA and protein carbonyls), and apo E polymorphism as variables, were also applied to help in predicting the shared variability relative to each clinical group *(35)*.

Apo E allele frequencies were estimated using the gene-counting method, and a χ^2 goodness-of-fit test was applied to evaluate the genetic Hardy–Weinberg equilibrium *(36)*. Apo E categories were created as follows: E2 (ε2/ε3), E3 (ε3/ε3), and E4 (ε3/ε4 and ε4/ε4) to explore the allelic effect and to increase statistical power *(37)*. The differences in apo E genotypes between the studied groups were observed using a χ^2 association test.

RESULTS

Groups Description and Clinical Characterization

Table 1 presents clinical and biochemical characteristics of the study groups. Genders were pooled because no major differences were found between them (data not shown). Furthermore, the majority of females were at the postmenopausal stage and this fact contributes to the similar probability of both genders for the occurrence of atherosclerotic events. Also, Pedersen et al. *(38)* have reported no evident differences between females and males based on the prevalence and extent of atherosclerosis.

Body mass index classified the mean value for the reference group as a marginally normal one (Table 1), with 69% normal and 31% overweight persons. In turn, the risk group was composed of 32.5% normal, 50% overweight, and 17.5% obese subjects and the pathological group by 25% normal, 62.5% overweight, and 12.5% obese patients. Patients and risk subjects had higher blood pressures, but no differences were observed either for systolic or diastolic blood pressures between these two groups (Table 1). Higher values were found for total and LDL cholesterol levels in the serum of risk subjects and of patients relative to the reference group (Table 1). Serum triglycerides levels were enhanced in risk subjects and marginally higher in patients compared to levels of the reference ones (Table 1). Total proteins in plasma were increased in patients compared to risk subjects and the same trend was verified in relation to the reference group, although not reaching statistical significance ($p = 0.069$) (Table 1). As seen in Table 1, medicine intake was more pronounced in the patho-

Table 1
Baseline Characteristics for Each Group

	Subject Groups		
	Reference	Risk	Pathological
Parameters	($n = 16$)	($n = 40$)	($n = 16$)
Anthropometric Variables			
Females/Males	10 / 6	26 / 14	10 / 6
Age (yrs)	50 ± 7	56 ± 6	64 ± 9
BMI (kg/m^2)	24.9 ± 2.4	27.1 ± 4.6	26.6 ± 3.2
Blood Pressure (mm Hg)			
Systolic BP	117 ± 12[a,b]	136 ± 21[a]	139 ± 18[b]
Diastolic BP	71 ± 8[a,b]	82 ± 13[a]	82 ± 10[b]
Hypertension (%)	0	65	87.5
Serum Lipids (mg/dl)			
Total Cholesterol	171 ± 11[a,b]	222 ± 30[a]	213 ± 47[b]
HDL Cholesterol	55 ± 10	62 ± 15	64 ± 17
LDL Cholesterol	101 ± 14[a,b]	139 ± 29[a]	130 ± 44[b]
Triglycerides	75 ± 16[a]	108 ± 41[a]	107 ± 77
Hyperlipidemia (%)	0	82.5	50
Plasma Proteins (mg/dl)	80 ± 9	77 ± 10[c]	86 ± 10[c]
Medicine Intake (%)			
Statins	0	0	37.5
Antihypertensives	0	22.5	56
Antiplatelet agents	0	0	19

Note: Data are expressed as mean ± standard deviation, except when otherwise indicated. Values in each row sharing the same superscript are significantly different (*t*-test, $p < 0.05$).

logical group, being the conjugation of the three therapies verified in 6% of the patients.

Markers of Antioxidant Defenses and Oxidative Damage

The activities of SOD in erythrocytes and GPx in whole blood and the plasma levels of total thiols kept unchanged across the three groups (Table 2). Serum α-tocopherol concentration was increased in atherosclerotic patients (Table 2). Ceruloplasmin levels in serum were similar for the three groups (Table 2). Figure 1 shows the variations of total MDA serum levels and plasma protein carbonyl contents among the three groups. Total MDA was similar between patients and references, but reached the highest values

Table 2
Activities of SOD in Erythrocytes and GPx in Whole
Blood, Levels of Plasma Total Thiols, Serum α-Tocopherol,
and Cp for Each Group

	Subject Groups		
Parameters	**Reference** ($n = 16$)	**Risk** ($n = 40$)	**Pathological** ($n = 16$)
SOD (U/g Hb)/100	32 ± 11	33 ± 9	29 ± 9
GPx (U/g Hb)	39 ± 14	34 ± 15	35 ± 12
Total Thiols (μmol/l)	427 ± 55	433 ± 57	453 ± 47
α-Tocopherol (μmol/l)	21 ± 5^a	23 ± 6^b	$35 \pm 16^{a,b}$
Ceruloplasmin (mg/dl)	34 ± 14	34 ± 10	32 ± 7

Note: Data are expressed as mean ± standard deviation. Values in
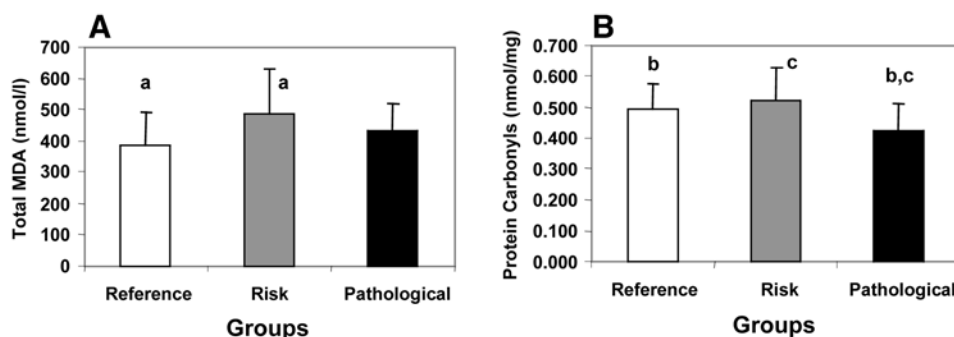each row sharing the same superscript are significantly different (*t*-
test, $p < 0.05$).

Fig. 1. Markers of oxidative damage for each group: **(A)** total MDA in serum;
(B) protein carbonyls in plasma. Bars sharing the same superscript are signifi-
cantly different (*t*-test, $p < 0.05$).

in risk subjects, this variation being statistically significant relative to the ref-
erence group (Fig. 1A). In turn, the level of protein carbonyls was the lowest
in the plasma of patients (Fig. 1B).

Elemental Analysis

Table 3 shows that the concentrations of K, either in plasma or in
blood cells, were higher in patients in relation to risk subjects and in blood
cells in relation to reference ones. Ca concentration in plasma was elevated
in the pathological group (Table 3). Fe, Cu, and Zn levels remained
unchanged across the three groups regardless of the biological compart-

Table 3
Elemental Concentrations (mg/L) in Plasma
and Blood Cell Fractions for Each Group

	Subject Groups					
	Reference		Risk		Pathological	
	Plasma	Blood Cells	Plasma	Blood Cells	Plasma	Blood Cells
Elements	($n = 16$)	($n = 16$)	($n = 16$)	($n = 17$)	($n = 16$)	($n = 16$)
K	119 ± 21	2508 ± 409^b	109 ± 24^e	2374 ± 376^d	131 ± 23^e	$2924 \pm 448^{b,d}$
Ca	69 ± 11^c	25 ± 7	64 ± 12^c	25 ± 9	$78 \pm 8^{e,c}$	27 ± 8
Fe	1.3 ± 0.4	946 ± 53	1.3 ± 0.3	978 ± 87	1.5 ± 0.8	958 ± 75
Cu	1.3 ± 0.3	1.2 ± 0.3	1.3 ± 0.1	1.1 ± 0.3	1.3 ± 0.2	1.1 ± 0.3
Zn	1.4 ± 0.4	12 ± 2	1.1 ± 0.2	13 ± 2	1.2 ± 0.3	12 ± 2
Se	0.059 ± 0.022^a	0.136 ± 0.072	0.086 ± 0.021^a	0.153 ± 0.097	0.073 ± 0.022	0.148 ± 0.029

Note: Data are expressed as mean ± standard deviation. Values in each row sharing the same superscript are significantly different (*t*-test, $p < 0.05$).

Table 4
Distribution of apo E Genotypes for Each Group

| | Subject Groups | | | | | |
| | Reference | | Risk | | Pathological | |
	n	%	*n*	%	*n*	%
Observed frequencies						
ε2/ε3	1	6.25	3	7.89	2	12.50
ε3/ε3	14	87.50	30	78.95	7	43.75
ε3/ε4	1	6.25	4	10.53	5	31.25
ε4/ε4	0	0.00	1	2.63	2	12.50
χ^2 statistic[a]	0.071		3.124		1.552	
P - value	0.965		0.210		0.460	
Relative allele frequencies						
ε2	0.031		0.039		0.063	
ε3	0.938		0.882		0.656	
ε4	0.031		0.079		0.281	

[a] χ^2: Statistic between observed and expected values under Hardy–Weinberg equilibrium.

ment (Table 3). Risk subjects presented higher levels of Se in plasma relative to reference ones (Table 3).

Distribution of apo E Genotypes

The distribution of apo E genotypes in this study is in Hardy–Weinberg equilibrium (Table 4). The genotypes ε2/ε2 and ε2/ε4 were not found, although all three alleles were represented in our samples (Table 4). The

majority of subjects within each group were homozygous ε3/ε3 (Table 4). However, an increase in the occurrence of the risk genotypes for atherogenesis (ε3/ε4 and ε4/ε4) was observed from the reference group to patients, and an inverse trend was verified for the common ε3/ε3 genotype (Table 4). This last observation is reinforced by the allelic frequencies. Moreover, the proportion of the E3 genotypes category was proved to be different between the risk group and the others (reference/risk: $\chi^2 = 5.8$, df = 1, $p = 0.05$; risk/pathological: $\chi^2 = 14.3$, df = 1, $p = 0.05$). The same observation could be applied for the E4 genotypes category between the reference and pathological groups ($\chi^2 = 4.5$, df = 1, $p = 0.05$).

Multivariate Analysis

The variations of the parameters based on atherosclerosis disease status were assessed by discriminant analysis (Fig. 2). The first function ($p = 0.000$) to which the variables total cholesterol, systolic blood pressure, diastolic blood pressure, total MDA, HDL cholesterol and triglycerides were related to (discriminant loadings 0.562, 0.271, 0.255, 0.237, 0.184, and 0.180, respectively) explained 62% of the variations and separated the reference group from the others. The second discriminant function ($p = 0.000$) was associated with α-tocopherol, protein carbonyls, total thiols, and apo E polymorphism (discriminant loadings –0.545, 0.281, –0.254, and –0.167, respectively) explained the remaining 38% of the variations and segregated the pathological group from the risk group (Fig. 2).

DISCUSSION

Oxidative processes and inflammation are key components of atherosclerosis, from fatty streak formation to plaque rupture and thrombosis. Recent clinical studies have identified potentially clinically useful markers of oxidative stress and inflammation *(17)*. In this study, we report on the usefulness of the relationship between those parameters and the major risk factors (including genetic markers) in humans from health to atherosclerosis disease.

Variations in Antioxidant Defenses and Oxidative Markers

Both SOD and GPx activities, either of patients or of subjects from the risk group, were not different from those of reference subjects. SOD is an important antioxidant enzyme and, contrary to our findings, some studies have reported decreased SOD activity in the erythrocytes of subjects suffering from ischemia *(39)*. It is believed that low SOD activity contributes to higher levels of the superoxide radical, which can be implicated in the pathogenesis of atherosclerosis *(40)*. The protective effect of GPx in particular on lipoperoxidation is reinforced because this enzyme not only eliminates hydrogen peroxide but also converts some lipid hydroperoxides to
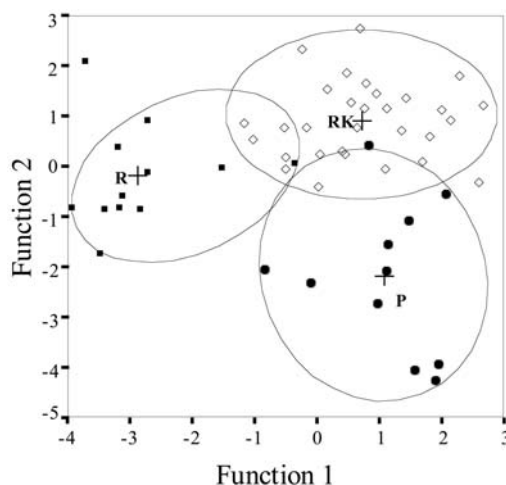
Fig. 2. Scatterplot of the canonical discriminant functions, indicating the centroids (cross) for each group (R, square: reference group; RK, diamond: risk group; P, circle: pathological group) (for details see the Materials and Methods section).

nontoxic alcohols, acting as a chain-breaking antioxidant *(41)*. In studies on atherosclerotic obliterans patients, a decline in the antioxidative barrier of plasma because of reduced GPx activity has been reported *(42)* and experimental evidence exists for a weak glutathione-related enzymatic antioxidant shield in human atherosclerotic plaques, resulting from the absence of GPx activity in atherosclerotic tissue *(43)*. In contrast, Weinbrenner et al. *(44)* suggested that oxidative stress might occur in patients with stable coronary heart disease, based on enhance activities of SOD in erythrocytes and GPx in whole blood, even in patients clinically stable and under medical treatment, like the patients of our study. Thus, contradictory findings exist for these enzyme activities in the atherosclerotic condition. The comparison of data from different studies must be made carefully because, in general, the experimental conditions are not identical, including some of the major risk factors associated with this pathology, such as cigaret smoking (Lopes et al., unpublished data) and medicine intake. In fact, recent data suggested that statins, in addition to their lipid lowering activity, can reduce the production of reactive oxygen species and increase the resistance of LDL to oxidation *(45)*.

Thiols are compounds containing sulfhydryl residues, and in biological systems, they are involved in protein synthesis and structure and play an important role in redox-sensitive signal transduction, immune regulation, and coordinating the antioxidant defense network *(46)*. Disturbances in tissue thiol homeostasis have been linked to several human health disorders *(46)*. In our study, the levels of total thiols in plasma were similar in patients and in reference subjects. However, patients with coronary artery

disease have been described as having decreased concentrations of plasma protein thiols *(47)*.

Vitamin E is the major lipid-phase antioxidant protecting against oxidative lipid damage *(5)*. It is thought to be effective in preventing atherosclerosis *(12)*, because a local or generalized diminution of α-tocopherol concentration caused by dietary or oxidative factors can stimulate cell growth and lead to atherosclerosis progress *(48)*. However, supplements of dietary vitamin E in clinical trials have not prevented consistently cardiac attacks in humans with established coronary heart disease, and vitamin E might become an oxidant agent *(2,49,50)*. Such contradictory results have questioned the role of vitamin E as a protective agent in human atherosclerosis *(51)*. In the present study, the highest levels of serum α-tocopherol were found in atherosclerotic patients, but this finding disagrees with others (as discussed later in this subsection) and could be justified by a healthy diet recommended by physicians. In turn, the serum level of α-tocopherol for the risk group was similar to that of the reference one, which is not in accordance with data reported elsewhere on decreased serum α-tocopherol concentration in subjects with various coronary risk factors including male gender, higher age, smoking habits, altered serum lipid profile, and obesity *(52)*. It is not clear whether the vitamin E decrease is a result of the ongoing atherosclerotic process by itself or contributes to its further aggravation *(12)*. In several clinical trials, patients with coronary heart and artery diseases and after stroke presented unchanged α-tocopherol status *(12,47,53)*.

In the present study, a positive correlation between total MDA and protein carbonyls ($r = 0.33$; $p < 0.05$) was found, supporting the view that different biomolecules can be damaged by oxidants. No differences were found in MDA concentration between healthy and atherosclerotic subjects, which agrees with data published by Weinbrenner et al. *(44)* for males with coronary heart disease. However, previous studies have shown that subjects with ischemic heart disease, unstable angina, stable angina, or myocardial infarction have higher levels of MDA *(11,54)*. One possible explanation for our results is the presence of both genders in the pathological group because estrogens could act as a confounding factor on the circulating levels of MDA, as stated by Fabbi et al. *(4)*. Also, the apparent absence of lipid peroxidation in these patients might be a result of the tight and successful regulation of lipids contents by statins, mainly total cholesterol, whose mean value, although increased, did not reach a hazardous level (above 250 mg/dL) *(4,45)*. Notwithstanding, higher values of total MDA were found in risk subjects with hyperlipidemic profile compared to reference ones, which could be eventually explained by the absence of statins intake. In addition, this result supports our own previous report in which the hyperlipidemia condition (without atherosclerotic events) led to higher lipoperoxidation levels, quantified through TBARS method (Lopes et al., unpublished data).

The evaluation of the carbonyl groups in plasma proteins is a respected marker of free-radical reaction intensity *(27,55)* in aging and in

various diseases *(56–58).* In our study, lower levels of protein carbonyls were found in the pathological group, which had a slightly higher protein concentration compared to the reference group. This result is not in accordance with previous findings that defend the accumulation of protein carbonyls in vascular lesions as a common phenomenon in most, if not all, types of vascular damage *(56).* However, in our study, the higher plasma protein level in the pathological group could lead to a carbonyl/protein ratio lower than that observed in the other groups. In turn, higher carbonyls and lower protein concentrations were found in the plasma of risk subjects, suggesting, for this group, signs of free-radical attack. These results are in fact corroborated by the negative correlation observed between total proteins and protein carbonyls ($r = -0.74$; $p < 0.05$).

Variations in Elemental Status

In this study, potassium and calcium were slightly elevated in patients, suggesting alterations in elemental homeostasis probably the result of changes in cellular ion transport related to the atherosclerotic process. Thus, the variations found for these elements might reflect an electrolytic imbalance. On the other hand, in vitro studies have reported an intracellular calcium rise due to oxidized LDL *(59),* which promotes the activation of T-cells *(60)* and could establish an interesting relationship among the oxidative stress, the inflammatory process, and the electrolytic balance.

Iron blood levels remained unchanged, but total proteins correlated positively with this element in plasma ($r = 0.33$; $p < 0.05$). This association could be justified by the existence of several iron-containing proteins, such as ferritin, transferrin, haemoglobin, myoglobin, or the hemin moiety *(61).* Recent studies have demonstrated that iron deposition is prominent in human atherosclerotic lesions, and animal experiments have further revealed that the severity of atherosclerosis can be markedly influenced by iron overload or deficiency *(62).* In fact, iron has been inconsistently associated with cardiovascular disease in spite of the plausible hypothesis as to how transition metal might accelerate the progression of atherosclerosis *(63).* Free iron is deleterious to cells because it catalyzes the generation of hydroxyl radicals from superoxide and hydrogen peroxide via the Fenton reaction *(62).*

In this study, unchanged blood levels of copper and iron in atherosclerotic patients agrees with the maintenance of serum Cp concentration. Cp is a α_2-globulin acting as a multicopper oxidase playing a role in iron metabolism, through its ferroxidase activity *(42,61,64).* The physiologic importance of this protein might include antioxidant activity by promoting iron mobilization or scavenging free radicals and, thus, preventing metal-catalyzed free-radical tissue damage. On the other hand, the putative role of Cp as a risk factor for atherosclerosis and cardiovascular disease has been attributed to its pro-oxidant activity to modify LDL *(61,64,65).* Previous studies on atherosclerosis obliterans showed that Cp activity and copper concentration in serum were increased and correlated

in this pathology *(42,66)*. This correlation supports in part the positive associations found in our study between Cp and copper (in plasma and blood cells: $r = 0.73$, $r = 0.45$; $p < 0.05$, respectively), although the absolute Cp concentration has been measured instead of its activity.

Zinc levels, either in plasma or in blood cells, were similar for all the studied groups. In contrast, Isra and Majewski *(66)* have found zinc levels greatly enhanced in the serum of atherosclerosis obliterans patients. The meaning of this finding is not clear, as several studies propose zinc to be antiatherogenic, through either its inhibition of LDL oxidation or its role in inhibition of the oxidative-stress-responsive factors involved in destruction of vascular endothelial cell integrity *(3,67)*. Furthermore, severe and moderate zinc deficiency has been reported to cause oxidative damage to proteins, lipids, and DNA in rat testes, which might be the result of iron accumulation or a reduction in zinc-dependent antioxidant processes *(68)*.

Concentrations of selenium in plasma can provide useful indicators of human selenium intake and status *(69)*. In this study, risk subjects presented higher levels of selenium in plasma than the reference ones, which might be related to the presence of approximately 80% of hyperlipidemic subjects in the risk group *(70)*. This association could in part be the result of the binding of selenium to lipoproteins, mainly LDLs, which are recognized as having significant amounts of selenium *(71)*. Moreover, epidemiological studies are known to be controversial with respect to the association of this element and the occurrence of cardiovascular diseases *(72)*, although a protective role has been attributed to selenium in reducing the risk for heart diseases and cancer *(73,74)*.

Variations in apo E Polymorphism

In recent years, several polymorphisms playing a role in the development and progression of atherosclerosis have been identified in relation to blood coagulation, endothelial function, and lipid metabolism *(75)*, as is the case of apo E. The serum cholesterol concentration is in part determined by the common apo E polymorphism and the ε4 allele appears to be associated with elevated serum cholesterol concentration *(6,76–78)* and to contribute to an increased risk for coronary artery disease and atherosclerosis *(37,79,80)*. In this study, the high-risk genotypes for atherogenesis (ε3/ε4 and ε4/ε4) were found in a higher percentage in patients, similarly to results of Stengard et al. *(81,82)* and to those of Pirim et al. *(83)*. These hazardous genotypes were also found in risk subjects. These findings agree with the above-mentioned association between the ε4 allele and the incidence of atherosclerosis. Moreover, intervention trials in humans as well as experimentation in animal models have demonstrated a connection between apo E and atherosclerosis. It is the case of experiments with apo E-null mutant mice that are hypercholesteremic animals, which rapidly developed atherosclerotic lesions, either when kept on an atherogenic diet or on a normal chow *(84)*.

FINAL REMARKS

The maintenance of some antioxidant defenses in atherosclerotic subjects observed in this study could be consequence of medical treatment. Nevertheless, a multivariate model applied to all the subjects clearly discriminated the three groups at study (i.e., the healthy group from the group in a steady state of risk disease and from the atherosclerotic one). These findings point out the need for further coordinated studies using complementary markers, both at biochemistry and genetics levels, in order to enlighten our knowledge about atherosclerosis molecular mechanisms.

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