

Review Article

The assessment of oxidative stress in clinical practice and its importance in nutrition

N. REGANO¹, E. L. IORIO², A. GUGLIELMI³, S. MAZZUOLI¹, A. FRANCAVILLA⁴, S. FREGNAN¹, G. LEOGRANDE¹, F.W. GUGLIELMI¹

¹Section of Gastroenterology and Day Hospital of Artificial Nutrition, San Nicola Pellegrino Hospital, Trani (Bari) - Italy

²International Observatory of Oxidative Stress, Salerno - Italy

³Section of General Surgery, II University "Francesco Paccione", Bari - Italy

⁴Section of Gastroenterology, Department of Emergency Medicine and Organ Transplantation, University of Bari, Bari - Italy

ABSTRACT: *An increased production of oxidative chemical species (OCS) and/or a decreased efficacy of antioxidant systems (AOS) can lead to the breakdown of the oxidative balance, thus generating the so-called oxidative stress, which is generally recognized as playing a relevant pathogenic role in early aging and in several inflammatory and/or degenerative diseases including atherosclerosis and hypertension (and their consequences, such as stroke and myocardial infarction), Alzheimer's disease, Parkinson's disease, and cancer. In particular, the currently available scientific evidence indicates that there is a very close relationship between oxidative stress and nutrition. In a vicious circle where genetic factors and abnormal lifestyles favor the onset of oxidative damage, which in turn may decrease the bioavailability of antioxidants and amplify initial lesions, it is not easy to establish whether OCS are the cause or the effect of the observed disease. However, one thing seems evident: the identification, by means of reliable analytical tools, of an impairment of the oxidative balance is the right premise for any rational attempt to correct the disequilibrium between OCS production and AOS efficacy and hence to contribute, in a more scientifically solid and not purely empirical manner, to "add life to years and years to life". The aim of this review was to analyze, with reference to the scientific literature, the analytical performance and clinical applications of currently available tests for the routine assessment of the oxidative balance, especially in the field of nutrition. The biochemical technology has given clinicians and nutritionists the opportunity to identify and quantify many markers of oxidative stress, which are currently used with the general purpose of preventing oxidative damage, diagnosing and monitoring oxidative stress and, finally, evaluating the indications and effectiveness of various antioxidant supplementations and therapeutic interventions. (Nutritional Therapy & Metabolism 2008; 26: 149-62)*

KEY WORDS: *Oxidative chemical species (OCS), Antioxidant systems (AOS), Oxidative stress, Biochemical markers, Antioxidant supplements*

INTRODUCTION

In all living organisms there exists a delicate balance between the production and the elimination of oxidative chemical species (OCS) (1). The production of OCS – which include radical and nonradical oxygen-, nitrogen-, carbon-, chlorine-, and sulphur-centered species – can depend on both exogenous agents (e.g., radiation, chemicals, bacteria) and endogenous phenomena (e.g., activation of specific enzymatic or non-enzymatic reactions) (2). The level and activity of antioxidant systems (AOS) – which include endogenous (e.g., antioxidant enzymes, like catalases and peroxidases) and exogenous agents

(e.g., vitamins and vitamin-like compounds) directed against OCS – are related to genetic and environmental factors (mainly lifestyle) (3). An increased production of OCS and/or a decreased efficacy of AOS can lead to the breakdown of the oxidative balance, thus generating the so-called oxidative stress (4).

Oxidative stress is generally recognized as playing a pathogenic role in early aging and in several inflammatory and/or degenerative diseases including atherosclerosis and hypertension (and their consequences, such as stroke and myocardial infarction), Alzheimer's disease, Parkinson's disease, and cancer (5). An imbalance between prooxidant and antioxidant factors seems to be in-

volved even in the fields of nutrition, food safety and allergy, gastroenterology and hormonal/metabolic diseases.

Oxidative stress is not a “disease” in the traditional sense of the word. It is the unwanted effect of the breakdown of a biochemical equilibrium. Therefore it can impact, often deceitfully, upon the onset and/or course of several basic diseases. As it is not a classical disease, oxidative stress does not exhibit a specific clinical picture but hides itself behind the symptoms and signs of the basic disease. Therefore, oxidative stress can be found only if the clinician submits the patient to specific biochemical tests.

At long last, research now offers clinicians and nutritionists the opportunity to identify and quantify many markers of oxidative stress, which are currently used with the general purpose of preventing oxidative damage, diagnosing and monitoring oxidative stress, and evaluating the indications and effectiveness of antioxidant supplementations and/or therapeutic interventions (6). Some of these markers have even been proposed as being predictive of disease (7).

The aim of this review was to analyze, with reference to the scientific literature, the analytical performance and clinical applications of currently available tests for the routine assessment of the oxidative balance, especially in the field of nutrition.

OXIDATIVE STRESS AND ITS IMPACT ON NUTRITION AND METABOLIC DISEASES

Oxidative stress is a pathological condition triggered by the damaging action of abnormally increased amounts of OCS on the cells and tissues of the body. It is the direct consequence of an increased generation of OCS and/or a reduced physiological activity of antioxidant defenses against OCS, like free radicals (1, 5).

Free radicals are single or grouped atoms having at least one outer orbit “occupied” by one single electron (“unpaired”) instead of a couple of electrons (“lone pair”) (1). Antioxidants are chemical or biological agents able to neutralize the potentially damaging action of free radicals (8). Some antioxidants (e.g., the enzymes superoxide-dismutase and catalase) are endogenous, that is, they are normal components of the body, while others (e.g., vitamins C and E) are exogenous and must be taken from the external environment, e.g., by eating fruits and vegetables (8).

Our body, even in normal conditions, produces a defined amount of free radicals due to the physiological cell metabolism (1, 9). For instance, the synthesis of some hormones involves the generation of free radicals

whilst polymorphonuclear leukocytes exploit the production of free radicals to kill bacteria, thus helping the body against infections (1, 3). Other free radicals, such as nitric oxide, are fundamental in the body’s homeostasis because they modulate important functions including vascular tone, platelet aggregation, cell adhesion, and so on (10). Free radicals (and other OCS) have been defined as “irreplaceable journey companions” of cell life (11). The causes believed to be responsible for an increased production of free radicals may have different origins, which can be of a physical, chemical or biological nature.

In its healthy state, the body is able to prevent damage by free radicals because of the natural defense system of antioxidants, whose name indicates the ability of these agents to counteract the oxidant action of free radicals and other OCS (8). A reduced effectiveness of such a system is substantially ascribable to an absolute or relative deficiency of antioxidants, independently of the involved mechanism.

Free radicals are potentially dangerous because they have the spontaneous tendency to fill their unfilled outer orbit with a second electron (1, 2). Indeed, the presence of 2 electrons in the same orbit is the condition of maximal energetic stability. Therefore, when a free radical is close to a target molecule, having one or more available electrons, such as the molecule of an unsaturated fatty acid (e.g., arachidonic acid), it immediately pulls out the electron from the target molecule. Due to this effect, the so-called oxidant action, the original free radical loses its potential dangerousness whilst the newly generated molecule is damaged and becomes a new free radical, thus perpetuating, if no antioxidants are available, the initial reaction to other molecules including carbohydrates, lipids, amino acids, peptides, proteins, nucleotides, and nucleic acids (a chain effect) (12).

The amount and quality of nutrients taken – as a function of genetic, immunological and psychoneurological-hormonal factors, lifestyle (exercise, cigarette smoking, alcohol use), concomitant diseases and drug intake – affects the oxidative balance in our body (1, 13, 14). For instance, oil-based foods can contain large amounts of lipoperoxides, which can increase the level of OCS in our body (15). We should also consider the potential impact on health of oxidized compounds in animals for human consumption, with a potential risk depending on the modality of rearing cattle, pigs, poultry, rabbits, fish, etc. (16-20). On the other hand, depending on environmental factors (acid rain, inadequate soil), many technological processes (refinement, temperature changes, packaging, storage), pharmacological treatments (pesticides) and cooking modalities (types of oven) can variously impact the amount of biologically

active antioxidants in the foods (21, 22). Moreover, the delicate balance between prooxidant and antioxidant factors can be impaired even by quantitative changes of nutrients. In particular, a calorie excess – often related to alcohol abuse and inadequate exercise – can increase *per se* the mitochondrial activity, thus generating more oxygen-centered OCS by the aerobic route (respiratory chain) while restriction of calorie intake may be beneficial also in light of the aging process (23). Overweight or obesity may further increase the production of OCS, often by activating inflammatory processes (24, 25).

The increased production of OCS, frequently due to impairment of the effectiveness of AOS, can lead to the production and release of many OCS in the blood, with extension of the oxidative damage from one tissue to the whole body.

One of the most common mechanisms by which OCS, after overcoming the antioxidant defenses, can attack the biochemical components of our body is the generation of so-called hydroperoxides (ROOH), a class of reactive oxygen metabolites (ROMs) (26). In this pathophysiological model – due to exogenous stressors (physical, chemical and biological agents) and/or metabolic activity (particularly in the plasma membrane, the mitochondria, the endoplasmic reticulum and cytosol) – the cell starts to produce increasing amounts of free radicals, among which the very powerful hydroxyl radical (HO[•]), one of the most potentially dangerous reactive oxygen species (ROS) (1). Indeed, hydroxyl radicals can “hit” every kind of molecule including carbohydrates, lipids, amino acids, peptides, proteins, nucleotides, and nucleic acids. As a consequence of this action, every molecule

TABLE I - HUMAN DISEASES MOST FREQUENTLY ASSOCIATED WITH OXIDATIVE STRESS (from reference 6, modified)

Aceruloplasminemia	Down's syndrome	Parkinson's disease
Acute and chronic alcoholic liver diseases	Eclampsia	Periodontal disease
Acute autoimmune myocarditis	End-stage renal disease	Peritoneal dialysis
Acute chest syndrome of sickle cell disease	Erectile dysfunction	Photoaging
Acute pancreatitis	Friedreich's ataxia	Preeclampsia
Acute respiratory distress syndrome	Heart failure	Primary biliary cirrhosis
Alcoholic liver disease	Helicobacter pylori infection/inflammation	Professional bronchopulmonary diseases
Alzheimer's disease	Hemodialysis	Progeria
Amyotrophic lateral sclerosis	Hepatic cirrhosis	Psoriasis
Arterial/systemic hypertension	Human immunodeficiency virus infection	Psoriatic arthritis
Asbestosis	Huntington's disease	Pulmonary hypertension
Asthma	Hyperbaric diseases	Radiotherapy side effects
Ataxia telangiectasia	Hypercholesterolemia	Reactive arthritis
Atherosclerosis	Hyperhomocysteinemia	Renal cell carcinoma
Atopic dermatitis	Hyperlipidemia	Respiratory distress syndrome
Brain ischemia	Idiopathic pulmonary fibrosis	Retinopathy of prematurity
Bronchopulmonary dysplasia	Interstitial lung disease	Retrolentacular fibroplasia
Burns	Ischemia-reperfusion injury	Rheumatic disease
Cancer (several kinds)	Juvenile chronic arthritis	Rheumatoid arthritis
Cardiopulmonary bypass	Kidney transplantation	Sarcoidosis
Cardiovascular diseases	Leukemia	Sepsis
Cataract	Lung cancer	Sickle cell disease
Cellulitis	Lung injury	Sleep apnea
Chemotherapy side effects	Macular degeneration	Spherocytosis
Chronic fatigue syndrome	Male infertility	Spinal cord injury
Chronic hepatitis C	Ménière's syndrome	Stroke
Chronic kidney disease	Meningitis	Synucleinopathies
Chronic obstructive pulmonary disease	Mild cognitive impairment	Systemic amyloidosis
Chronic renal failure	Multiple sclerosis	Systemic lupus erythematosus
Colitis	Myelodysplastic syndromes	Systemic sclerosis (scleroderma)
Coronary artery disease	Myocardial infarction	Thrombophilia
Creutzfeldt-Jakob disease	Myocarditis	Tauopathies
Crohn's disease	Neonatal bronchopulmonary dysplasia	Tuberculosis
Cutaneous leishmaniasis	Obesity	Unstable angina
Cystic fibrosis	Osteoarthritis	Uremia
Diabetes mellitus type 1	Osteoporosis	Venous insufficiency
Diabetes mellitus type 2	Pancreatitis	Werner's syndrome
Dislipidemia	Parkinsonisms	

loses an electron and becomes, in turn, a radical.

A radical chain reaction starts, leading to the generation of hydroperoxides if molecular oxygen (by respiration) is present. Although hydroperoxides are relatively stable chemical species, they have the potential to generate free radicals and to oxidize other molecular targets. For this reason the cell pulls the hydroperoxides into the external environment, i.e., the extracellular matrix and finally the extracellular fluids, including blood, cerebrospinal fluid, pleural fluid, etc.

When a condition of ischemia is induced due to prolonged vasoconstriction or partial thrombus, the reduced availability of oxygen inside the microcirculation (hypoxia) compels the cell to activate the anaerobic metabolism, with the release into the small blood vessels of acidic metabolites, including lactate. The consequent lowering of the pH may induce a conformational change of transition metal-carrier proteins, including transferrin and ceruloplasmin. The low-pH-induced conformational change of transferrin then causes the release from the carrier of iron, which acts as a catalyst in the so-called Fenton reaction where hydroperoxides are broken into alkoxyl (RO•) and hydroperoxyl (ROO•) radicals (27). Both radicals are able to oxidize the endothelium surface or the circulating lipids and cholesterol, thus favoring atherosclerosis. In this context we should consider as “bad” the oxidized cholesterol, independently of its being bound to low- or high-density lipoproteins (28, 29). It is evident, though,

that hydroperoxides are not only the witnesses or markers of oxidative stress (due to their origin from the cell) but also potential amplifiers of the initial damage to the whole body (due to their ability to circulate in the extracellular fluids) (30).

Every nutritional antioxidant regimen must be carefully evaluated by the clinician or the nutritionist as a function of the individual response. It is, in fact, important to highlight the role that every individual, with his or her biological patrimony, can play in conditioning the response to foods and antioxidant supplements proposed to maintain or reach a satisfying oxidative balance, also with the aim of preventing common invalidating diseases (31-33). In this respect, we should carefully consider the role of the whole gastrointestinal apparatus, including the oral cavity (34-36).

In the small intestine, for instance, diseases such as celiac disease can find in oxidative stress a biunivocal relation (37, 38). The immunological disease underlying the illness may increase the production of OCS in the mucosa and this phenomenon in turn may decrease the absorption of antioxidants, thus favoring a condition of oxidative stress (increased production of oxidants/decreased efficacy of antioxidant systems). On the other hand, in the large bowel, every pathological change in the intestinal bacterial flora due to an unbalanced diet or antibiotics may lead to an impairment of oxidative balance and therefore to tissue damage (39).

TABLE II - MAIN TESTS TO EVALUATE OXIDATIVE BALANCE (IN BLOOD)

Tests to measure oxidant capacity/potential		Tests to measure antioxidant capacity/potential	
Total capacity/potential	d-ROMs test TOS/TMP	Total capacity/potential	TAS, OXY-ADS, TRAP/ORAC FRAP, BAP, CUPRAC
Specific capacity/potential	AGE MDA/TBAR Conjugated dienes Isoprostanes Lipoperoxides Hydroxyalkenals Pentane/ethane* Ox-LDL AOPP Protein carbonyl 8-OH-dG Myeloperoxidase Chemiluminescence NPBI	Specific capacity/potential	Superoxide dismutase Glutathione peroxidase Catalase Ascorbate Carotenes Tocopherols Albumin Uric acid Bilirubin Ceruloplasmin (?) Transferrin Ferritin Haptoglobin Plasma thiols

8-OH-dG, 8-hydroxy-2'-deoxyguanosine; AGE, advanced glycation end products; AOPP, advanced oxidation protein product; BAP, biological antioxidant potential; CUPRAC, cupric reducing antioxidant capacity; FRAP, ferric reducing ability of plasma; MDA, malondialdehyde; NPBI, non-protein-bound iron; ORAC, oxygen radical absorbance capacity; Ox-LDL, oxidatively modified low-density lipoproteins; OXY-ADS, OXY-adsorbent; TAS, total antioxidant status; TMP, trimethylphosphorothioate; TOS, total oxidant status; TRAP, total peroxyl radical trapping capacity assay; TRBAR, thiobarbituric reactive species

*On exhaled breath. *On polymorphonuclear leukocytes.

Lastly, we should also consider the importance of the oral cavity. It seems to have been ascertained that a local change in oxidative balance can favor periodontal disease, which in turn may lead to risk of cardiovascular diseases, where inflammation and oxidative stress may play a major pathogenic role (34-36, 40). Therefore, both the quality and the amount of food, by impairing the oral oxidative balance, may affect distant organs via oxidative stress.

The relationship between oxidative stress and nutrition becomes evident when we consider the most common metabolic disorders, such as metabolic syndrome and type 2 diabetes mellitus, where hyperglycemia, insulin resistance and inflammation are the most relevant determinants of oxidative stress (41). Moreover, an increased production of OCS is closely related to the complications of diabetes (cardiovascular diseases, neuropathy, etc.) (42).

These findings suggest that very close relationships exist between oxidative stress and nutrition. In a vicious circle where abnormal lifestyles favor the onset of local and systemic oxidative damage (which in turn may decrease the bioavailability of antioxidants with amplification of initial lesions), it is not easy to establish whether OCS are the cause or the effect of the observed disease. However, one thing seems evident: the identification by means of reliable analytical tools of the impairment of the oxidative balance is the right premise for every rational attempt to correct the imbalance between OCS production and AOS efficacy and hence to contribute, in a more scientifically solid and not purely empirical manner, to “adding life to years and years to life”.

OXIDATIVE STRESS MEASUREMENT

Principles

According to the generally accepted definition of oxidative stress given above, an increased production of OCS and/or a decreased efficacy of the antioxidant defense system inside or outside the cells may lead to the (per)oxidation of a number of biomolecules with generation of (per)oxidized by-products (e.g., hydroperoxides, chloramines, advanced glycosylation end products, isoprostanes, 8-OH-dG) (43). This may be followed by an increase in (per)oxidized by-products and/or a reduced concentration/activity of antioxidants either in tissues or extracellular fluids, which will represent the optimal specimens in which to evaluate the oxidative stress (6).

When a condition of oxidative stress is generated in a living organism, a number of OCS may accumulate at levels above the physiological limits in tissues and/or biological fluids. The first analytical approach therefore involves

the direct measurement of such oxidant(s) in a biological specimen. This goal can be achieved by using electron spin resonance (44) for radical OCS like hydroxyl or peroxyl radicals, or other photometric/fluorescent methods for nonradical OCS like hydrogen peroxide. When direct measurement of OCS is not possible, different methods, referred to as fingerprinting, must be applied (45). According to this approach, a radical is inferred from the molecular nature of the damage it causes to biological molecules (45). When the oxidative stress is great enough to overcome the antioxidant defense, the reactive chemical species can theoretically damage every component of the cell, including lipids, amino acids, proteins, and nucleic acids, thus generating oxidized by-products. These damaged molecules – or the products resulting from their breakdown – are the “fingerprinting”. In other words, oxidative damage is presumed to happen in vivo when it generates identifiable and quantifiable specific by-products in vitro. These by-products are assumed to be biomarkers of oxidative status (45). Notably, some of these “biomarkers”, like hydroperoxides, can also act as “amplifiers” of oxidative damage, which underscores the importance of detecting these molecules in order to reduce not only the effect but also the cause of oxidative stress (30, 46).

The evaluation of antioxidant defenses – which is apparently easier than the quantification of OCS – is generally possible by direct methods evaluating the activity of enzymes (e.g., superoxide dismutase, catalases and peroxidases) or water/lipid-soluble antioxidants (e.g., vitamin C and E) by means of photometry or fluorescence. For the evaluation of both oxidant and antioxidant capacity, some tests provide a global idea of the oxidant or antioxidant status (e.g., d-ROMs test and Total Antioxidant Status, respectively), while others provide the quantification of a specific enzymatic activity or concentration (e.g., measurement of glutathione peroxidase activity or serum levels of tocopherols, respectively).

On this basis we chose to classify the most commonly available methods for oxidative stress assessment into 2 main categories: tests to evaluate the oxidative status and tests to evaluate the antioxidant status. In each category we will further distinguish, when adequate, direct from indirect methods and global from selective methods. Further classifications can be made depending on the biological source (e.g., plasma, exhaled breath, etc.).

Methods to assess oxidant capacity and potential

Global assessment methods

Total oxidant status is generally evaluated by exploiting the ability of a biological sample (e.g., blood

serum or plasma) to oxidize a chromogenic substrate whose color change is quantified photometrically. The most common substrates for routine analysis having the property to change their color due to the oxidant action of a biological sample are N,N-diethyl-paraphenylenediamine and xylenol orange. The former is used in the d-ROMs test, the latter in the FOX test.

Although initially interpreted as a method to evaluate the levels of hydroperoxides, the d-ROMs test is a suitable test to evaluate total oxidant status in a serum or plasma sample or other extracellular fluid, including cerebrospinal fluid and pleural effusions (47- 49). This test is based mainly on the application in vitro of a phenomenon that occurs in vivo (in the microcirculation). In fact, the dilution of the blood sample (serum/plasma) in an acidic buffered solution induces – as observed in vivo when a condition of ischemia with microacidosis occurs – the release from the transferrin of iron ions, which as free ions can catalyze the blood hydroperoxide breakdown and generate free radicals (RO[•], alkoxyl, and ROO[•], hydroperoxyl radicals). When the colorless oxidizable chromogenic substrate (N,N-diethyl-paraphenylenediamine) is added to this solution, the highly unstable newly generated free radicals (RO[•] and ROO[•]) pull out an electron from the aromatic amine, which becomes a colored radical cation. The latter is relatively stable and can be detected and measured. The chromogen is originally colorless and becomes pink to red when it releases an electron. Based on the pink color intensity, which is proportional to the radical cation concentration, it is possible, using a photometer (calculation of the absorbance change at 505 nm, $\Delta_{\text{abs}}/\text{min}$), to evaluate the concentration of free radicals and hence the concentration of hydroperoxides initially present in the biological sample; the use of an adequate biochemical standard, i.e., control serum with a known value, is essential for a correct measurement (30). The proof of this principle was provided years ago and subsequently confirmed by an expert team of the Italian National Research Council (CNR) validating the test by electron spin resonance spectrometry, which is the technique universally recognized as the gold standard to study radical species in vitro (30, 50). Thanks to this comparison it was demonstrated that the signal obtained by performing the d-ROMs test in a flat cell of an electron spin resonance spectrometer can be fully overlapped by the one obtained by following, in parallel, the course of the same reaction with a photometer. However, pretreatment of the serum sample with a chelating agent such as ethylenediaminetetraacetic acid (EDTA), thereby making the iron unusable for catalysis, is followed by a significant reduction but not annulment of the ESR/photometric signal. This experimental finding indicates that at least a

part of the absorbance change at 505 nm, as detected by performing the d-ROMs test, is not due to hydroperoxides but also to other OCS and/or enzymatic activity (30). For instance, the so-called chloramines, which are believed to be reliable markers of the hypochlorous–acid-induced oxidative damage on peptide or protein amine groups, can contribute to the absorbance change of the d-ROMs test (51). Moreover, since pretreatment of serum samples with sodium azide, a compound believed to be an inhibitor of the (iron)oxidase activity of ceruloplasmin, decreases the absorbance change, it is possible that the d-ROMs test evaluates, although minimally, the oxidation of N,N-diethyl-paraphenylenediamine apparently due to the ceruloplasmin at low pH (30). Interestingly, this ceruloplasmin activity seems to be related to the vascular damage rather than to a protective effect (52). In any case, by indicating the possibility that the d-ROMs test can measure more than one class of oxidants, which in turn are derived from different metabolic pathways, these findings reinforce the clinical significance of the d-ROMs test as a reliable method to obtain a global and suitable measure of the “total” serum/plasma oxidant status (30, 53). The normal range is 250 to 300 CARR U (Carratelli Units, name after the inventor), where 1 CARR U is equivalent to 0.08 mg/dL hydrogen peroxide. The result of many studies indicate that the d-ROMs test is a reliable, precise, and repeatable test with acceptable within-run and between-run coefficients of variation (CV), either with manual or automatic procedures (1-3%), including a multi-well analytical system (54, 55). The lowest limit of sensitivity is estimated to be 17 CARR U. The maximal linearity is within the range of 50 to 500 CARR U. The test is not subject to analytic interference by most common serum analytes, including triglycerides (up to 28.2 mmol/L), hemoglobin (up to 0.068 mmol/L) and bilirubin (up to 171 mmol/L) (54). The d-ROMs test has proved useful in many clinical trials on nutrition and metabolism in humans (56) and animals (18-20, 57).

Total oxidant status can be measured also by exploiting the redox properties of xylenol orange. In the FOX test, the oxidant capacity of a serum or plasma sample is quantified by the amount of xylenol orange generated by ferric ions, as produced from ferrous ions by the sample oxidants (58). The test proved to be reliable not only on plasma but also in vitro on parenteral nutrition solutions (59).

Specific or selective assessment methods

The oxidant capacity and potential can be measured more selectively by quantifying specific radical or non-radical OCS.

Radical species, which are generally short-living species, can be measured directly by means of electron spin resonance or nuclear magnetic resonance spectroscopy, which includes a double approach, i.e., the direct quantification of free radicals and the indirect measurement by spin trapping methods (60). Specific methods are now available also for biological nitric oxide (61).

Unfortunately, although electron spin resonance/nuclear magnetic resonance spectroscopy remains the gold standard for direct measurement of free radicals, it is not suitable – at the moment – in clinical practice because of its limited availability and relatively high costs.

Nonradical species such as hydrogen peroxide can be measured in plasma or other extracellular fluids (including exhaled breath) by means of photometric methods based on the use of catalase (62).

Indirect or fingerprinting methods can be further classified according to the chemical nature of the original hit molecule, e.g., carbohydrates, lipids, amino acids, peptides, proteins, and nucleic bases. In particular, exposure to increased levels of sugars may induce the non-enzymatic glycation of lipids, proteins or nucleic acids, thus generating – through some key intermediates such as methylglyoxal – the so-called advanced glycation end products, which are prevalent in (but not exclusive for) the diabetic vasculature and contribute to the development of atherosclerosis (63). Advanced glycation end products are by-products that are able to block nitric oxide activity in the endothelium and cause the production of ROS.

Lipids due to the presence of one or more double bonds between carbon atoms in their mono- and polyunsaturated fatty acid moieties, respectively, are particularly susceptible to the oxidative attack (1). The main targets of OCS are either membrane or serum/plasma lipids.

Increased oxidant levels or activity may affect the plasma membrane integrity mainly by isomeric conversion and lipoperoxidation processes, which can be monitored by different analytical approaches. The isomeric conversion is the inversion of the naturally occurring *cis* conformation to the non-natural and pathological *trans* conformation at the level of a double bond between 2 carbon atoms. It seems that this change is mainly due to an excess of thiyl radicals ($R-S^{\bullet}$) (64). The isomeric inversion may reduce the distance between phospholipids and increase the membrane rigidity, thus impairing the metabolic exchanges between the cell and its environment. Now the distribution of all fatty acids including the *trans* component in the plasma membrane can be evaluated by means of the lipidomic approach, that is, by means of the mass spectrometric or gas chromatographic

determination of the erythrocyte plasma membrane fat profile (64, 65). This new analytical approach is very intriguing because it virtually allows the clinician to establish the need for polyunsaturated fatty acids and to select which kind of fatty acids are required by every individual for personalized supplementation.

Peroxidation of membrane lipids can be very damaging because it leads to alterations in the biological properties of the membrane, including the degree of fluidity; it may also lead to inactivation of membrane-bound receptors or enzymes, which in turn may impair normal cellular function and increase tissue permeability (66). Moreover, lipid peroxidation generates many oxidized and sometimes reactive by-products that are able to amplify the initial oxidative damage (12). Most of these by-products are detectable in tissues and biological fluids, and may therefore be useful as biomarkers of oxidative stress (6). Lipid peroxidation, which plays a major role in arteriosclerosis, inflammation, and mitochondrial function, is a complex process consisting of 3 stages: initiation, propagation, and termination. For each stage, there are many available methods to quantify the progress of this process and the evidence for its existence (6). For instance, because lipid peroxidation causes loss of substrates, such as unsaturated fatty acid chains, the measurement of lipid content before and after exposure may give an indication of peroxidation. In addition, because oxygen is consumed during the propagation stage, measurement of its uptake by oxygen electrodes may serve as a tool for evaluating the progress of oxidation (6). According to the classical radical chain reaction of lipid peroxidation, after the abstraction of a hydrogen atom by a reactive species, rearrangement of the fatty acid radical occurs. This process is characterized by the early formation of a conjugated diene, which can be easily monitored by UV spectroscopic means (67). In the last stage of the peroxidation process, peroxides are decomposed to a variety of relatively stable decomposition products including α and β -unsaturated reactive aldehydes such as malondialdehyde (MDA), 4-hydroxy-2-nonenal (HNE), and 2-propenal (acrolein) (6). All of these are termed thiobarbituric reactive species (67). This method, which produces an easily measurable pink color, is one of the most widely used assays to assess peroxidation in the whole organism. It does not, however, provide early information and is not completely specific, at least not for MDA, which should be measured as “free MDA” (68). The end products of other aldehydes, e.g., hexanal, can be evaluated by liquid chromatography-mass spectrometry (6).

Among the by-products of lipid oxidation are also F_2 -isoprostanes, a large family of F_2 -prostaglandin-like compounds derived from the non-enzymatic oxidation

of arachidonic acid (6). Once produced, they are released into the bloodstream, where they are rapidly metabolized – although not as rapidly or as extensively as prostaglandins – and eliminated. Their rapid disappearance from plasma may hamper practical application (6). However, *ex vivo* or *in vitro* F₂-isoprostanes are relatively stable and can be measured in plasma or urine as a reliable marker of oxidant status in several clinical conditions including acute and chronic inflammation, ischemia-reperfusion injury, liver disease, diabetes, and atherosclerosis; they are particularly useful in the monitoring of the antioxidant response to dietary supplements (6, 69). Measurement of F₂-isoprostanes is therefore believed to be one of the most reliable approaches for the assessment of oxidative stress derived from lipid peroxidation at the moment (6). Different analytical approaches have been developed, among which the association of mass spectrometry with gas chromatography is considered the gold standard because it allows to identify the exact chemical nature of the different kinds of isoprostanes and to distinguish these by-products from other prostanoids. These methods, however, require extensive preparation of the material (e.g., phospholipid extraction and alkaline hydrolysis) and/or expensive instrumentation (6). Novel immune assays based also on RIA technology, although less specific, have therefore been proposed for routine analyses (6).

The last stage of the lipoperoxidation process can be evaluated by measuring exhaled hydrocarbon gases, such as pentane and ethane, using gas chromatography (70).

The oxidative attack on lipids may also impair circulating lipoproteins, especially low-density lipoproteins (LDL), with generation of oxidized by-products (7, 71). Such oxidatively modified LDLs (ox-LDL) are more potent proatherosclerotic mediators than the native unmodified LDLs. Indeed, ox-LDL may alter endothelium, decreasing the gene expression of endothelial cell nitric oxide synthase, and activate inflammatory cells, thereby facilitating the release of a number of growth factors from monocytes/macrophages (7). Many methods are now available to quantify ox-LDL in plasma; they are based on the detection of ox-LDL by means of antibodies or on the quantification of antibodies against ox-LDL (71). More recent evidence shows that even high density lipoprotein (HDL), widely considered as a protection factor against cardiovascular risk, can undergo oxidation to ox-HDL by myeloperoxidase-derived hypochlorous acid, thus raising again the question that the so-called *bad* cholesterol should be identified in all types of oxidized cholesterol, independently of its binding to LDL or HDL (72). More studies need to evaluate the possible predictive role of total plasma lipoperoxides in cardiovascular diseases. In the meantime, many ex-

perimental methods are now available to quantify ox-LDL (73).

Besides sugars and lipids, OCS can variously impair protein integrity and thus produce a number of oxidized by-products, some of which have been proposed as markers of oxidative stress. In this scenario, the oxidation of several amino-acid side chains (e.g., lysine, arginine, proline and threonine) may generate the so-called protein carbonyls, whose detection is exploited as a reliable approach to evaluate the effect of oxidative stress on proteins (74). Similar information may be provided by measuring advanced oxidation protein products (AOPP) using spectrophotometry on a microplate reader (6). Potentially useful is also the evaluation of oxidatively modified amino acids, peptides and proteins by chlorination, nitration, hydroxylation, and glutathionylation reactions (6).

Finally, increased oxidative stress may affect the base moiety of nucleic acids, particularly the guanosine residues, thus generating 8-hydroxy-2'-deoxyguanosine (8-OH-dG), whose level is considered an index of oxidative DNA damage (6). This modified nucleobase can be measured in urine by coupling mass spectrometry to gas-liquid chromatography, considered as the gold standard, and/or antibody-based techniques (75). Interestingly, the level 8-OH-dG decreased after carotenoid intake in women previously treated for breast cancer (76). Of course, DNA can also be damaged by OCS, like nitrogen-centered species, undergoing mainly nitration and deamination of purines, but the methods for the measurement of the relative by-products need more refinement and validation before they can be routinely applied to human materials (6).

Other methods to evaluate oxidative status

Infections and other reactive processes like autoimmune disorders can activate polymorphonuclear leukocytes, thus inducing the so-called “respiratory burst” (1). In this process bacteria, lipopolysaccharides and autoantibodies activate the NADPH oxidase with generation of superoxide anion. The latter is converted to hydrogen peroxide by superoxide dismutase. Both superoxide anion and hydrogen peroxide are useful ROS able to kill bacteria. Any excess hydrogen peroxide is scavenged by the catalase(s). However, when the infectious or inflammatory burden is high, the excess of such ROS, including singlet oxygen, may lead to oxidative tissue damage (1). In particular, hydrogen peroxide can react with chloride ions and generate hypochlorous acid, a powerful oxidant, by the activation of myeloperoxidase. Hypochlorous acid in turn may hit every amine group, finally generating oxidized by-products like chloramines

(51). On the other hand, hydrogen peroxide may also generate the powerful oxidant hydroxyl radical via the Fenton reaction if free iron is available (27).

Some new tests have been developed to measure the above phenomena and to provide new reliable markers of oxidative stress. In particular, the respiratory burst can be evaluated by performing a chemiluminescence assay on leukocytes *ex vivo* (77). Briefly, leukocytes are separated from plasma and other blood cells and incubated alone or with specific stimuli such as phorbol ester. The subsequent emission of photons is quantified after amplification of the signal with luminol. Baseline and post-stimulus chemiluminescence is interpreted as a measure of the ability of leukocytes to produce ROS. Unfortunately this approach provides only a measure of leukocytes' respiratory burst and is of limited use in free radical research (78).

The evaluation of myeloperoxidase activity seems to be more promising and was recently proposed as a reliable marker in cardiovascular diseases because of its relationship to HDL oxidation (79). Alternatively, measurement of chloramines may provide a surrogate marker of hypochlorous-acid-induced oxidation (51).

Finally, in inflammatory conditions the detection of free iron, the only substance able to catalyze the peroxide breakdown via the Fenton reaction, should be considered as a reliable method to evaluate oxidative stress (27, 80).

Methods to assess antioxidant capacity/potential/activity

Global assessment methods

One of the most important "first lines" of the antioxidant defense system is located in the extracellular fluids. Particularly in the blood, many organic molecules, either exogenous (from diet and supplements) or endogenous (from the cell metabolism), may contribute to protecting our body against reactive chemical species, thus preventing or minimizing the unwanted side effects of oxidative stress (81). Among these are proteins (mainly albumin), bilirubin, uric acid, cholesterol, antioxidant vitamins (beta-carotene, ascorbate, tocopherols), food antioxidants (polyphenols, bioflavonoids), protein and nonprotein thiols, and many others (81).

It is conceivable that increased levels of OCS may cause the depletion of one or different antioxidant species in serum or plasma. Such depletion can be measured using a variety of biochemical techniques. However, determination of total low-molecular-weight antioxidants rather than individual antioxidants should precede any other approach because all these chemical species

work in concert against oxidants.

According to this principle, a number of tests aimed at evaluating total antioxidant capacity (TAC) or total antioxidant status (TAS) have been proposed to monitor the body's ability to counteract the potentially damaging effects of OCS either in healthy or ill subjects, even after dietary antioxidant supplements (82, 83).

Methods to evaluate the overall antioxidant serum/plasma barrier in clinical practice are generally based on the reducing or oxidizing properties of a number of organic molecules or transition metals, respectively.

The methods based on reducing organic molecules involve a 2-step reaction where the source of antioxidants, i.e., plasma or serum, interacts with a source of oxidants in the presence of a chromogenic oxidizable substrate (81). The antioxidant capacity of the biological sample is evaluated on the basis of its ability to inhibit the oxidation of the chromogen, as monitored photometrically (83). The most common tests based on this principle are the classical TAS (Randox), the OXY-Adsorbent test (Diacron International), and the ORAC assay.

In the classical TAS, hydrogen peroxide reacts with metmyoglobin (source of oxidants, with peroxidase activity) and oxidizes 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) (organic oxidizable substrate) to its blue-green colored radical cation (ABTS^{•+}); the absorbance can be photometrically measured at 600 nm (84). The addition of serum or plasma to be tested causes inhibition of this color production to a degree that is proportional to the antioxidant capacity of the biological sample. Many compounds may contribute to such antioxidant activity: albumin (43%), uric acid (33%), ascorbate (9%), alpha-tocopherol (3%), bilirubin (2%) and unknown chemical species or activities (10%) (85). Results are expressed as mmol/L of Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid) equivalents. The reference value in apparently healthy adult people is 1.54 ± 0.02 mmol/L (85).

The OXY-Adsorbent test developed by Carratelli is similar to the classical TAS, the only differences being the source of oxidants, i.e., hypochlorous acid, and the organic chromogenic substrate, i.e., N,N-diethylparaphenylenediamine, which can be monitored at 505 nm (86, 87). Because the oxidant power of hypochlorous acid is higher than that of metmyoglobin, the OXY-Adsorbent test may detect more classes of antioxidants than the classical TAS. Results are expressed as mmol/L of (absorbed) hypochlorous acid. The reference value in apparently healthy adult people is more than 350 mmol/L; in other words, normally, 1 mL of human plasma is able to adsorb at least 350 mol of hypochlorous acid (86, 87).

The oxygen radical absorbance capacity (ORAC), con-

ceptually similar to the OXY-Adsorbent test, is the prototype of the so-called total peroxy radical trapping capacity (TRAP) assay (81). According to the original version of this method, thermal decomposition of azo-initiators generates at a constant rate peroxy radicals, which are quantified by measuring the maximum oxygen uptake, the generation of luminol-enhanced chemiluminescence, or the decay in phycoerythrin fluorescence (88). In particular, the ORAC test measures the antioxidant scavenging activity of a serum or plasma sample against peroxy radical induced by 2,2'-azobis(2-amidinopropane) dihydrochloride (AAPH) at 37°C by using beta-phycoerythrin as a fluorescent probe (88).

Finally, the total oxidant scavenging capacity (TOSC) assay, which can be considered as an evolution of the TRAP method, allows to evaluate the "adsorbent" capacity of a biological sample against 3 powerful oxidants, hydroxyl radical, peroxy radical, and peroxyxynitrite, by using 2-keto-4-methylthiobutyric acid (KMBA) as the oxidizable substrate and by chromatographically monitoring the ethylene produced (6, 89).

The methods based on the reduction of a transition metal evaluate the capacity or potential of a serum or plasma sample as a function of its ability to reduce iron or copper from Fe(III) to Fe(II) or Cu(II) to Cu(I), respectively. The reaction can be monitored photometrically thanks to a specific chromogen.

The reduction of iron is the principle of the FRAP assay and BAP test. The ferric reducing ability of plasma (FRAP) assay, designed by Benzie and Strain, is based on the reduction of ferric tripyridyltriazine (Fe^{III}-TPTZ) to ferrous tripyridyltriazine (Fe^{II}-TPTZ), which develops an intense blue color photometrically measurable by setting the wavelength at 593 nm (90). Calibration can be performed by means of solutions with known concentrations of Fe^{II} (FeSO₄·7H₂O). The antioxidant compounds contributing to the ferric reducing ability of plasma are uric acid (60%), ascorbate (15%), proteins (10%), alpha-tocopherol (5%), bilirubin (5%), and other unknown antioxidants (5%) (90). The normal range measured in apparently healthy Chinese subjects is between 612 and 1634 μmol/L (90).

The biological antioxidant potential (BAP) test, developed by Carratelli and first described by Dohi and coworkers, is based on the ability of a colored solution containing a source of ferric ions adequately bound to a special chromogenic substrate (a thiocyanate derivative) to decolor when ferric ions are reduced to ferrous ions, as occurs when a reducing/antioxidant system such as a plasma sample is added (91). Values above 2200 M reduced iron are considered optimal in humans (91). This test proved very promising in monitoring the antioxidant efficacy of food and supplements (unpublished data).

The reduction of copper by a serum or plasma sample is the principle of the cupric reducing antioxidant capacity (CUPRAC) assay. This test uses neocuproine (2,9-dimethyl-1,10-phenanthroline), the Cu(I) complex of which absorbs at 450 nm. A dilution curve generated by uric acid standards is used to convert sample absorbance to uric acid equivalents (92).

Specific or selective assessment methods

Different biochemical techniques have been developed to identify and quantify any specific component of the serum/plasma barrier to oxidation. These assays are designed to measure either endogenous (albumin, ferritin, transferrin, ceruloplasmin, uric acid, bilirubin, melatonin) or exogenous non-enzymatic compounds (carotenoids, tocopherols, ascorbate, polyphenols, bioflavonoids, selenium, zinc, copper, magnesium). However, ceruloplasmin may also exhibit prooxidant properties (52).

Sometimes it can be useful to measure also the by-products of these antioxidants after oxidative attack, like allantoin (derived from oxidation of uric acid) or ascorbate (derived from dehydroascorbate oxidation), or specific ratios (e.g., ascorbate/dehydroascorbate or reduced/oxidized glutathione).

In particular, the plasma level of thiols, according to the -SHp test developed by Carratelli, can be measured on the basis of the ability of thiol groups to develop a photometrically evaluable colored complex (maximum absorbance peak 405 nm) when reacted with 5,5'-dithiobis-2-nitrobenzoic acid (DTNB) (86, 93). The thiol titer directly parallels the color intensity. The range in healthy people is 450-650 mmol/L (86). Decreased values directly correlate with a lowered efficacy of the thiol antioxidant barrier. The -SHp test has been demonstrated to be a reliable assay to monitor the effectiveness of thiol-based supplements in Down's syndrome (94).

Enzymatic antioxidants, mainly superoxide dismutase (SOD), glutathione peroxidase (GPx) and glutathione transferase (GTx) can be measured in whole blood or serum/plasma by specific photometric assays. SOD activity in erythrocytes is measured using xanthine as superoxide anion generator. Results are expressed as IU/g hemoglobin. In apparently healthy adult men and women, SOD activity is 954.30 ± 198.0 IU/g Hb and 967.60 ± 226.1 IU/g Hb, respectively (85). GPx activity in erythrocytes is evaluated on the basis of a NADP⁺ shift at 340 nm. Results are expressed as IU/g hemoglobin. In apparently healthy adults GPx activity is 51.5 ± 13.0 IU/g Hb (85). GPx activity proved to be a reliable predictive marker of cardiovascular events in a large cohort of individuals at 5 years of follow-up (95). GTx activity in erythrocytes is also quantified on the basis of a NADP⁺ shift at 340 nm. Results are

expressed as IU/g hemoglobin. In apparently healthy adults GTx activity is 111.3 ± 16.5 MU/L (i.e., 0.841 ± 0.107 MU/g Hb).

Mixed methods

More recently, new tests able to measure the oxidant and antioxidant capacity of a serum or plasma sample have been developed. One of these assays is based on 3,3',5,5'-tetramethylbenzidine and its cation, used as a redox indicator participating in 2 simultaneous reactions (96).

CONCLUSIONS

A MEDLINE search for "oxidative stress" yielded more than 50,000 results (January 2008), underlining the importance of oxidative imbalance in the physiopathology of many diseases including metabolism and nutrition disorders. At the moment, although OCS can be considered either the cause or effect of tissue damage, growing evidence suggests a role of oxidative stress as an emerging health risk factor. In this scenario many studies are aimed at translating the evidence of basic research to the clinic and gaining full understanding of the role of free radicals and antioxidants in healthy and ill people.

Current evidence indicates that the oxidative attack on biomolecules produces one or more subclasses of oxidized metabolites which may accumulate in body tissues and/or fluids, thus suggesting oxidative damage. On the other hand, a loss of antioxidants may mirror indirectly a tissue lesion from OCS.

On this basis we believe that biochemical evaluation of the oxidative balance is a prerequisite for establishing whether a subject is suffering from oxidative stress, because this "transversal" condition is not associated with specific symptoms and clinical signs (97). Therefore, any treatment or supplement should be performed or given only after biochemical evidence has demonstrated that the body level of oxidants is increased and/or the amount or activity of antioxidants decreased.

In this review we analyzed the most common available methods and assays to evaluate oxidative balance in the clinical routine. Some of the described tests are able to evaluate the oxidant capacity while others are focused on the antioxidant capacity of tissues or extracellular fluids, mainly blood.

The state of the art suggests that single measurement of oxidant status or antioxidant status would be sufficient, but a battery of measurements, many of which we described, will be necessary to adequately assess oxidative stress in biological systems.

Further studies should clarify the physiological role of

oxidants and antioxidants, and extensive research will be needed to find the "ideal" biomarker. Meanwhile, the analytical approach suggested in this paper may lead clinicians to appreciate the practical importance of oxidative stress and help them understand how to act correctly in the choice and monitoring of antioxidant treatments. The current trend to prescribe antioxidants before a biochemical test has documented the real need for a supplement, especially in the presence of a nutrition or metabolic disorder, is not convincing.

Financial support: none

Conflict of interest: none declared

Abbreviations:

8-OH-dG: 8-hydroxy-2'-deoxyguanosine
AAPH: dihydrochloride
ABTS: 2,2-azino-bis (3-ethylbenzthiazoline-6-sulfonic acid)
ABTS^{•+}: blue-green colored radical cation
AGE: advanced glycation end products
AOPP: advanced oxidation protein products
AOS: antioxidant system
BAP: biological antioxidant potential
CARR U: Carrattelli Units
CNR: National Research Council
CUPRAC: cupric reducing antioxidant capacity
CV: coefficient of variation
DTNB: 5,5'-dithiobis-2-nitrobenzoic acid
EDTA: ethylenediaminetetraacetic acid
Fe^{II}-TPTZ: ferrous tripyridyltriazine
Fe^{III}-TPTZ: ferric tripyridyltriazine
FRAP: ferric reducing ability of plasma
GPx: glutathione peroxidase
GTx: glutathione transferase
HDL: high-density lipoprotein
HNE: 4-hydroxy-2-nonenal
HO: hydroxyl radical
KMBA: 2-keto-4-methylthiobutyric acid
LDL: low-density lipoprotein
MDA: malondialdehyde
OCS: oxidative chemical species
ORAC: oxygen radical absorbance capacity
Ox-LDL: oxidatively modified LDL
OXY-ADS: OXY-adsorbent
R-S: thiyl radical
RO: alkoxy radical
ROMs: reactive oxygen metabolites
ROO: hydroperoxyl radical
ROOH: hydroperoxides
ROS: reactive oxygen species
SOD: superoxide dismutase
TAC: total antioxidant capacity
TAS: total antioxidant status
TMP: trimethylphosphorothioate
TOS: total oxidant status
TOSC: total oxidant scavenging capacity
TRAP: total peroxy radical trapping capacity
TRBAR: thiobarbituric reactive species

Address for correspondence:
Prof. Francesco William Guglielmi
Unità Operativa di Gastroenterologia & Day-Hospital Nutrizione Artificiale
Ospedale "San Nicola Pellegrino"
Viale Padre Pio
70059 Trani (BA), Italy
e-mail: guglielmifw@libero.it

REFERENCES

- Halliwell B, Gutteridge JMC. Free radicals in biology and medicine, 2nd ed. Oxford: Clarendon Press, 1989.
- Gardes-Albert M. Physico-chemical aspects of reactive oxygen species. *Ann Pharm Fr* 2006; 64: 365-72.
- Valko M, Leibfritz D, Moncol J, Cronin MT, Mazur M, Telser J. Free radicals and antioxidants in normal physiological functions and human disease. *Int J Biochem Cell Biol* 2007; 39: 44-84.
- Delattre J. Introduction: from molecular oxygen to oxidative stress and radical biochemistry. *Ann Pharm Fr* 2006; 64: 363.
- Favier A. Oxidative stress in human diseases. *Ann Pharm Fr* 2006; 64: 390-6.
- Dalle-Donne I, Rossi R, Colombo R, Giustarini D, Milzani A. Biomarkers of oxidative damage in human disease. *Clin Chem* 2006; 52: 601-23.
- Ridker PM, Brown NJ, Vaughan DE, Harrison DG, Mehta JL. Established and emerging plasma biomarkers in the prediction of first atherothrombotic events. *Circulation* 2004; 109 (Suppl IV): IV6-19.
- Cadenas E, Packer L. Handbook of antioxidants. New York: Marcel Dekker Inc, 1996.
- Beaudeau JL, Peynet J, Bonnefont-Rousselot D, Therond P, Delattre J, Legrand A. Cellular sources of reactive oxygen and nitrogen species. Roles in signal transcription pathways. *Ann Pharm Fr* 2006; 64: 373-81.
- Ignarro LJ. Nitric oxide: a unique endogenous signaling molecule in vascular biology. Nobel Lecture, December 8, 1998. In: Nobel lectures: Physiology or medicine 1996-2000; 178-98.
- Cooper KH. Antioxidant revolution. Nashville (TN): Thomas Nelson Publishers, 1997.
- Therond P. Oxidative stress and damages to biomolecules (lipids, proteins, DNA). *Ann Pharm Fr* 2006; 64: 383-9.
- Spence JD. Nutrition and stroke prevention. *Stroke* 2006; 37: 2430-5.
- Sies H, Stahl W, Sevanian A. Nutritional, dietary and postprandial oxidative stress. *J Nutr* 2005; 135: 969-72.
- Pironi L, Guidetti M, Zolezzi C, et al. Peroxidation potential of lipid emulsions after compounding in all-in-one solutions. *Nutrition* 2003; 19: 784-8.
- Bagni M, Civitareale C, Priori A, et al. Pre-slaughter crowding stress and killing procedures affecting quality and welfare in sea bass (*Dicentrarchus labrax*) and sea bream (*Sparus aurata*). *Aquaculture* 2007; 263: 52-60.
- Castellini C, Mugnai C, Dal Bosco A. Meat quality of three chicken genotypes reared according to the organic system. *Ital J Food Sci* 2002; 4: 401-2.
- Brambilla G, Cantafora A. Metabolic and cardiovascular disorders in highly inbred lines for intensive pig farming: how animal welfare evaluation could improve the basic knowledge of human obesity. *Ann Ist Super Sanità* 2004; 40: 241-4.
- Brambilla G, Civitareale C, Ballerini A, et al. Response to oxidative stress as a welfare parameter in swine. *Redox Rep* 2002; 7: 159-63.
- Brambilla G, Ballerini A, Civitareale C, et al. Oxidative stress as a bio-marker of estrogen exposure in healthy veal calves. *Anal Chim Acta* 2003; 483: 281-8.
- Boskou D. Losses of natural antioxidants and vitamins during deep-fat frying. *Forum Nutr* 2003; 56: 343-5.
- Xianquan S, Shi J, Kakuda Y, Yueming J. Stability of lycopene during food processing and storage. *J Med Food* 2005; 8: 413-22.
- Gredilla R, Barja G. Minireview: the role of oxidative stress in relation to caloric restriction and longevity. *Endocrinology* 2005; 146: 3713-7.
- Joseph JA, Shukitt-Hale B, Casadesus G, Fisher D. Oxidative stress and inflammation in brain aging: nutritional considerations. *Neurochem Res* 2005; 30: 927-35.
- Kalantar-Zadeh K, Balakrishnan VS. The kidney disease wasting: inflammation, oxidative stress, and diet-gene interaction. *Hemodial Int* 2006; 10: 315-25.
- Girotti AW. Lipid hydroperoxide generation, turnover, and effect or action in biological systems. *J Lipid Res* 1998; 39: 1529-42.
- Halliwell B, Gutteridge JMC. Role of free radicals and catalytic metal ions in human disease: an overview. *Methods Enzymol* 1990; 186: 1-85.
- Hurtado I, Fiol C, Gracia V, Caldú P. In vitro oxidised HDL exerts a cytotoxic effect on macrophages. *Atherosclerosis* 1996; 25: 39-46.
- Ansell BJ, Fonarow GC, Fogelman AM. High-density lipoprotein: is it always atheroprotective? *Curr Atheroscler Rep* 2006; 8: 405-11.
- Alberti A, Bolognini L, Macciantelli D, Carratelli M. The radical cation of N,N-diethyl-para-phenylendiamine: a possible indicator of oxidative stress in biological samples. *Res Chem Intermed* 2000; 26: 253-67.
- Marotta F, Weksler M, Naito Y, Yoshida C, Yoshioka M, Marandola P. Nutraceutical supplementation: effect of a fermented papaya preparation on redox status and DNA damage in healthy elderly individuals and relationship with GSTM1 genotype: a randomized, placebo-controlled, cross-over study. *Ann NY Acad Sci* 2006; 1067: 400-7.
- Kaliora AC, Dedoussis GV, Schmidt H. Dietary antioxidants in preventing atherogenesis. *Atherosclerosis* 2006; 187: 1-17.
- Borek C. Dietary antioxidants and human cancer. *Integr Cancer Ther* 2004; 3: 333-41.
- Sculley DV, Langley-Evans SC. Periodontal disease is associated with lower antioxidant capacity in whole saliva and evidence of increased protein oxidation. *Clin Sci (Lond)* 2003;

- 105: 167-72.
35. Buchmann R, Hasilik A, Van Dyke TE, Lange DE. Amplified crevicular leukocyte activity in aggressive periodontal disease. *J Dent Res* 2002; 81: 716-21.
 36. Battino M, Bullon P, Wilson M, Newman H. Oxidative injury and inflammatory periodontal diseases: the challenge of antioxidants to free radicals and reactive oxygen species. *Crit Rev Oral Biol Med* 1999; 10: 458-76.
 37. Odetti P, Valentini S, Aragno I, et al. Oxidative stress in subjects affected by celiac disease. *Free Radic Res* 1998; 29: 17-24.
 38. De Stefano D, Maiuri MC, Simeon V, et al. Lycopene, quercetin and tyrosol prevent macrophage activation induced by gliadin and IFN-gamma. *Eur J Pharmacol* 2007; 566: 192-9.
 39. Reid GM. *Candida albicans* and selenium. *Med Hypotheses* 2003; 60: 188-9.
 40. Al-Zahrani MS, Kayal RA, Bissada NF. Periodontitis and cardiovascular disease: a review of shared risk factors and new findings supporting a causality hypothesis. *Quintessence Int* 2006; 37: 11-8.
 41. Abraham NG, Brunner EJ, Eriksson JW, Robertson RP. Metabolic syndrome: psychosocial, neuroendocrine, and classical risk factors in type 2 diabetes. *Ann NY Acad Sci* 2007; 1113: 256-75.
 42. Graves DT, Kayal RA. Diabetic complications and dysregulated innate immunity. *Front Biosci* 2008; 3: 1227-39.
 43. Ogino K, Wang DH. Biomarkers of oxidative/nitrosative stress: an approach to disease prevention. *Acta Med Okayama* 2007; 61: 181-9.
 44. Kopáni M, Celec P, Danisovic L, Michalka P, Biró C. Oxidative stress and electron spin resonance. *Clin Chim Acta* 2006; 364: 61-6.
 45. Karlsson J. Antioxidants and exercise. *Human Kinetics Publishers*, 1997.
 46. Miyamoto S, Ronsein GE, Prado FM, et al. Biological hydroperoxides and singlet molecular oxygen generation. *IUBMB Life* 2007; 59: 322-31.
 47. La Torre F, Orlando A, Silipigni A, Giacobello T, Pergolizzi S, Aragona M. Incremento dei radicali liberi dell'ossigeno e dei loro derivati in pazienti neoplastici chemio e radiotrattati. *Minerva Medica* 1996; 86: 1-4.
 48. Yamanaka G, Kawashima H, Suganami Y, et al. Diagnostic and predictive value of CSF d-ROM level in influenza virus-associated encephalopathy. *J Neurol Sci* 2006; 243: 71-5.
 49. Papageorgiou E, Kostikas K, Kiropoulos, Karetsi E, Mpatavanis G, Gourgoulialis KI. Increased oxidative stress in exudative pleural effusions: a new marker for the differentiation between exudates and transudates? *Chest* 2005; 128: 3291-7.
 50. Alberti A, Della Bona MA, Bolognini L, Carratelli M, Macchiantelli D. Assessing oxidative stress in living organisms by ESR spectroscopy. *Proc Third European ESR Meeting, Leipzig, Germany*, 1997; 2.
 51. Hawkins CL, Davies MJ. Reaction of HOCl with amino acids and peptides: EPR evidence for rapid rearrangement and fragmentation reactions of nitrogen-centered radicals. *J Chem Soc Perkin Trans* 1998; 2: 1937-45.
 52. Shukla N, Maher J, Masters J, Angelini GD, Jeremy JY. Does oxidative stress change ceruloplasmin from a protective to a vasculopathic factor? *Atherosclerosis* 2006; 187: 238-50.
 53. Banfi G, Malavazos A, Iorio EL, et al. The iron-dianisidine/xylene orange assay in comparative oxidative stress assessment. Some possible shortcomings. *Eur J Appl Physiol* 2005; 97: 506-8.
 54. Iamele L, Fiocchi R, Vernocchi A. Evaluation of an automated spectrophotometric assay for reactive oxygen metabolites in serum. *Clin Chem Lab Med* 2002; 40: 673-6.
 55. Hayashi I, Morishita Y, Imai K, Nakamura M, Nakachi K, Hayashi T. High-throughput spectrophotometric assay of reactive oxygen species in serum. *Mutat Res* 2007; 631: 55-61.
 56. Komatsu F, Kagawa Y, Sakuma M, et al. Investigation of oxidative stress and dietary habits in Mongolian people, compared to Japanese people. *Nutr Metab (Lond)* 2006; 3: 1-18.
 57. Ballerini A, Civitareale C, Fiori M, Regini M, Betti M, Brambilla G. Traceability of inbred and crossbred Cinta Senese pigs by evaluating the oxidative stress. *J Vet Med A Physiol Pathol Clin Med* 2003; 50: 113-6.
 58. Firuzi O, Mladenka P, Ricciari V, et al. Parameters of oxidative stress status in healthy subjects: their correlations and stability after sample collection. *J Clin Lab Anal* 2006; 20: 139-48.
 59. Silvers KM, Darlow BA, Winterbourn CC. Lipid peroxide and hydrogen peroxide formation in parenteral nutrition solutions containing multivitamins. *J Parenter Enteral Nutr* 2001; 25: 14-7.
 60. Swartz HM, Khan N, Khramtsov VV. Use of electron paramagnetic resonance spectroscopy to evaluate the redox state in vivo. *Antioxid Redox Signal* 2007; 9: 1757-71.
 61. Kleschyov AL, Wenzel P, Munzel T. Electron paramagnetic resonance (EPR) spin trapping of biological nitric oxide. *J Chromatogr B Analyt Technol Biomed Life Sci* 2007; 851: 12-20.
 62. Frew EK, Jones P, Scholes G. Spectrophotometric determination of hydrogen peroxide and organic hydroperoxide at low concentrations in aqueous solution. *Anal Chem Acta* 1983; 155: 139-50.
 63. Goldin A, Beckman JA, Schmidt AM, Creager MA. Advanced glycation end products: sparking the development of diabetic vascular injury. *Circulation* 2006; 114: 597-605.
 64. Ferreri C, Chatgililoglu C. Geometrical trans lipid isomers: a new target for lipidomics. *Chembiochem* 2005; 6: 1722-34.
 65. German JB, Gillies LA, Smilowitz JT, Zivkovic AM, Watkins SM. Lipidomics and lipid profiling in metabolomics. *Curr Opin Lipidol* 2007; 18: 66-71.
 66. Vance DE, Vance JE. *Biochemistry of lipids, lipoproteins and membranes*, 4th ed. Amsterdam: Elsevier, 2002.
 67. Moore K, Roberts LJ 2nd. Measurement of lipid peroxidation. *Free Radic Res* 1998; 28: 659-71.
 68. Veglia F, Cighetti G, De Franceschi M, et al. Age- and gender-related oxidative status determined in healthy subjects by means of OXY-SCORE, a potential new comprehensive index. *Biomarkers* 2006; 11: 562-73.
 69. Sicilia T, Mally A, Schauer U, Pähler A, Völkel W. LC-MS/MS methods for the detection of isoprostanes

- [iPF(2alpha)-III and 8,12-iso-iPF(2alpha)-VI] as biomarkers of CCl₄-induced oxidative damage to hepatic tissue. *J Chromatogr B Analyt Technol Biomed Life Sci* 2008; 861: 48-55.
70. Pabst F, Miekisch W, Fuchs P, Kischkel S, Schubert JK. Monitoring of oxidative and metabolic stress during cardiac surgery by means of breath biomarkers: an observational study. *J Cardiothorac Surg* 2007; 2: 37-45.
71. Itabe H, Ueda M. Measurement of plasma oxidized low-density lipoprotein and its clinical implications. *J Atheroscler Thromb* 2007; 14: 1-11.
72. Koller E, Volf I, Gurvitz A, Koller F. Modified low-density lipoproteins and high-density lipoproteins. From investigation tools to real in vivo players. *Pathophysiol Haemost Thromb* 2006; 35: 322-45.
73. Matsunaga T, Koyama I, Hokari S, Komoda T. Detection of oxidized high-density lipoprotein. *J Chromatogr B Analyt Technol Biomed Life Sci* 2002; 781: 331-43.
74. Dalle-Donne I, Aldini G, Carini M, Colombo R, Rossi R, Milzani A. Protein carbonylation, cellular dysfunction, and disease progression. *J Cell Mol Med* 2006; 10: 389-406.
75. Malayappan B, Garrett TJ, Segal M, Leeuwenburgh C. Urinary analysis of 8-oxoguanine, 8-oxoguanosine, fapy-guanine and 8-oxo-2'-deoxyguanosine by high-performance liquid chromatography-electrospray tandem mass spectrometry as a measure of oxidative stress. *J Chromatogr A* 2007; 1167: 54-62.
76. Thomson CA, Stendell-Hollis NR, Rock CL, Cussler EC, Flatt SW, Pierce JP. Plasma and dietary carotenoids are associated with reduced oxidative stress in women previously treated for breast cancer. *Cancer Epidemiol Biomarkers Prev* 2007; 6: 2008-15.
77. Dahlgren C, Karlsson A. Respiratory burst in human neutrophils. *J Immunol Methods* 1999; 232: 3-14.
78. Ricevuti G, Mazzone A, Fossati G, et al. Assay of phagocytic cell functions. *Allerg Immunol (Paris)* 1993; 25: 55-66.
79. Shao B, Oda MN, Oram JF, Heinecke JW. Myeloperoxidase: an inflammatory enzyme for generating dysfunctional high density lipoprotein. *Curr Opin Cardiol* 2006; 21: 322-8.
80. Perrone S, Bracci R, Buonocore G. New biomarkers of fetal-neonatal hypoxic stress. *Acta Paediatr Suppl* 2002; 91: 135-8.
81. Prior RL, Cao G. In vivo total antioxidant capacity: comparison of different analytical methods. *Free Radic Biol Med* 1999; 27: 1173-81.
82. Huang D, Ou B, Prior RL. The chemistry behind antioxidant capacity assays. *J Agric Food Chem* 2005; 53: 1841-56.
83. Yeum KJ, Russell RM, Krinsky NI, Aldini G. Biomarkers of antioxidant capacity in the hydrophilic and lipophilic compartments of human plasma. *Arch Biochem Biophys* 2004; 430: 97-103.
84. Romay C, Pascual C, Lissi EA. The reaction between ABTS radical cation and antioxidants and its use to evaluate the antioxidant status of serum samples. *Braz J Med Biol Res* 1996; 29: 175-83.
85. Habdous M, Herbeth B, Vincent-Viry M, et al. Serum total antioxidant status, erythrocyte superoxide dismutase and whole-blood glutathione peroxidase activities in the Stanislas Cohort: influencing factors and reference intervals. *Clin Chem Lab Med* 2003; 41: 209-15.
86. Carratelli M, Porcaro R, Ruscica M, De Simone E, Bertelli AAE, Corsi MM. Reactive oxygen metabolites (ROMs) and prooxidant status in children with Down's syndrome. *Int J Clin Pharmacol Res* 2001; 21: 79-84.
87. Trotti R, Carratelli M, Barbieri M, et al. Oxidative stress and thrombophilic condition in alcoholics without severe liver disease. *Haematologica* 2001; 86: 85-91.
88. Cao G, Alessio HM, Cutler RG. Oxygen-radical absorbance capacity assay for antioxidants. *Free Radic Biol Med* 1993; 14: 303-11.
89. Franzoni F, Ghiadoni L, Galetta F, et al. Physical activity, plasma antioxidant capacity, and endothelium-dependent vasodilation in young and older men. *Am J Hypertens* 2005; 18: 510-6.
90. Benzie IFF, Strain JJ. The ferric reducing ability of plasma (FRAP) as a measure of "antioxidant power": the FRAP assay. *Anal Biochem* 1996; 239: 70-6.
91. Dohi K, Satoh K, Ohtaki H, et al. Elevated plasma levels of bilirubin in patients with neurotrauma reflect its pathophysiological role in free radical scavenging. *In Vivo* 2005; 19: 855-60.
92. Apak R, Güçlü KG, Özyürek M, Karademir SE. Novel total antioxidant capacity index for dietary polyphenols and vitamins C and E, using their cupric iron reducing capability in the presence of neocuproine: CUPRAC method. *J Agric Food Chem* 2004; 52: 7970-81.
93. Ellman G, Lysko H. A precise method for the determination of whole blood and plasma sulfhydryl groups. *Anal Biochem* 1979; 93: 98-102.
94. Gualandri W, Gualandri L, Demartini G, et al. Redox balance in patients with Down's syndrome before and after dietary supplementation with alpha-lipoic acid and L-cysteine. *Int J Clin Pharmacol Res* 2003; 23: 23-30.
95. Blankenberg S, Rupprecht HJ, Bickel C, et al; AtheroGene Investigators. Glutathione peroxidase 1 activity and cardiovascular events in patients with coronary artery disease. *N Engl J Med* 2003; 349: 1605-13.
96. Alamdari DH, Paletas K, Pegiou T, Sarigianni M, Befani C, Koliakos G. Novel assay for the evaluation of the prooxidant-antioxidant balance, before and after antioxidant vitamin administration in type II diabetes patients. *Clin Biochem* 2007; 40: 248-54.
97. Iorio EL, Cinquanta L, Pisano R. A diagnostic algorithm to manage oxidative stress. *Australasian J Cosmet Surg* 2006; 2: 26-30.

Received: April 1, 2008

Accepted: September 25, 2008