Elevated Levels of Oxidative Stress as a Prognostic Predictor of Major Adverse Cardiovascular Events in Patients with Coronary Artery Disease

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Aims: To evaluate the prognostic significance of oxidative stress on the rate of major adverse cardiovascular events (MACEs: cardiac and all-cause death, nonfatal myocardial infarction, coronary revascularization-PTCA/CABG) in CAD.

Methods: We studied 97 angiographically proven CAD patients (78 males, age: 67 ± 11 years, mean ± SD). Reactive oxygen metabolites and total antioxidant status, assessed by commercially assays (d-ROMs and OXY-Adsorbent Test; Dacron, Grosseto, GR, Italy), were used to calculate the oxidant/antioxidant balance. Patient data were collected from the Institute’s electronic databank, which saves demographic, clinical, instrumental and follow-up data of all patients admitted to our department.

Results: Kaplan-Meier survival estimates showed a significantly worst outcome in patients presenting with elevated oxidative stress levels (>75th percentile, \( p < 0.01 \)). Multivariate Cox models showed that a higher level of oxidative stress was an independent predictor of developing MACEs (hazard ratio = 2.1, confidence intervals 1.2-3.6, \( p < 0.01 \)).

Conclusion: Oxidative stress may represent a useful additional tool in the prediction of MACE in CAD.

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Key words; Oxidative stress, Atherosclerosis, Coronary artery disease, Cardiovascular events, Follow-up

Introduction

There is general agreement that oxidative stress is a relevant determinant involved in the pathogenesis and development of a variety of chronic and degenerative diseases, including aging, cancer and cardiovascular disease. In particular, an enhanced oxidative stress status plays a significant role in the onset and progression of atherosclerosis\(^1\).\(^2\).

However, the majority of data have been obtained by experimental or observational studies in humans. Poor data on the capacity of oxidative stress biomarkers to predict CV event are available, and this is still largely under debate\(^3\). In particular, an important limitation of the available results in this field is the lack of measures of the antioxidant status.

Accordingly, the aim of the present study was to evaluate the role of the oxidative stress status as a predictor of CV events in a cohort of patients with angiographically proven coronary artery disease (CAD). For this purpose, reactive oxygen metabolites and the total antioxidant status were assessed by commercially available assays (d-ROMs and OXY Adsorbent Test, respectively; Dacron, Grosseto, GR, Italy)\(^4\).\(^5\). The global oxidative stress index (Oxidative-INDEX), including both the oxidative and antioxidant counterparts, and therefore potentially more complete, was calculated\(^6\).

Materials and Methods

Subjects
Subjects selected to participate in the study were 97 consecutive inpatients (78 men, age: 67 ± 11 years,
mean ± SD) with angiographically proven CAD, admitted to the Coronary Care Unit of the Clinical Physiology Institute-CNR in Pisa.

Data were collected from the Institute's electronic databank (installed in 1975), which saves demographic, clinical, laboratory, instrumental and 10-year follow-up data of all consecutive patients admitted to our department5). Information on left ventricular function was obtained by echocardiography or by left ventricular angiography. Database information on smoking habit, a family history of ischemic heart disease, arterial hypertension (systolic blood pressure > 140 mmHg and/or diastolic pressure > 90 mmHg or by the use of antihypertensive medication), diabetes (fasting plasma glucose > 126 mg/dL or use of antidiabetic treatment), obesity (body mass index > 30 kg/m²), and dyslipidemia (defined when total cholesterol concentration was ≥ 200 mg/dL, or triglyceride concentration ≥ 150 mg/dL, or current use of lipid-lowering drugs) were coded in a dichotomized fashion. Smoking habit was coded by grouping patients into never smokers (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 taking vitamins and/or receiving antioxidant therapies.

Follow-Up

For this study, follow-up events were collected retrospectively7). Patients were followed up for a maximum of 48 months from the time of enrollment. 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 to the outpatient clinic. The clinical events recorded during the follow-up and analyzed for the prediction of events were death, non-fatal myocardial infarction, and coronary revascularization (angioplasty PTCA or surgery 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, cardiac arrest, death attributable to congestive heart failure, or myocardial infarction in the absence of any other precipitating factor. 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 less than 2 weeks before subsequent analysis.

Analytical Method

Reactive oxygen metabolites were measured in serum samples using the d-ROM test and (Diacron), as we previously described in detail6, 8). In brief, the D-ROM test is based on the ability of transition metal to catalyze, in the presence of peroxides, the generation of free radicals, which are trapped by an alchalmine. The reaction of alchalmine yields a colored radical detectable at 505 nm. The results were expressed as arbitrary units (AU). The limit of quantification for this assay (as concentration corresponding to the mean value of 10 determinations of the zero calibrator + 2 SD) was determined to be 40 AU, as we previously reported8).

The standard calibration curves were linear up to 475 AU, with correlation coefficients greater than 0.99. Samples with different concentrations were evaluated to estimate within- and between-run coefficients of variation (CVs). Within-run imprecision was assessed by evaluating the results of 10 samples analyzed on the same day for three different concentration levels. Between-run imprecision was evaluated over 10 days by analysis of d-ROM in the same serum sample for three different concentration levels. The range resulted in 2.9-4.7% and 1.4-4% for between- and within-run imprecision, respectively8).

Recovery, achieved by adding different amounts of known concentration to six different aliquots of two serum samples, ranged between 97% and 105%8).

The storage effect was evaluated using 10 fresh serum samples and their aliquots maintained at 4°C after 24-h storage, and no significant difference was observed. Moreover, samples showed a very good degree of stability over a 3-month storage period at -80°C6, 8).

The total antioxidant capacity was estimated by using the Oxy-adsorbent test (Diacron). Injury from excessive availability of free radicals is neutralized by numerous molecules, including components with reducing action (e.g. vitamins, uric acid, etc) as well as other components, such as proteins. This assay is based on the endogenous antioxidant capacity to oppose the oxidant action of added hypochlorous acid (HClO). Specifically, the effectiveness of the anti-oxidant barrier against free radicals is estimated by simulating a massive attack by HClO, added in excess to the system5). After 10 min, residual HClO undergoes
a reaction with an alkyl-substituted aromatic amine (A-NH2, solubilized in a chromogenic mixture). This amine is oxidized by HClO, giving a colored product, which is detected photometrically. The concentration of the colored species is inversely related to the antioxidant capacity of tested samples. In practice, the lower the concentration detected, the higher the antioxidant power of the tested sample.

The standard calibration curves for the OXY test were linear up to 440 μmol HClO/mL, with correlation coefficients greater than 0.99. Samples with different concentrations were evaluated to estimate within- and between-run coefficients of variation (CVs), according to the procedure described for d-ROMs, obtaining results ranging between 5.4-9.3% and 2.1-2.6% for inter- and intra-assay coefficients of variation, respectively.

To evaluate the effect of storage, measurements were performed using fresh serum samples (n=10) and repeated on aliquots of the same samples after 24-h storage at 4°C, and no significant statistical differences were observed; however, sample concentration progressively decreases over time, with a significant reduction over a long storage period, with a significant mean loss of 15-20% after 2 years at −80°C.

The global score of the oxidative stress status was assessed in order to evaluate both the oxidant and the antioxidant counterparts. To use variables with different measurement units and variability, the standardized values of the oxidant and antioxidant indices were calculated using the following formula: 

$$svvar = \frac{(vvar - mvar)}{dsvar}$$

where svvar represents the standard value of a certain parameter, vvar its original value, and mvar and dsvar the mean and standard deviation of the parameter. The difference between the ROMs standardized variable and the OXY standardized variable is the oxidative-INDEX.

### Statistical Analysis

Data are expressed as the mean ± S.E.M. Statistical analysis included Student’s t test, and χ2 test, using the statistical package Statview, version 5.0.1 (SAS Institute, Abacus Concept, Inc., Berkeley, CA). 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 CV events. A p value ≤0.05 was considered significant.

### Results

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

During a mean follow-up period of 38 ± 36 months, 66 (68%) patients had major adverse cardiovascular events. Specifically, 24 patients died; there were 15 cardiac deaths and 6 patients had nonfatal myocardial infarctions; 36 patients also underwent major vascular procedures (22 PTCA and 14 CABG).

The baseline level of oxidative stress was significantly higher in those who had MACEs than in those who did not (0.3 ± 0.23 vs −0.6 ± 0.3, p < 0.05). Further characteristics of the study participants who had cardiovascular events and those who did not are listed in Table 2.

The Kaplan-Meier survival estimates showed a significantly worst outcome in patients presenting with elevated oxidative stress levels (> 75th percentile, corresponding to 1.127) for all the endpoints considered (p < 0.01, Fig. 1).

According to the Cox model, elevated oxidative stress at entry had a hazard ratio corresponding to 2.2 (95% confidence interval 1.3-3.7, p < 0.01). Among other parameters considered, multivessel disease was also associated with the occurrence of MACE on univariate analysis (Table 3).

In the multivariate Cox regression model, elevated levels of oxidative stress were significantly associated with a higher risk for MACEs during follow-up (HR = 2.1, CI 1.2-3.6, p = 0.0057; Table 3).

### Table 1. Baseline characteristics of the study population (n=97 patients)

| Characteristic                        | Number (%)
|--------------------------------------|------------|
| Age (> 69 yrs-50th percentile)       | 46 (47)    |
| Males                                | 78 (80)    |
| Hypertension                         | 56 (58)    |
| Type 2 Diabetes                      | 32 (33)    |
| Dyslipidemia                         | 51 (53)    |
| Smoking habit                        |            |
| Current smokers                      | 9 (9)      |
| Ex smokers                           | 27 (28)    |
| Ejection fraction < 40%              | 36 (37)    |
| Coronary angiography                 |            |
| One-vessel disease                   | 41 (42)    |
| Multi-vessel disease                 | 56 (58)    |
| Prior myocardial infarction          | 50 (52)    |
| Oxy-index (>1.127-75th percentile)   | 24 (25)    |
Oxidative stress has a recognized role in the onset and progression of atherosclerotic processes.

In particular, we and others have demonstrated that isoprostanes, markers of lipid peroxidation and reduced antioxidant capacity, are related to an increased risk for cardiovascular disease and correlated with the number of cardiovascular risk factors. Other evidence indicates that thiobarbituric acid reactive substance (TBARS, indicators of lipid peroxidation) levels are correlated with endothelial dysfunction and coronary artery disease. Specifically, our previous data indicated associations between d-ROMs, OXY-adsorbent and Oxidative-INDEX and the presence and severity of CAD, and the relationship between these biomarkers of oxidative stress and the extent and number of risk factors, including the presence of diabetes, dyslipidemia, hypertension, and smoking habit, and with inflammatory biomarkers. Interestingly, a significant inverse correlation between d-ROMs and OXY-adsorbent tests was also observed in our CAD patient cohort.

A recently published review studied the role of oxidative stress biomarkers as predictors of CV events. Data are available on oxidized (Ox)-LDL, myeloperoxidase (MPO), lipid peroxidation and protein oxidation indices.

Of the 26 studies measuring Ox-LDL, 16 have shown it to be an independent predictor of cardiovascular events and 10 have not. Also, the association of MPO levels with CAD risk in stable patients is still unclear, although a very recent study conducted on a large cohort of stable CAD patients indicated that MPO concentrations may provide an independent prognostic value for the prediction of long-term MACEs. Nonetheless, comparisons among studies are still difficult due to differences in the tests used and methodological aspects for both biomarkers, and the difficulty of defining reference values related to large differences in the median concentrations between the populations studied. Lipid peroxidation remains the most common measurement to estimate oxidative stress in vivo. Different lipid peroxidation

### Table 2. Clinical characteristics of patients with and without adverse events

| Characteristic                        | no  | yes  | \( p \) values |
|---------------------------------------|-----|------|---------------|
| Age (>69 yrs-50th percentile)         | 11  | 35   | 0.11          |
| Males                                 | 26  | 52   | 0.5           |
| Hypertension                          | 18  | 38   | 0.66          |
| Type 2 Diabetes                       | 6   | 26   | 0.051         |
| Dyslipidemia                          | 15  | 36   | 0.53          |
| Smoking habit (current or past)       | 10  | 26   | 0.5           |
| Ejection fraction <40%                | 9   | 27   | 0.26          |
| Coronary angiography                  |     |      |               |
| One-vessel disease                    | 20  | 21   | 0.02          |
| Multi-vessel disease                  | 11  | 45   |               |
| Prior myocardial infarction           | 12  | 38   | 0.08          |
| Oxy-index (>1.127-75th percentile)    | 3   | 21   | 0.01          |

Data are the number (%) of patients.

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**Discussion**

Oxidative stress has a recognized role in the onset and progression of atherosclerotic processes.

Fig. 1. Kaplan-Meier survival curves according to oxidative stress levels with major events (MACEs) as end points.
Biomarkers, including F2alpha-isoprostanes, lipid hydroperoxides, fluorescent products of lipid peroxidation, oxidation resistance assay, oxysterols and TBARS, have been utilized. In this field, only one study, which utilized F2alpha-isoprostanes, did not find oxidative stress to be an independent predictor of CVD. Of note, subjects enrolled in the study were not patients with stable CAD. Conversely, protein oxidation biomarkers measured in AMI patients were not predictive of all-cause mortality over a 5-year follow-up period. Interestingly, the aspect of antioxidants has been neglected in all of these studies. This limitation is crucial, because oxidative stress occurs when enzymatic and non-enzymatic antioxidants cannot fully neutralize the ROS produced, so that unquenched ROS remain long enough to cause further reactions. Thus, the oxidative stress status can be better evaluated considering both pro- and anti-oxidant counterparts in an attempt to elucidate the complexity of the reaction linked to the oxidative redox balance.

In this context, the d-ROMs and Oxy-adsorbent tests, here used to calculate a global index which accounts for the two oxidative counterparts simultaneously, represented well-investigated indices of in vivo oxidative stress, whose characteristics and effectiveness have been largely evaluated in previous studies by us and others. Other than being reliable and feasible tools for assessment of the oxidative stress status, their advantages also include easy and rapid execution, low cost, and the possibility to be performed without the necessity of special equipment or skilled operators. The adoption of a global index gives unique parameters easy to compute and interpret, potentially increasing the accuracy and completeness of the information.

Clearly, the methods used here to estimate oxidative stress are indirect; however, this disadvantage is common to almost all available assays that estimate derived products, the half-life of the free radicals being extremely short and their reactivity extreme. In fact, techniques available for the direct estimation of oxidative stress required special equipment and are expensive and time-consuming, characteristics which make these procedures reserved for specialized research laboratories and not for large-scale application. Another sensitive stage concerns sampling and storage, because sample handling represents a possible crucial source of result variability when evaluating the oxidative stress status. Sample maintenance at room temperature or successive freezing-thawing cycles can determine auto-oxidation. This has been demonstrated for MPO samples, because leucocytes continue to release MPO in samples not kept on ice. Accordingly, we focused particular attention in the preanalytical phase: samples were collected on ice, immediately centrifuged and stored at \(-80^\circ C\) for less than 2 weeks before subsequent testing.

The number of enrolled subjects is limited; however, the significance of our data is reinforced by strict criteria used to select a well-defined and characterized cohort of patients and the homogeneity of clinical treatment. Moreover, it should also be considered that the long follow-up time allows the observation of a consistent number of events.

In conclusion, our study suggests that the global OXY-index, as an assessment of the oxidative stress status, represents a significant independent predictor of MACEs in patients with stable CAD, and may represent an adjunctive prognostic parameter useful in the prediction of the risk for cardiovascular events in CAD.

### Table 3. Cox predictive model for MACEs

| Predictors                                      | Univariate Analysis | Multivariate Analysis |
|------------------------------------------------|---------------------|-----------------------|
|                                                | Hazard ratio  | 95% CI | p value | Hazard ratio  | 95% CI | p value |
| Oxy-Index (>1.127-75th percentile)                 | 2.2            | 1.31-3.74 | 0.003   | 2.1            | 1.2-3.6 | 0.006   |
| Multivessel disease                              | 1.94          | 1.1-3.3  | 0.013   | 1.86          | 1.1-3.14 | 0.021   |
| Type 2 diabetes                                  | 1.4            | 0.9-2.3  | ns      | 1.4            | 0.9-2.2  | ns      |
| Prior myocardial infarction                      | 1.3            | 0.9-2    | ns      | 1.3            | 0.9-2    | ns      |
| Age (>69 yrs-50th percentile)                    | 0.9            | 0.6-1.5  | ns      | 0.9            | 0.7-1.5  | ns      |
| Males                                           | 0.9            | 0.6-1.5  | ns      | 0.9            | 0.7-1.7  | ns      |
| Hypertension                                    | 0.9            | 0.7-1.5  | ns      | 0.9            | 0.7-1.5  | ns      |
| Dyslipidemia                                    | 1.1            | 0.7-1.7  | ns      | 1.1            | 0.7-1.5  | ns      |
| Smoking habit (current or past)                  | 1.2            | 0.9-2    | ns      | 1.2            | 0.9-2    | ns      |
| Ejection fraction <40%                           | 0.9            | 0.7-1.6  | ns      | 0.9            | 0.7-1.5  | ns      |

CI = confidence interval
Conflicts of Interest

The authors have no financial and personal relationships with any source that could influence their work. The authors have no affiliation with any organization with a financial interest, direct or indirect, in the subject matter or materials discussed in the manuscript. No sources of funding were declared.

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