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Age- and gender-related oxidative status determined in healthy subjects by means of OXY-SCORE, a potential new comprehensive index

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Abstract
Oxidative stress has been related to various diseases, gender and ageing, and has been measured by various markers. The authors developed a procedure to compute a global oxidative stress index (OXY-SCORE), reflecting both oxidative and antioxidant markers in healthy subjects. Its performance was tested in relation to age and gender and in coronary artery disease (CAD) patients. Eighty-two healthy subjects and 20 CAD patients were enrolled. Plasma free and total malondialdehyde (F- and T-MDA), glutathione disulphide/reduced form ratio (GSSG/GSH) and urine isoprostanes ($iPF_{2\alpha}$-III) levels were combined as oxidative damage markers (damage score). GSH, α- and γ-tocopherol (TH) levels, and individual antioxidant capacity were combined as antioxidant defence indexes (protection score). The OXY-SCORE was computed by subtracting the protection score from the damage score. Among single parameters, T-MDA and $iPF_{2\alpha}$-III significantly correlated with age; only GSH and both tocopherols correlated with male gender in healthy subjects. The OXY-SCORE was positively associated with age ($p = 0.004$) and male gender ($p = 0.03$). As expected, the OXY-SCORE was higher in CAD with a very significant p-value (< 0.0001), after adjusting for age, gender and smoking. Combining different markers can potentially provide a powerful index in the evaluation of oxidative stress related to age, gender and CAD status.

Keywords: *Oxidative stress markers, pro/antioxidant global indices, ageing, coronary artery disease (CAD)*

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Introduction

The role of oxidative stress in the pathogenesis of a variety of chronic and degenerative diseases, such as tumours, cardiovascular and neurodegenerative diseases, has been extensively investigated (Maytin et al. 1999, Cavalca et al. 2001, Maccioni et al. 2001, Aliev et al. 2002, Bjelakovic et al. 2004, Shi et al. 2004, Valko et al. 2004, Vassalle et al. 2004, Rundle et al. 2005). In addition, changes in endogenous oxidative balance have also been related to the ageing process since 1956 (Harman 1956). Several

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following studies postulated different mechanisms for age related oxidative damage and contributed to the development of the free-radical theory of ageing (Cutler 1982, Beckman & Ames 1998, Asif et al. 2000, Droge 2002, Sohal 2002, Yu & Chung 2006).

Oxidative stress occurs in cells when the production of free radicals overwhelms the antioxidant defence system, and the consequent oxidant damage leads to alterations in gene expression, protein structure and function, lipid and membrane integrity. Reactive oxygen species (ROS) are continuously generated during the normal processes of cell metabolism, and may be induced by external sources such as ultraviolet light radiation, smoke, ozone, chemotherapeutic agents and toxins. Under physiological conditions, the crucial and delicate balance of ROS is maintained by endogenous antioxidants and free-radical scavengers. Alterations in oxidant/antioxidant status can be detected measuring a variety of variables. The direct detection of reactive species, the determination of biological damage indices such as malondialdehyde (MDA), glutathione disulphide (GSSG), and isoprostanes may provide indexes of pro-oxidant status. Conversely, individual antioxidant capacity (IAC), cellular antioxidant enzymes, reduced glutathione (GSH), and vitamin E may provide indexes of antioxidant status.

Studies based on the measurement of individual antioxidant/pro-oxidant indexes have led to conflicting results concerning the effect of age (Aejmelaeus et al. 1997, Garcia-Arumi et al. 1998, Ji 1999, Mecocci et al. 1999, 2000, Hernanz et al. 2000, Kostka et al. 2000, Kasapoglu & Ozben 2001, Mendoza-Nunez et al. 2001, Stadtman 2002, Mutlu-Turkoglu et al. 2003, Pastore et al. 2003), or gender (Aejmelaeus et al. 1997, Kasapoglu & Ozben 2001, Proteggente et al. 2002, Marra et al. 2002, Pastore et al. 2003, Vassalle et al. 2004, Liu et al. 2004).

Indeed, no single parameter can yet be recommended as a gold standard for defining individual redox status. In our opinion, individual markers are able to describe only partially the oxidative status and are associated with a large intra- and inter-subject variability. Thus we believe it would be useful to bring together multiple measures of both damage and antioxidant factors, in order to obtain a comprehensive score with higher sensitivity to physiological and pathological alterations.

We here propose OXY-SCORE, which includes a variety of individual markers of oxidative damage and defence, as a comprehensive index of oxidative stress status. Evidence is provided that this index is highly sensitive to age- and gender-related differences in oxidative balance in healthy subjects. Its ability to discriminate patients with coronary artery disease (CAD) from healthy subjects has also been tested.

Materials and methods

Subjects

Eighty-two healthy volunteers (51 male and 31 female) were enrolled among the hospital staff, after giving their written informed consent. Subjects taking drugs, vitamin, antioxidant supplements, synthetic estrogens as contraceptives or hormone replacement therapy were excluded. All of the subjects were asymptomatic and their healthy status was confirmed by clinical assessment and laboratory analyses (clinical chemistry profile, complete blood cell and differential counts, urinalysis).

Twenty consecutive patients with angiographically documented CAD were enrolled as a validation sample after giving their written informed consent to a protocol

approved by the Institutional Review Board of Centro Cardiologico Monzino. None of the patients had undergone a myocardial infarction in the previous 6 weeks, or suffered from unstable angina. The patients were compared with a random subgroup of 40 subjects extracted from our healthy individuals, frequency matched for age and gender.

Sample collection

Whole blood. Peripheral blood samples were collected on ice from fasting subjects into ethylenediamine tetra-acetic acid (EDTA)-containing tubes (9.3 mmol l^{-1}; Vacutainer Systems, Becton, Dickinson Co., Franklin Lake, NJ, USA), immediately precipitated with 10% trichloroacetic acid in 1 mmol l^{-1} EDTA solution, and stored at -80°C until analysis.
Plasma. Plasma was obtained from peripheral blood anticoagulated with EDTA after centrifugation (3000*g* for 10 min at 4°C) within 30 min. Samples were stored at -80°C until analysis.
Urine. Overnight urine samples were added with the antioxidant 4-hydroxy-tempo (1 mmol l^{-1}; Sigma-Aldrich Chemical Co., St Louis, MO, USA) and stored at -80°C before extraction.

Free and total MDA assay

Plasma F- and T-MDA levels were determined by the reference method based on gas chromatography-mass spectrometry (GC-MS) technique, with synthesized dideuterated MDA added as the internal standard (Cighetti et al. 1999). T-MDA was evaluated after alkaline hydrolysis (NaOH 1 mol l^{-1}) at 60°C before the derivatization step. The intra- and inter-assay CVs were 1.2 and 1.5% for F-MDA and 2.0 and 2.1% for T-MDA, respectively.

Isoprostane analysis

Urinary $iPF_{2\alpha}$-III levels were determined by means of a previously described enzyme-immunoassay method (Wang et al. 1995) using a commercially available kit (SPI-BIO, Saclay, F; Cayman Chemical Co., Ann Arbor, MI, USA) after double solid-phase extraction of urine added with of [^{3}H]-$PGF_{2\alpha}$ as the internal standard. The intra- and inter-assay CVs were 4.9 and 10.2%, respectively.

GSH and GSSG measurements

Whole blood GSH and GSSG levels were measured by HPLC. Briefly, whole blood samples were centrifuged at 14 000*g* for 1 min, and GSH and GSSG (20 µl injections) were separated using a Discovery® C18 5 mm RP column (4.6 × 250 mm) (Supelco, Bellafonte, PA, USA), eluted at 30°C with a mobile phase (50 mmol l^{-1} NaH_2PO_4, 0.05 mmol l^{-1} octane sulfonic acid, and 2% acetonitrile, adjusted to pH 2.7 with phosphoric acid) at a flow rate of 1 ml min^{-1}. Analysis was carried out using an ESA CoulArray detector (5600A) (ESA Inc., Chelmsford, MA, USA) with electrodes set at 400, 700, 750 and 800 mV. The peak areas were integrated using commercial software (ESA CoulArray for Windows). Under these conditions, GSH and GSSG elute at 5.08 and 9.3 min, respectively. The sample concentrations were calculated from calibration curves using standard GSH and GSSG solutions (Sigma).

Calibration of the analytical procedure gave a linear signal over the GSH range of 3.125–50.0 μmol l^{-1} ($r = 0.9999$) and the GSSG range of 1.56–25.0 μmol l^{-1} ($r = 0.9998$), with a limit of quantitation (LOQ) of 0.265 and 0.426 μmol l^{-1}, respectively. The intra- and inter-assay CVs were 1.1 and 5.9% for GSH, and 3.9 and 12.5% for GSSG, respectively.

Vitamin E measurement

α- and γ-Tocopherol (TH) concentrations were detected by HPLC after previous organic extraction according to Perugini et al. (2000). Briefly, 0.1-ml plasma samples were deproteinized with ethanol and extracted in hexane. After evaporation to dryness under vacuum, the residue was dissolved in methanol (0.2 ml), and an aliquot of the organic extract (25 μl) was injected into a C18 reversed-phase column (3.5 μm, 150 × 4.6 mm, Simmetry 300™; Waters Corporation, Mildford, MA, USA) and eluted with methanol (100%) as mobile phase at a flow rate of 1 ml min^{-1}. The retention times of α- and γ-TH were 4.82 and 4.34 min, respectively, as determined by a Jasco (Jasco Corporation Tokyo, Japan) FP15-20 fluorescent detector ($\lambda_{exc} = 292$ nm, $\lambda_{em} = 335$ nm). Kroma System3000 software (Bio-Tek Kontron Instruments MI, Italy) was used for chromatogram integration. The data were calculated after comparison with calibration curves using pure α- and γ-TH standard solutions (Sigma). The calibration of the analytical procedure gave a linear signal over the α-TH range of 5–40 μmol l^{-1} ($r = 0.9997$) and over the γ-TH range of 0.1–10 μmol l^{-1} ($r = 0.9998$), with an LOQ of, respectively, 0.38 and 0.14 μmol l^{-1}. The intra- and inter-assay CVs were 3.3 and 4.0% for plasma α-TH, and 3.3 and 4.7% for γ-TH, respectively.

Individual antioxidant capacity (IAC)

Plasma IAC was assessed using a commercially available spectrophotometric assay (OXY-adsorbent test, Diacron®, GR, Italy), which measures the plasma capability to neutralize a massive oxidative action of hypochlorous acid on alkyl-substituted aromatic amine in a chromogenic mixture (*N*,*N*-diethylparaphenilendiamine). The decrement of absorbance was measured at 505 nm and the antioxidant capacity was expressed as μmol HClO ml^{-1} of sample. The intra- and inter-assay CVs were 2.2 and 6.3%, respectively.

Statistical analysis

Variables with markedly skewed distributions (T- and F-MDA, GSSG/GSH, $iPF_{2\alpha}$-III, and α- and γ-TH) were log-transformed before score computation and analysis. After transformation, the null hypothesis of normality was never rejected by the Wilks–Shapiro test. Except for GSH, univariate Pearson correlations were assessed using the log-transformed data. The associations with age, gender, CAD and smoking were assessed by means of analysis of covariance (ANCOVA). The ability of the scores to discriminate CAD patients from healthy subjects was tested by applying logistic regression and computing the area under the ROC curve. All analyses were performed using SAS statistical package v.8 (SAS Institute, Cary, NC, USA).

Results

The characteristics of healthy subjects and CAD patients are reported in Table I. Since the healthy subjects were younger than CAD patients (mean age 48.5 and 64.3 years, respectively), a subgroup of 40 healthy subjects was selected, which was comparable with CAD patients in terms of gender (26 males; 65%) and age distribution (mean age 61.1 years).

Individual marker analyses in healthy subjects

Plasma or urinary analyte levels are reported in Table II, stratified by gender and age. ANCOVA revealed a significant age-related variation in the case of T-MDA and iPF$_{2\alpha}$-III, and a nearly significant age-related variation in GSSG/GSH (rising with age) (panel A); significant gender differences were found only for GSH and tocopherols (higher in females) and a nearly significant difference in GSSG/GSH (higher in males) (panel B).

Positive correlations were observed between the two forms of MDA ($r=0.44$, $p=0.001$) and between the two tocopherol isoforms ($r=0.40$, $p=0.0005$), and significant negative correlations between GSH and T-MDA ($r=-0.52$, $p=0.003$), and between GSSG/GSH and α-TH ($r=-0.26$, $p=0.04$), stratified by age and gender.

Summary score computation and analysis in healthy subjects

Three summary indexes of oxidative status were generated: a score of oxidative damage, combining pro-oxidant factors (damage score, DS), a score of antioxidant defences (protection score, PS), and a global score of oxidative balance (OXY-SCORE). The details for the computation of the three scores are reported in Appendix 1.

The mean DS, PS and OXY-SCORE values, stratified by age and gender, are shown in Table III. Multivariable analysis, adjusting for age, gender and smoking,

Table I. Clinical characteristics of the subjects.

Variable	Healthy subjects ($n=82$)	Healthy control subgroup ($n=40$)	CAD patients ($n=20$)	$p^\star$
Males, *n* (%)	51 (62.2)	26 (65.0)	13 (65.0)	0.69
Age, mean (range)	48.5 (24–77)	61.1 (49–77)	64.3 (47–81)	0.19
Active smokers, *n* (%)	7 (8.3)	2 (5)	9 (19.2)	<0.001
Body mass index	24.2±2.2	24.0±3.6	27.3±1.7	<0.001
Total cholesterol (mg dl^{-1})	210.9±22.6	219.0±28.2	202.4±74.5	0.22
HDL-C (mg dl^{-1})	58.6±5.7	57.6±10.8	34.6±9.9	<0.001
LDL-C (mg dl^{-1})	131.9±15.2	136.6±26.9	128.7±73.4	0.45
Previous AMI (%)			8 (40.0)	
Diabetes (%)			5 (25.0)	
Hypertension (%)			15 (75.0)	
Ejection Fraction (%)			59.5±10.9	

Qualitative variables are expressed as the mean±standard deviation. The healthy control subgroup was chosen among healthy subject to match CAD patients.
*CAD patients versus healthy control subgroup.
HDL, high-density lipoproteins; LDL, low-density lipoproteins; AMI, acute myocardial infarction.

Table II. Pro- and antioxidant factors stratified by for age class (panel A) and gender (panel B).

Panel A

Analyte	Young: 24–50 years, $n=42$			Old: 51–77 years, $n=40$			$p^{\star}$
	Mean	SD	Median	Mean	SD	Median	
T-MDA (μmol l^{-1})	3.27	1.13	3.03	4.76	1.99	4.16	0.04
F-MDA (μmol l^{-1})	0.501	0.207	0.460	0.614	0.316	0.580	0.34
$iPF_{2\alpha}$-III (pmol $mmol^{-1}$ creatinine)	174	109.9	192	301.7	143.1	315.5	0.04
GSSG/GSH	0.059	0.043	0.044	0.089	0.093	0.061	0.09
GSH (μmol g^{-1} Hb)	70.52	18.25	68.76	65.95	15.76	65.19	0.18
IAC (μmol HClO ml^{-1})	304.5	36.18	299	303.1	38.6	298.5	0.55
α-TH (μg ml^{-1})	14.28	3.95	13.33	14.99	7.44	11.93	0.36
γ-TH (μg ml^{-1})	0.41	0.13	0.4	0.42	0.36	0.37	0.95

Panel B

Analyte	Females, $n=31$			Males, $n=51$			$p^{\star\star}$
	Mean	SD	Median	Mean	SD	Median	
T-MDA (μmol l^{-1})	3.38	1.62	2.82	3.62	1.24	3.5	0.68
F-MDA (μmol l^{-1})	0.48	0.22	0.45	0.55	0.23	0.52	0.39
$iPF_{2\alpha}$-III (pmol $mmol^{-1}$ creatinine)	270.5	144.8	290	208.6	138.9	212.5	0.34
GSSG/GSH	0.049	0.04	0.03	0.083	0.079	0.06	0.06
GSH (μmol g^{-1} Hb)	77.34	17.72	79.18	63.68	15.21	62.09	0.002
IAC (μmol HClO ml^{-1})	296.9	29.57	292	307.9	40.62	300	0.25
α-TH (μg ml^{-1})	17.82	7.69	14.2	12.91	3.72	12.36	0.0004
γ-TH (μg ml^{-1})	0.49	0.26	0.41	0.38	0.17	0.36	0.04

*Adjusted for gender and smoking status by ANCOVA; **Adjusted for age and smoking status by ANCOVA.
T-MDA and F-MDA, total, free malondiadehyde; $iPF_{2\alpha}$-III, isoprostane; GSSG and GSH, disulphide, reduced form of glutathione; IAC, individual antioxidant capacity; α-TH and γ-TH, α-, γ-tocopherol; SD, standard deviation.

Table III. Oxidative stress scores stratified by age (panel A) and gender (panel B), as assessed by analysis of covariance (ANCOVA).

Panel A

	Young: 24–50 years, $n=42$			Old: 51–77 years, $n=40$			
Variable	Mean	SD	Median	Mean	SD	Median	$p^{\star}$
DS	−0.061	0.721	−0.13	0.376	0.854	0.263	0.002
PS	−0.003	0.576	0.053	−0.121	0.714	−0.195	0.03
OXY-SCORE	−0.054	1.026	−0.324	0.365	1.132	0.438	0.004

Panel B

	Females, $n=31$			Males, $n=51$			
Variable	Mean	SD	Median	Mean	SD	Median	$p^{\star\star}$
DS	−0.097	0.748	−0.318	0.246	0.813	0.232	0.10
PS	0.132	0.632	0.175	−0.177	0.631	−0.190	0.04
OXY-SCORE	−0.210	1.016	−0.493	0.370	1.089	0.284	0.03

DS, damage score; PS, protection score.
*Adjusted for gender and smoking status by ANCOVA; **Adjusted for age and smoking status by ANCOVA.

showed that DS was significantly and positively correlated with age ($p=0.002$) but gender differences were not significant ($p=0.10$); PS was significantly and negatively correlated with age ($p=0.03$) and was higher in females ($p=0.04$). Finally, OXY-SCORE correlated more significantly with both age ($p=0.004$) and gender ($p=0.03$), indicating a higher oxidative damage in males and in older subjects. The interaction between age and gender, as assessed by ANCOVA, was not significant ($F=0.19$, $p=0.66$), but the two regression lines showed a certain degree of convergence (Figure 1), which suggests that the apparent protection against oxidative stress observed in females tends to disappear with ageing. There was a significant negative correlation between the PS and DS ($r=-0.39$, $p=0.001$), persisting after adjustment for age and gender ($F=6.4$, $p=0.01$).

Scores in CAD patients

After adjusting for age, gender, body mass index (BMI), high-density lipoproteins (HDL)-cholesterol and smoking status, OXY-SCORE was significantly higher in CAD patients than in controls (2.70 ± 0.41 versus 0.66 ± 0.29, $p<0.001$). The two partial scores were also different in the two groups, only DS reaching significance (DS: 1.94 ± 0.27 versus 0.65 ± 0.27, $p<0.007$; PS: -0.77 ± 0.24 versus -0.22 ± 0.18, $p=0.12$). No significant differences were observed between male and female CAD patients.

When the capacity of each score to discriminate CAD patients from matching controls was tested by logistic regression, the area under the ROC curve was 0.78 for PS, 0.94 for DS, and 0.96 for OXY-SCORE. The almost complete separation of OXY-SCORE distributions in the CAD patients and controls is shown in Figure 2.

When control subjects and CAD patient were both included in the analysis, and after adjusting for age, gender and CAD, the active smokers had a higher OXY-SCORE ($+0.52\pm0.30$, $p=0.09$) and DS ($+0.24\pm0.21$, $p=0.26$), and a lower PS (-0.34 ± 0.17, $p=0.05$).

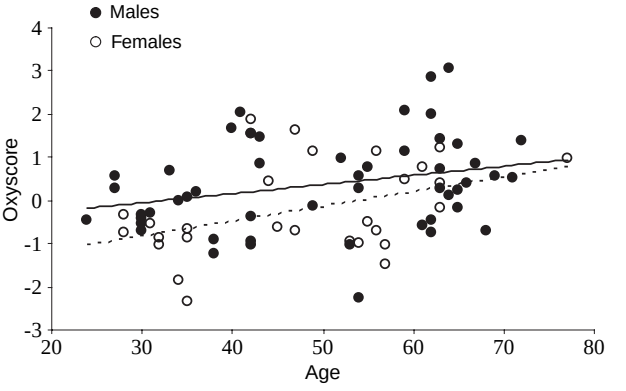

Figure 1. Linear regression of OXY-SCORE versus age in male (—) and female (----) healthy subjects ($n = 82$). The correlation coefficients were significant in both groups ($r = 0.28$, $p = 0.049$; $r = 0.36$, $p = 0.04$).

Discussion

Oxidative damage occurs when the complex equilibrium between pro- and antioxidants is altered. The body contains various antioxidant molecules for use against free-radical injury, of which glutathione and vitamin E are critical for maintaining cell redox balance (Vassalle et al. 2004). A number of distinct pathophysiological conditions affect the different pro- and antioxidants systems, depending on the evoked oxidative stress and the involved antioxidant defence. The oxidative stress hypothesis is one of the prevailing theories of ageing. Although the causative role of oxidative stress in ageing remains unknown, accumulated evidence identifies the increased oxidative stress with age as a source of damage to cellular structure and function (Yu & Chung 2001, Kim et al. 2004, Poon et al. 2004). However, *in vivo* studies based on laboratory measurements of oxidative or antioxidant markers have led to conflicting results (Aejmelaeus et al. 1997, Garcia-Arumi et al. 1998, Ji 1999, Mecocci et al. 1999, 2000, Hernanz et al. 2000, Kostka et al. 2000, Kasapoglu & Ozben 2001, Mendoza-Nunez et al. 2001, Droge 2002, Stadtman 2002, Mutlu-Turkoglu et al. 2003, Pastore et al. 2003), and the same is true for studies considering gender as an independent variable affecting oxidative parameters (Aejmelaeus et al.

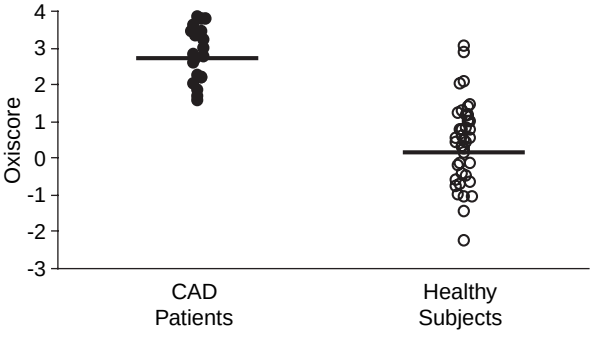

Figure 2. OXY-SCORE distributions in 20 coronary artery disease (CAD) patients and 40 matched controls: $p < 0.0001$ after adjusting for age, gender, body mass index (BMI), smoking habits and high-density lipidaemia (HDL)-cholesterol.

1997, Kasapoglu & Ozben 2001, Marra et al. 2002, Proteggente et al. 2002, Pastore et al. 2003, Liu et al. 2004, Vassalle et al. 2004).

Two main reasons may explain these discrepancies: first, the complexity of the biochemical pathways involved in the maintenance of plasma homeostasis; second, the analytical variability associated with the measurement of single markers. The comprehensive index of oxidative status that we developed may, thus, partially circumvent both these problems: it accounts simultaneously for parameters included in different pathways and, being a mean value, its variability is reduced with respect to single analytes. Indeed, we showed that this comprehensive index of oxidative status is possibly more sensitive to age and gender variations than single conventional parameter of oxidative stress.

To generate this index we selected four common markers of oxidative damage, and four accepted antioxidant factors. Specifically, T- and F-MDA were selected because they are indexes of lipoperoxidation, thus representing chronic or recent oxidative injury (Cighetti et al. 1999); isoprostanes are well accepted markers of *in vivo* oxidation (Lawson et al. 1999); the ratio disulphide/reduced forms of GSH is a marker of cellular unbalance toward oxidation. Among markers of antioxidant status IAC is an index of the total antioxidant system, GSH and TH were selected as representative of hydrophilic and lipophilic compartments of the body. Furthermore, GSH plays a pivotal role in the regeneration of other antioxidants and tocopherols act as both potent peroxyl radical scavengers and chain-breaking antioxidants, regulating both enzyme activity and membrane fluidity.

In our sample of healthy subjects pro-oxidant variables were, in most cases, higher in males and in older subjects, while antioxidant defence markers were higher in females and in younger subjects, as previously reported by others (Mecocci et al. 1999, Kostka et al. 2000). However, only T-MDA, $iPF_{2\alpha}$-III, GSH and TH reached, in some of the comparisons, full statistical significance. A different scenario emerged considering the three scores devised. DS (combining F- and T-MDA, $iPF_{2\alpha}$-III, and GSSG/GSH) was very significantly associated with age and marginally with gender; PS (combining GSH, α- and γ-TH, and IAC) was significantly associated with gender and age. Each score provided adequate statistical power to describe at least in part population differences in terms of age and gender. The comprehensive OXY-SCORE behaved as the most powerful index, reflecting both age and gender variation, with full statistical significance. Its correlation with age was present in both genders, with an upward-shifted distribution in males.

Another interesting finding is the close negative correlation between DS and PS, which suggests that less protected individuals tend to have higher damage scores. DS and PS remained inversely related also after adjustment for age and sex, which suggests that direct (mechanistic?) interactions between the pro- and antioxidant systems may occur. Alternatively, other unknown (genetic or environmental) factors may act on both systems.

As a validation test, we analysed the ability of OXY-SCORE to discriminate healthy subjects and CAD patients, whose severe oxidative stress has been reported by various authors (Maytin et al. 1999, Cavalca et al. 2001, Vassalle et al. 2004). Even with a limited sample size and after adjustment for age, gender and HDL-cholesterol, CAD patients showed higher OXY-SCORE values (together with decreased PS and increased DS), with very high statistical significance. The more than 95% areas

under the ROC curves provide an estimate of both sensitivity and specificity of OXY-SCORE.

It should be mentioned, however, that our sample size was inadequate to assess the effect of smoking habits on oxidative stress, but current smokers tended to have higher values of OXY-SCORE.

Conclusions

Even in a straightforward situation in which healthy subjects only differ in terms of age and gender, the pool of pro- and antioxidant factors show a very complex response pattern. We showed that oxidative stress evaluation can be improved by simultaneously considering pro-oxidant aspects and the decrease in antioxidant capacity, and we believe that OXY-SCORE can meet these requirements. It is relatively easy to compute and has valuable statistical characteristics, such as a fairly normal distribution. In our population, it behaved as expected, showing higher values in the elderly, males and CAD patients.

Needless to say, the algorithm to compute OXY-SCORE should be considered as a working hypothesis. Adding or deleting variables from its formula is a very easy procedure. Its precision and completeness are expected to increase with the number of parameters included, but a reasonable compromise should be found between power and feasibility. Further work identifying new candidate parameters (such as markers of DNA and protein damage, e.g. 8-hydroxyguanosine, dityrosine, etc.) or selecting the most informative variable subset, is needed to test at which extent the proposed OXY-SCORE gives an exhaustive estimate of the pro/anti-oxidant status in both healthy subjects and patients with CAD.

Moreover OXY-SCORE still needs to be tested for its predictivity with respect to cardiovascular risk factors, e.g. smoking status, hyperlipidaemia, etc., or in subjects carrying known polymorphisms of the genes involved in oxidative pathways.

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Appendix 1: Computation of scores

First, variables with markedly skewed distributions (F- and T-MDA, $iPF_{2\alpha}$-III, GSSH/GSH, α–TH and γ–TH) are log-transformed; only GSH and IAC do not need log-transformation.

Subsequently, pro- and antioxidant variables are standardized according to the usual formula:

$$z_{ij} = \frac{(x_{ij} - m_j)}{s_j},$$

where x_{ij} and z_{ij} are the original measure and the standardized value of variable j for subject i; and m_j and s_j are the mean and the standard deviation of variable j. The problem of combining factors with different measurement units and different variability is therefore solved. Here means and standard deviations calculated in the healthy population are used, but any other reference population would have suited just as well. The damage score (DS) is computed as the average of the standardized pro-oxidant factors. The protection score (PS) is computed in a similar way by averaging the standardized antioxidant variables. An example of DS and PS computation on an illustrative subject is reported in Table IV.

The global OXY-SCORE is computed by subtracting the PS from the DS.

Table IV. Example of computation of damage (DS) and protection (PS) scores of an illustrative subjects using means and standard deviations obtained in our healthy population.

	Healthy population		Illustrative subject			
Variable	Mean	SD	Raw values	Standardized values	Scores	
Pro-oxidant						
F-MDA (log)	−0.42	0.22	−0.3	0.54	0.51	DS
T-MDA (log)	0.39	0.21	0.4	0.05		
$iPF_{2\alpha}$-III (log)	2.30	0.33	2.5	0.62		
GSSG/GSH (log)	−1.29	0.35	−1	0.85		
Anti-oxidant						
IAC	303.9	36.6	300	−0.11	−0.63	PS
GSH	69.1	17.2	50	−1.11		
α-TH (log)	1.14	0.15	1	−0.96		
γ-TH (log)	−0.43	0.20	−0.5	−0.33		