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Oxidative stress as a predictor of cardiovascular events in coronary artery disease patients

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Abstract

Background: Enhanced oxidative stress has been associated with atherosclerosis and coronary artery disease (CAD). However, the predictive value of circulating oxidative stress biomarkers for cardiovascular events (CE) in patients with CAD has remained poorly understood.
Aim: To assess the prognostic significance of reactive oxygen metabolites, estimated as index of oxidative stress in serum samples by means of a commercial kit (ROMs, Diacron, Italy) on the rate of mortality and major adverse CE (MACE) in CAD.
Methods: A study of 93 consecutive patients with angiographically documented CAD (75 males, age: 68±10 years, mean±SD) was made during a mean follow-up of 66 months until the occurrence of one of the following CE: cardiac and all cause death, non-fatal myocardial infarction and coronary revascularization [percutaneous transluminal coronary angioplasty (PTCA) and coronary artery bypass grafting (CABG)]. Patient data were retrospectively collected from the Institute's electronic databank that saves demographic, clinical, instrumental and follow-up data of all patients admitted to our department.
Results: The Kaplan-Meier survival estimates showed a significantly worst outcome in patients presenting elevated ROM level (>75th percentile, corresponding to 481 AU) (log rank=11, 7.5, 5.1; $p<0.001$, $p<0.01$, $p<0.05$ for cardiac and all cause death and MACEs, respectively). In a multivariate Cox regression model, elevated oxidative stress remained a significant predictor of cardiac and all cause death [hazard ratio (HR) 3.9, 95% confidence interval, 95% (CI) 1.4–11.1, p=0.01; HR=2.6, 95% CI 1.1–6.2, p=0.02) and MACE (HR=1.8, 95% CI 1.1–3.1, p=0.03)].
Conclusions: The estimation of ROMs may represent an additional prognostic tool in the assessment of CE in CAD patients.

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Introduction

There is general agreement on the fact that oxidative stress represents a relevant determinant involved in the pathogenesis and development of a variety of chronic and degenerative diseases, including aging, cancer and atherosclerosis. In particular, an enhanced oxidative stress status plays a significant role in the onset and progression of atherosclerotic damage (1, 2). However, the predictive value of oxidative stress biomarkers for cardiac events in patients with coronary artery disease (CAD) is still poorly understood (3).

Recently, a simple method to estimate reactive oxygen metabolites in serum samples by means of a commercial kit (ROMs, Diacron, Italy) has been developed (4, 5), giving reliable results in terms of assessment of the oxidative stress status and monitoring of antioxidant treatment strategies (6, 7). In particular, we previously evidenced that elevated levels of ROMs are associated with cardiovascular risk factors and CAD, and found ROMs as independent predictors of death in patients with cardiovascular disease (8–11).

Thus, in the present study we tested the hypothesis that elevated levels of ROMs, evaluated as index of the oxidative stress status, may predict mortality and major adverse cardiovascular events (MACE) in patients with CAD.

Materials and methods

Subjects

Subjects selected to participate in the study were 93 consecutive inpatients (75 males, age: 68±10 years, mean±SD) with angiographically documented CAD, admitted to the Coronary Care Unit of the CNR-Clinical Physiology Institute in Pisa.

Patient data were collected from the Institute's electronic databank that saves demographic, clinical, instrumental and 10-year follow-up data of all consecutive patients admitted to our department from January 2001 (12). Information on left ventricular function was obtained by echocardiography or by left ventricular angiography. Database information on smoking habit, family history of ischemic heart disease, arterial hypertension (systolic blood pressure>140 mm Hg and/or diastolic pressure >90 mm Hg or by the use of antihypertensive medication), diabetes (fasting plasma glucose >126 mg/dL corresponding to 7 mmol/L or use of antidiabetic treatment), obesity (body mass index >30 kg/m^2), and dyslipidemia (defined when total cholesterol concentration was ≥200 mg/dL corresponding to 5.18 mmol/L, or triglyceride concentration ≥150 mg/dL corresponding to 1.695 mmol/L, or current use of lipid-lowering drugs) were coded in a dichotomized fashion.

Smoking habit was coded by grouping patients into no-smokers (those who had never smoked), ex-smokers (who had quit smoking for at least 6 months) and current smokers. Medical therapy included ACE inhibitors, beta-blockers, lipid-lowering agents, diuretics, and aspirin. No patient was receiving vitamins and/or antioxidant therapies.

Informed consent was obtained from each patient. The authors confirm that they have complied with the World Medical Association Declaration of Helsinki regarding ethical conduct of research involving human subjects.

Follow-up

Follow-up data were obtained from at least one of the following four sources: review of the patient's record, telephone interview conducted by trained personnel, personal communication with the patient's physician or medical visit at the outpatient clinic. The clinical events recorded during the follow-up and analyzed for the prediction of MACE were cardiac and total mortality, non-fatal myocardial infarction, and coronary revascularization [percutaneous transluminal coronary angioplasty (PTCA) and coronary artery bypass grafting (CABG)]. The cause of death was derived from medical records or death certificates. The definition of cardiac death required the documentation of either significant arrhythmias, or cardiac arrest, or death attributable to congestive heart failure, or myocardial infarction in the absence of any other precipitating factor. Non-cardiac causes of death included two stroke, three malignancies, two infections, one acute abdomen and one death during surgical intervention. The diagnosis of myocardial infarction was based on the documentation of persistent electrocardiographic ST segment changes, or new Q-wave development, associated with biomarker increase.

Blood sampling and storage of samples

All subjects were studied in the morning and in a fasting state. Blood samples were drawn from the left antecubital vein, kept on ice and centrifuged within 15 min after blood collection at 2500 *g* for 15 min at 4°C. Then, serum samples were immediately stored at –80°C for <2 weeks before subsequent analysis.

Analytical method

The oxidative stress status was estimated in serum samples by using the d-ROMs test (Diacron, Italy), as we previously described in detail (5, 13). In brief, the d-ROMs test is based on the ability of transition metal to catalyse in the presence of peroxides with generation of free radicals that are trapped by an alchilamine. The reaction of alchilamine yields a coloured radical detectable at 505 nm. The results were expressed as arbitrary units (AU).

As we previously reported, the limit of quantification for the ROM test, defined as the concentration corresponding to the mean value of 10 determinations of the zero calibrator ±2 SD, resulted 40 AU (5). The standard calibration curves for the d-ROMs test are linear up to 475 AU, with correlation coefficients >0.99. Samples with different concentrations are evaluated to estimate within- and between-run coefficients of variation (CVs). Intra- and inter-assay coefficients of variation resulted always lower that 5% for ROMs. Recovery obtained after addition of different amounts of known concentration to six different aliquots of two serum samples ranges between 97% and 105% (5).

Statistical analysis

Data were expressed as the mean±SEM. Statistical analysis included Student's t-test, χ^2-test, and ANOVA analysis and Scheffe's test using the statistical package Statview, version 5.0.1 (SAS Institute, Abacus Concept, Inc., Berkeley, CA, USA). Cumulative event rates were estimated by Kaplan-Meier survival curves and probability values determined with the log-rank test. For survival analysis, only one event was considered in each patient. Statistical analysis also included Cox proportional hazard models to determine independent predictors of CE. A $p<0.05$ was considered statistically significant.

Results

Demographic and clinical characteristics of patients are summarized in Table 1.

ROMs were slightly skewed among the study participants, ranging from 157 AU to 883 AU (median 378.5 AU; skewness 0.9) (Figure 1). Patients with multivessel disease had higher levels of ROMs compared to one vessel disease (434±19 vs. 362±19 AU, $p\leq0.01$). Higher levels of ROMs were also associated with female sex (463±39 vs. 392±15, $p<0.05$).

Patients were followed for a period of 66±28 months, with occurrence of 66 events. Specifically, 24 (26%) patients died (15 cardiac death), 6 (9%) had a non-fatal MI and 36 underwent revascularization (22 PTCA and 14 CABG).

The cumulative survival rates free of events on the basis of Kaplan-Meier analysis for cardiac and all cause death and MACE are shown in Figure 2. The Kaplan-Meier survival estimates showed a significantly worst outcome in patients presenting elevated concentration of ROMs (>75th percentile, corresponding to 481 AU) for all the endpoints considered (log rank=11, $p<0.001$; 7.5, $p<0.01$; 5.1, $p<0.05$, for cardiac and all cause death and MACE, respectively).

The significant univariate effect of high levels of oxidative stress was seen as considering cardiac and total death (hazard ratio, HR=4.7, confidence interval, CI 1.7–13.1, $p<0.01$ and HR=3, CI 1.3–6.6, $p<0.01$, respectively) (Table 2). The other parameters associated with cardiac and all cause death at the univariate analysis are also shown in Table 2. In the multivariate Cox regression model, elevated oxidative stress showed an independent effect on cardiac (HR=3.9, CI 1.4–11.1, $p\leq0.01$) and total death (HR=2.6, CI 1.1–6.2, $p<0.05$) (Table 2).

The elevated concentration of ROMs, among all the variables considered, resulted the only determinant significantly associated with the occurrence of a MACE (Table 3).

Discussion

This study demonstrated that high levels of ROMs, as index of the oxidative stress status, can serve as an independent and significant predictor for CE in CAD patients.

Oxidative stress represents a recognized determinant in the onset and progression of endothelial dysfunction and atheroma formation (1). Indeed, elevated oxidative stress may cause marked cellular and tissue damage, inducing events such as lipid peroxidation, oxidation of proteins and DNA, and reducing NO production (1). However, most of the evidence linking oxidative stress to CAD is obtained in in vitro system, or in experimental studies on animals, or by observational human studies (3). Accordingly, disorders closely associated to the

Table 1 Baseline characteristics of the study population.

n	93
Age, years	68±10
Age (>69 years, 50th percentile)	44 (47)
Males	75 (81)
Hypertension	55 (59)
Type 2 diabetes	32 (34)
Dyslipidemia	50 (54)
Smoking habit	34 (36.5)
Current smokers	7 (7.5)
Ex-smokers	27 (29)
Ejection fraction, %	50.2±12.9
Ejection fraction <40%	20 (21)
Coronary angiography	
One-vessel disease	37 (40)
Multi-vessel disease	56 (60)
Prior myocardial infarction	50 (54)
ROMs, AU	406±137
ROMs (>481 AU, 75th percentile)	23 (25)

Data presented are mean value±SD or number (%) of patients.

atherosclerotic process, such as diabetes, hypertension, and dyslipidemia, have all been associated with elevated levels of different oxidative stress biomarkers (5, 7, 8, 13). Moreover, we and others have demonstrated that different oxidative stress parameters -including d-ROMs and biomarkers of lipid peroxidation and DNA damage- are related to increased risk for cardiovascular disease and correlated with the number of cardiovascular risk factors (9, 10, 13–16).

The extent of the relationship between oxidative stress and atherosclerosis and cardiovascular (CV) risk factors rose the hypothesis that antioxidant supplementation could reduce the global burden related to CAD (17). However, expectations about clinical benefits from the administration of antioxidants were hampered, although several biases can be assumed regarding the negative results of such clinical intervention trials (17).

Other than diet differences, age, stage of disease as well as sex and lifestyle, there are many other points to consider.

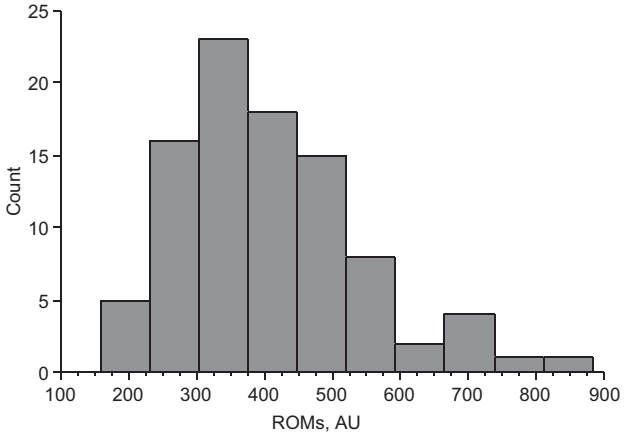

Figure 1 Distribution of ROM levels in the CAD patient population.

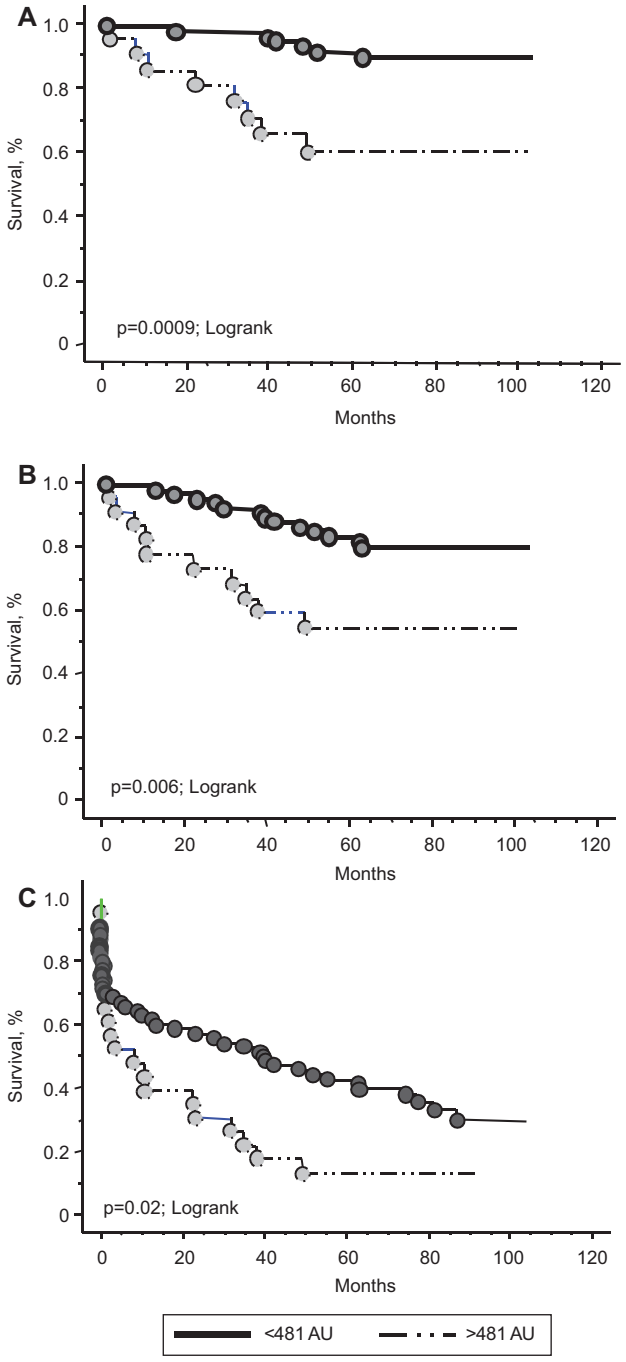

Figure 2 Kaplan-Meier survival curves according to oxidative stress (levels >75% percentile) considering cardiac death (A), all cause death (B) and MACE (panel C) as end points.

Generally, patient selection of these trials was based on the hypothesis that all patients could benefit from antioxidant supplementation, and not on biochemical evidence of oxidative stress (17). Conversely, it is important to consider the pre-existing oxidative stress status of each patient, as antioxidant supplementation could not be effective in patients in whom there was no increase in oxidative stress. In addition, monitoring of the trend in levels of oxidative stress biomarkers,

Table 2 Cox predictive model for cardiac and total death.

	Univariate analysis		Multivariate analysis	
	HR (95% CI)	p-Value	HR (95% CI)	p-Value
Cardiac death				
Hydroperoxides (>481 AU)	4.7 (1.7–13.1)	0.003	3.9 (1.4–11.1)	0.01
Age (>69 years, 50th percentile)	3.5 (1.1–10.8)	0.03	2.8 (1–9.1)	0.09
LVEF (<40%)	5.1 (1.9–14.2)	0.0016	4.2 (1.5–12)	0.006
Diabetes	3.3 (1.2–9.3)	0.03	2.6 (1–7.5)	0.07
Dyslipidemia	0.5 (0.5–1.3)	ns	–	
Gender	1.1 (0.5–3.8)	ns	–	
Hypertension	1.4 (0.6–4.2)	ns	–	
Smoking habit	1.6 (0.8–4.3)	ns	–	
Prior myocardial infarction	2 (0.8–5.7)	ns	–	
Total death				
Hydroperoxides (>481 AU)	3 (1.3–6.6)	0.009	2.6 (1.1–6.2)	0.02
Age (>69 years, 50th percentile)	2.5 (1.1–5.8)	0.035	2.5 (1–5.9)	0.04
LVEF (<40%)	3.2 (1.4–7.3)	0.005	2.3 (1.0–5.2)	0.05
Diabetes	2.6 (1.2–5.8)	0.02	2.9 (1.2–6.8)	0.014
Dyslipidemia	0.3 (0.5–1)	0.01	0.33 (0.5–0.9)	0.015
Gender	1.2 (0.6–3.4)	ns	–	
Hypertension	1.4 (0.6–3.3)	ns	–	
Smoking habit	1.5 (0.7–3.4)	ns	–	
Prior myocardial infarction	1.6 (0.8–3.7)	ns	–	

LVEF, left ventricular ejection fraction; HR, hazard ratio; CI, confidence interval; ns, not significant.

including d-ROMs, must also be included during the antioxidant treatment.

Vitamin E has been used in mostly clinical trials, but other antioxidants, or combination of more antioxidants, may be more effective (17). The dosage and duration of supplementation must be also considered. The presence of specific genetic variants of key enzymes in the detoxification of reactive oxygen species may be an important factor in the susceptibility of an individual, and should be evaluated in future studies.

The treatment of patients with other drugs can be another confounding factor, because different drugs may potentially affect the oxidative stress status, because some molecules retain antioxidant properties (e.g., calcium channel blockers). However, in the present study we did not observe any significant relationship between d-ROMs concentration and specific pharmacological therapies (data not shown).

Table 3 Cox predictive model for MACEs.

	Univariate analysis	
	HR (95% CI)	p-Value
Hydroperoxides (>481 AU)	1.8 (1.1–3.1)	0.03
Age (>69 years, 50th percentile)	1.2 (0.8–2)	ns
LVEF (<40%)	1.1 (0.8–1.9)	ns
Diabetes	1.3 (0.9–2.1)	ns
Dyslipidemia	1 (0.8–1.6)	ns
Gender	1.3 (0.8–2.3)	ns
Hypertension	0.8 (0.6–1.4)	ns
Smoking habit	1.3 (0.9–2.1)	ns
Prior myocardial infarction	1.2 (0.8–2)	ns

LVEF, left ventricular ejection fraction; HR, hazard ratio; CI, confidence interval; ns, not significant.

Although at the moment the clinical use of antioxidant supplementation is not recommended, the evaluation of their potential beneficial effects require more efforts. There is a large body of observational (epidemiological, case-control, or prospective and retrospective studies) data on the dietary antioxidant intake linked to prevention of CV risk factors and onset and progression of atherosclerosis (17). In this context, the evolution of natural dietary patterns with relevant effectiveness represents a challenge for the future, as these compounds may be crucial complementary treatment options for subjects at risk for CV disease.

The predictive value for cardiac events in CAD by using oxidative stress biomarkers, including oxidized-LDL, myeloperoxidase (MPO), and lipid and protein peroxidation measures, has been recently reviewed (3). Majority of studies suggested a role for oxidative stress biomarkers in the prediction of CE, although some evidences lack to find any association (3).

The observed differences may be due to the use of different parameters, heterogeneity of design and analysis and lack of the standardization method. The different approaches used to determine oxidative stress biomarkers in body fluids (for example gas chromatography/mass spectrometry, gas chromatography tandem mass spectrometry, or liquid chromatography tandem mass spectrometry, radioimmunoassay and enzyme immunoassay for F2-isoprostane) may induce great variation in the level measured (18–21). In this context, we paid particular attention to the preanalytical phase (see Blood sampling and storage of samples in the Materials and methods section). In fact, sample handling is extremely important to estimate oxidative stress in vivo and may represent another important source for conflicting results. Samples left at room temperature or which have been freeze-thawed can undergo

auto-oxidation (22). For example, MPO levels may be greatly affected by this factor, because leucocytes continue to release MPO in samples not maintained on ice (3).

The ROMs test, used for this study, is able to estimate hydroperoxides and other oxidizing agents in the sample, evidence of an early oxidative damage (23). These biomarkers have been previously found associated to various condition related to an elevated oxidative stress. In particular, ROMs have been previously found correlated with the presence and number of CV risk, the presence of CAD, and associated with inflammatory parameters in healthy subjects and CAD patients by us and others (5, 7, 10, 13, 24). In the present study, this biomarker appears also correlated with the severity of CAD, being higher in patients with multivessel disease.

We found that baseline ROM levels resulted significantly higher in women than in men. However, it is important to consider the advanced age of our patients, and the fact that mostly women were in a postmenopausal status, when the oxidative stress status has been observed higher in women than in men both in healthy and CAD patients (8, 9, 25).

The present study had some limitations. We performed only baseline measurements of oxidative stress, without evaluating whether the levels of these biomarkers changed over time. Moreover, our sample size was small, although the long follow-up time allows observing a consistent number of events. However, as such, the findings need to be confirmed in a larger sample size and prospectively designed studies. Nonetheless, these potential limitations do not invalidate our observations that are descriptors of a relevant pathophysiologic mechanism. It is also important to remind that, although the relatively low number of patients enrolled may represent a limitation, the importance of our results is reinforced by strict criteria used to select a well-defined and characterized subject population.

In conclusion, our study indicates a major role of elevated ROMs in the prediction of cardiac events, suggesting that the estimate of oxidative stress might represent an additive sensitive biomarker of prognostic stratification in patients with known CAD.

Conflict of interest statement

Authors' conflict of interest disclosure: The authors stated that there are no conflicts of interest regarding the publication of this article.
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