



The measurement of oxidative stress

*Reasoned and illustrated guide to the global assessment of oxidative stress
by means of the most frequently asked questions (FAQ)*

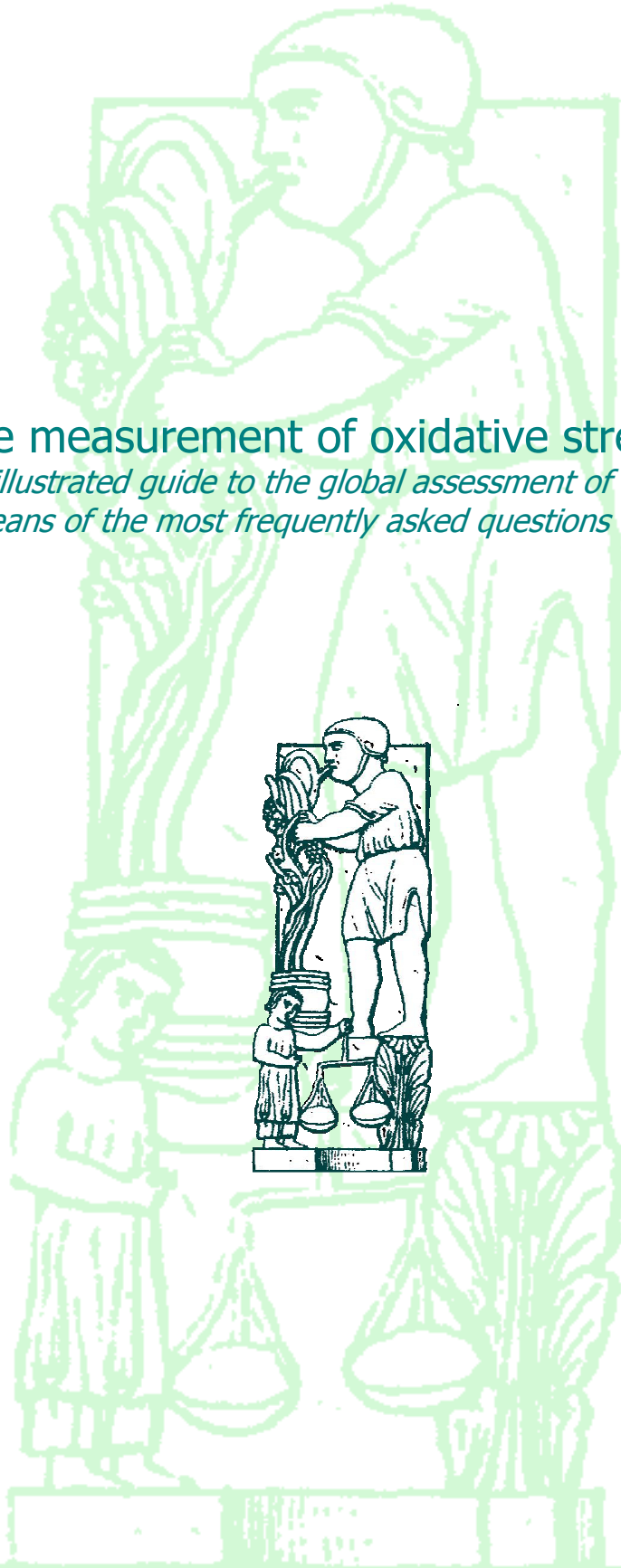






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1. The oxidative stress. A novel health risk.

Definition, pathophysiology, and clinical aspects.

In all living organisms, including Humans, takes place a delicate equilibrium between the production and the elimination – by antioxidant defense system – of the so-called "free radicals". The breaking of this balance, frequently named as "oxidative stress", may induce a cellular damage, with differing degrees of severity, leading ultimately, over time, to early aging and to many diseases

1. What is the oxidative stress?

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

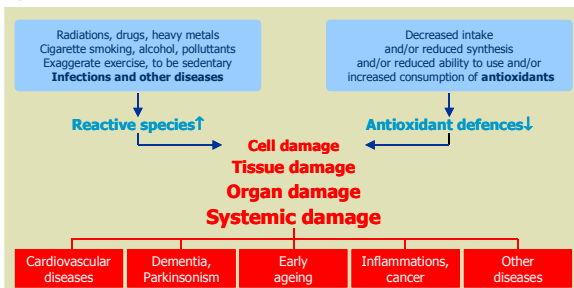


Figure 1. 1. The oxidative stress. The breaking of a balance.

2. What is a free radical?

Free radicals are single or grouped atoms having at least one external orbital "occupied" by one single electron ("unpaired") instead of a couple of electrons ("lone pair") (Figure 1. 2.).

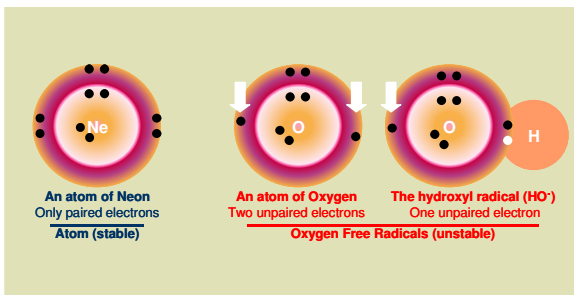


Figure 1. 2. Atoms and radicals.

3. What is an antioxidant?

Antioxidants are chemical or biological agents able to neutralize the potentially damaging action of free radicals (Figure 1. 3.). Some antioxidants (e. g. the enzymes superoxide-dismutase and catalase) are endogenous, i. e. are normal component of the body, while others (e. g. vitamins C and E) are exogenous, and must be

taken by the external environment, e. g. by means of fruits and vegetables.

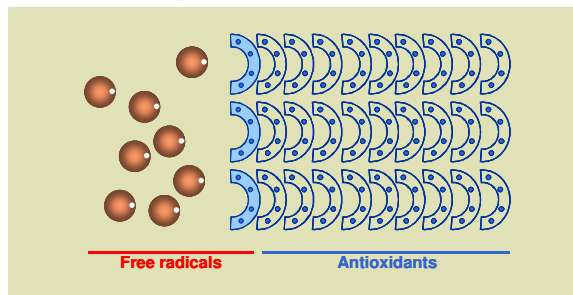


Figure 1. 3. Il sistema di difesa antiossidante.

4. What are the causes responsible for an increased production of free radicals?

The body, even in normal condition, produces a defined amount of free radicals, due to the physiological cell metabolism. 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. Other free radicals, such as Nitric Oxide (NO) are fundamental for the homeostasis of the body, because they modulate some important functions, including vascular tone, platelet aggregation, cell adhesion, and so on. According to this point of view, free radicals have been defined as "irreplaceable journey companions" of cell life. The causes believed to be responsible for an increased production of free radicals can be of different origins, e. g. physical, chemical and biological nature (Table 1. 1.).

Table 1. 1. Causes of increased production of free radicals

Etiology	Examples
Environment factors	Radiations, pollution
Physiological status	Pregnancy (?)
Life-style	Bad food, alcohol, cigarette smoke, inadequate exercise
Psychological factors	Emotional stress (?)
Diseases	Trauma, inflammation, infection, vascular diseases, cancer
Iatrogenic factors	Drugs, radio-therapy, radiological exams

5. What are the causes responsible for a reduced antioxidant defense?

In healthy condition the body is able to prevent free radicals because of the natural defense system of antioxidants, which name indicates the ability of these agents to counteract the oxidant action of free radicals (see below). A reduced effectiveness of such a system is substantially ascribable to an absolute or relative deficiency of antioxidants, independently of the involved mechanism (Table 1. 2.).

Table 1. 2. Causes of reduced antioxidant defenses

Etiology	Examples
Reduced intake of AO	Hypovitaminosis, monotonous diet
Reduced absorption of AO	Malabsorption syndromes, celiac disease
Reduced ability to utilize AO	Uptake and/or carrier deficiency
Deficiency of enzymatic AO	Genetic and/or iatrogenic factors

Abnormal consumption of AO	Abnormal production of oxidant species
Drugs intake/abuse	Microsomal overload
Diseases	Various
AO: antioxidants	

6. Why free radicals are potentially dangerous?

Free radicals are potentially dangerous because they have the spontaneous tendency to fill their unfilled external orbital with a second electron. Indeed, the presence of two electrons in the same orbital is the condition of maximal energetic stability. Therefore, when a free radical is near 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 (*Figure 1. 4*).

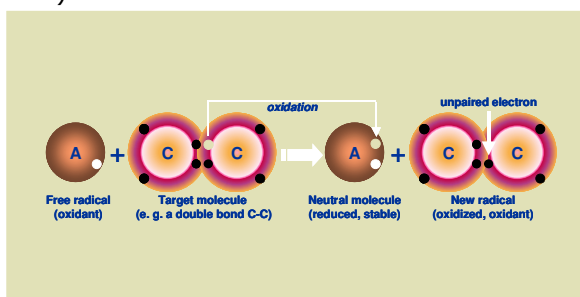


Figure 1. 4. The "oxidant" action of free radicals.

Due to this effect – "oxidant action" – the original free radical loses its potential dangerousness whilst the newly generated molecule is damaged and, in turn, it 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, nucleic acids and so on ("chain-effect").

7. What is the most common mechanism by which free radicals induce the typical molecular and cellular abnormality of oxidative stress?

One of the most common mechanisms by which free radicals, after overcoming the antioxidant defenses, attack the biochemical components of our body is the generation of so-called "hydroperoxides" (*Figure 1. 5*).

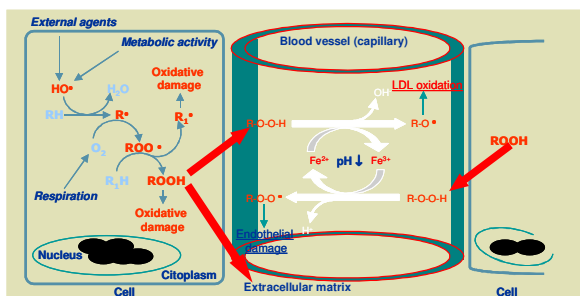


Figure 1. 5. The model of tissue damage by hydroperoxides

In this pathophysiological model, the cell, due to exogenous stressors (physical chemical and biological agents) and/or to its metabolic activity (particularly into the plasma membrane, the mitochondria, the endoplasmic reticulum and cytosol) starts to produce increasing amounts of free radicals, among which there is the very powerful hydroxyl radical (HO•), one of the most potentially dangerous reactive oxygen species (ROS).

Indeed, hydroxyl radicals can "attack" every kind of molecule (including carbohydrates, lipids, amino acids, peptides, proteins, nucleotides, nucleic acids and so on). As the consequence of this action, every molecule loses an electron and becomes, in turn, a radical.

Therefore a radical chain reaction starts, leading, in the presence of molecular oxygen (by respiration), to the generation of hydroperoxides (ROOH), a class of Reactive Oxygen Metabolites (ROMs). Although hydroperoxides are relatively stable chemical species, they have the potential to generate again free radicals and to oxidize other molecular targets. For this reason the cell pulls out the hydroperoxides in the external environment, i. e. in the extracellular matrix and finally in the extracellular fluids, including the blood, cerebro-spinal fluid, pleural fluid and so on.

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 anaerobic metabolism with the releasing into the small blood vessels of acidic metabolites, including lactate. The consequent lowering of pH may induce a conformational change of transition metal-carrier protein, including transferrin and ceruloplasmin. In turn, the low-pH induced conformational change of transferrin induces the release from the carrier of iron, which finally acts as a catalyst in the so-called Fenton's reaction, where hydroperoxides are broken into alkoxyl (RO•) and hydroperoxyl (ROO•) radicals. Both radicals are able to oxidize either the endothelium surface or the circulating lipoproteins, thus favoring the atherosclerosis.

It is evident 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 the ability to circulate in the extracellular fluids).

8. Are there possible relationships between the biochemical abnormalities of oxidative stress and the clinical picture?

The activation of specific cellular sites (plasma membrane, mitochondria, endoplasmic reticulum and cytosol) can be related to a specific pathophysiological, hence clinical pattern (*Table 1.3*).



Table 1. 3 Main pattern of oxidative stress (OS)

OS*	Site [†]	Mechanism [†]	ROS/ROMs	Relationships
I	Plasma	Generation of arachidonic acid	Hydroperoxides Superoxide anion	Reactive processes (inflammation)
	Membrane	NADPH oxidase activation	Superoxide anion	Reactive processes (inflammation)
II	Mitochondria	Metabolic activation	Superoxide anion Hydrogen peroxid	Hyperfeeding, inadequate exercise
		Mitochondrial dysfunction	Superoxide anion Hydrogen perox	Mitochondrial diseases (primary/secondary)
III	Microsomes	Cytochrome P ₄₅₀ /b ₅ activation	Various	Alcohol/drugs abuse, xenobiotics
IV	Cytosol	Xanthine oxidase activation	Superoxide an. Hydrogen perox	Ischemia-reperfusion damage
V	>1 of above	Multiples	Different kinds [‡]	Smoke, pollution, radiations

*I: OS by reactive changes of cell surface; II: OS by reduced effectiveness of cell respiration; III: OS by pharmaco-metabolic induction; IV: OS by intracellular changes of pO₂; V: OS by multiple mechanisms; [†] Prevalent. [‡]Charbon, Nitrogen, Chlorine, etc.

9. How does the oxidative stress clinically appear?

The oxidative stress, being a merely biochemical condition, generally doesn't exhibit any specific clinical symptoms nor clinical signs.

Therefore it will remain unknown, with unavoidable damage to the patient, until the clinician suspects its existence and decides to perform on the patient specific biochemical tests, i. e. the d-ROMs test and the BAP test.

Table 1. 4. reports the most common diseases which, according to the available literature, are associated with a condition of oxidative stress.

Table 1. 4. Human diseases most frequently associated to the oxidative stress

1. Aceruloplasminemia	38. Dislipidemia	76. Parkinsonisms
2. Acute and chronic alcoholic liver diseases	39. Down's syndrome	77. Parkinson's disease
3. Acute autoimmune myocarditis	40. Eclampsia	78. Periodontal disease
4. Acute chest syndrome of sickle cell disease	41. End-stage renal disease	79. Peritoneal dialysis
5. Acute pancreatitis	42. Erectile dysfunction	80. Photoageing
6. Acute Respiratory Distress Syndrome	43. Friedreich ataxia	81. Preeclampsia
7. Alcoholic liver disease	44. Heart failure	82. Primary biliary cirrhosis
8. Alzheimer's disease	45. Helicobacter pylori infection/inflammation	83. Professional broncopulmonary diseases
9. Amyotrophic lateral sclerosis	46. Hemodialysis	84. Progeria
10. Arterial/systemic hypertension	47. Hepatic cirrhosis	85. Psoriasis
11. Asbestosis	48. Human Immunodeficiency Virus infection	86. Psoriatic arthritis
12. Asthma	49. Huntington disease	87. Pulmonary hypertension
13. Ataxia telangiectasia	50. Hyperbaric diseases	88. Radio-therapy side effects
14. Atherosclerosis	51. Hypercholesterolemia	89. Reactive arthritis
15. Atopic dermatitis	52. Hyperhomocysteinemia	90. Renal cell carcinoma
16. Brain ischemia	53. Hyperlipidemia	91. Respiratory distress syndrome
17. Bronchopulmonary dysplasia	54. Idiopathic pulmonary fibrosis	92. Retinopathy of prematurity
18. Burns	55. Interstitial lung disease	93. Retrolenticular fibroplasy*
19. Cancer (several kinds)	56. Ischemia/Reperfusion injury	94. Rheumatic disease
20. Cardiopulmonary bypass	57. Juvenile chronic arthritis	95. Rheumatoid arthritis
21. Cardiovascular diseases	58. Kidney transplantation	96. Sarcoidosis
22. Cataract	59. Leukaemia	97. Sepsis
23. Cellulitis	60. Lung cancer	98. Sickle cell disease
24. Chemotherapy side-effect	61. Lung injury	99. Sleep apnea
25. Chronic fatigue syndrome	62. Macular degeneration	100. Spherocytosis
26. Chronic hepatitis C	63. Male infertility	101. Spinal cord injury
27. Chronic kidney disease	64. Ménière's syndrome	102. Stroke
28. Chronic Obstructive Pulmonary Disease	65. Meningitis	103. Synucleinopathies
29. Chronic renal failure	66. Mild cognitive impairment	104. Systemic amyloidosis
30. Colitis	67. Multiple sclerosis	105. Systemic lupus erythematosus
31. Coronary artery disease	68. Myelodysplastic syndromes	106. Systemic sclerosis (scleroderma)
32. Creutzfeldt-Jakob disease	69. Myocardial infarction	107. Thrombophily
33. Crohn disease	70. Myocarditis	108. Tauopathies
34. Cutaneous leishmaniasis	71. Neonatal bronchopulmonary dysplasia	109. Tuberculosis
35. Cystic fibrosis	72. Obesity	110. Unstable angina
36. Diabetes mellitus type 1	73. Osteoarthritis	111. Uremia
37. Diabetes mellitus type 2	74. Osteoporosis	112. Venous insufficiency
*Neonatal cataract	75. Pancreatitis	113. Werner syndrome
		114. Zellweger syndrome



Glossary

Alkoxy radical	Highly reactive oxygen-centered radical (RO [•]), generally deriving from the iron-catalyzed hydroperoxide breakdown.
Antioxidant	Every agent able to reduce the level or the activity of an oxidant agent.
Free radical	Atom or grouped atoms having one or more unpaired electrons in an external orbital.
Homeostasis	Ability of a living organism to adapt itself with a specific response to the changing environment conditions with the aim to maintain constant its features (e. g. metabolism activation with generation of energy when the external temperature is low in order to maintain the body integrity) .
Hydrogen peroxide	Non radical reactive oxygen specie (ROS), belonging to the peroxides (H ₂ O ₂).
Hydroperoxide	Reactive oxygen metabolite (ROM), having as general formula R-OOH, which is generated in living organisms and cells by the oxidation of a wide class of organic compounds (glucosides, amino acids, peptides, proteins, lipids, nucleotides, nucleic acids, and so on); according to this point of view every hydroperoxide is a reliable witness and therefore a suitable marker of oxidative damage; on the other hand, because every hydroperoxide can undergo the breakdown to free radicals (alkoxy, RO [•] , and hydroperoxy, ROO [•] radical) in the presence of transition ion metals (iron or copper), it is obvious that hydroperoxides are also a dangerous systemic amplifier of tissue oxidative damage.
Hydroxyl radical	Highly reactive oxygen-centered radical (HO [•]), generally deriving from the iron-catalyzed hydrogen peroxide (H ₂ O ₂) breakdown or the radiation-mediated water photolysis; it is one of the most dangerous and powerful oxidant in biological systems.
Lipoperoxide	Reactive oxygen metabolite (ROM), having as general formula L-OOH, which is generated in living organisms and cells by the oxidation of a lipid (L), generally unsaturated fatty acid.
Orbital	Area around the atom nucleus where there are a generally high, pre-determined probability to find an electron.
Oxidant or reactive chemical species	Generally unstable (=reactive) chemical species showing the ability to pull out one or more electrons/hydrogen atoms (reducing equivalents) from an organic substrate (e. g. amino acid, protein and so on); they are believed responsible of the so-called oxidative stress.
Oxidant	Every chemical agent able to acquire one or more electrons or hydrogen atoms (reducing equivalents) from another chemical specie (reducing agent).
Oxidation	The transfer of one or more electrons or hydrogen atoms (reducing equivalents) from a chemical specie (oxidant species) to another chemical specie (reducing agent).
Oxidative stress	Pathological condition due to an unbalance between the production and the elimination, by the antioxidant defense system, of reactive/oxidant chemical species; it is associated to more than one hundred diseases and it is considered an emerging health risk factor.
Peroxide	Reactive oxygen metabolite (ROM), having as general formula R-OO-R (two bonded oxygen atoms between two radicals), which is generated in living organisms and cells by the oxidation of a wide class of organic compounds; depending on the nature of the radical (R-) we distinguish two different kind of peroxides, i. e. hydroperoxides (R-OO-H) and lipoperoxides (L-OO-H).
Peroxy radical	Highly reactive oxygen-centered radical (ROO [•]), generally deriving from the iron-catalyzed (hydro)peroxide breakdown.
Plasma membrane	A synonymous of cell membrane, i. e. the membrane around the cell.
Reactive Oxygen Metabolites	A secondary class of oxidant/reactive chemical species, centered on the oxygen, deriving from reactive oxygen species; they include also the peroxides; however, according to some Authors ROMs is synonymous of ROS.
Reactive Oxygen Species	A primary class of oxidant/reactive chemical species, centered on the oxygen, which include either radical species (e. g. hydroxyl radical) or non-radical species (e. g. ozone); however, according to some Authors ROS is synonymous of ROMs.
Reducing equivalent	Chemical entity (electron or hydrogen atom) which constitutes the essence of the oxidative processes; indeed, the transfer of one or more reducing equivalent from a chemical specie (oxidant) to another specie (reducing) is named oxidation.
Reducing	Every chemical agent able to give one or more electrons or hydrogen atoms (reducing equivalents) to another chemical specie (oxidant agent).
Reduction	Acquisition of one or more electrons or hydrogen atoms (reducing equivalents) from a chemical specie (oxidant specie).
Scavenger	A kind of antioxidant able to inactivate directly the oxidant action of a free radical.
Transition metal	A metal belonging to a series between the II and the III group of the Periodic Table of Elements; transition elements, although having small differences in the penultimate energy level, show the same external energy level (two electrons) so that they share very similar properties in each series; biologically relevant transition metals are the iron (which exists as a ferrous or reduced form, Fe ²⁺ , and a ferric or oxidized form Fe ³⁺) and the copper (which exists as a rameous or reduced form, Cu ¹⁺ , and a rameic or oxidized form Cu ²⁺); both the metals, when available as free ions can catalyse the hydroperoxide breakdown to the alkoxy/hydroperoxy free radicals
Unpaired electron	Every orbital around the atom nucleus can host up to electrons, and this condition corresponds to the maximal stability; when in an orbital one single electron is present, such an electron is called "unpaired" and it confers to its atom ("free radical") a condition of instability or reactivity; indeed, every unpaired electron trends to complete a couple in its orbital (lone pairs) and therefore it will try "to pull up" an electron (or a reducing equivalent) from every chemical species having free electrons (e. g. a double bond between two carbon atoms, like in unsaturated fatty acids).
Unsaturated fatty acid	An organic acid having a long chain with one (monounsaturated, like oleic acid) ore more (polyunsaturated, like arachidonic acid) double bond between two atoms of carbon; the double bond, having two exposed electrons, is a preferential target of oxidant/reactive species, like free radicals



2. The oxidative stress. General diagnostic aspects.

The diagnosis of oxidative stress is exclusively based on the execution of specific biochemical tests, which must be able to demonstrate the imbalance between the production and elimination of free radicals in the body, which is the basis of the pathological condition.

10. With the aim of making a correct diagnosis, can oxidative stress be considered as a common disease?

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

11. Can the classical laboratory tests, e. g. total cholesterol, lipoproteins, Erythrocyte Sedimentation Rate/Velocity (ESR/ESV), C-Reactive Protein (CRP), uric acid, serum albumin, and so on, allow the clinician to establish a diagnosis of "oxidative stress"?

Over the last decades many biochemical tests have been proposed with the aim to have an idea, although indirect, of oxidative balance; however such tests proved to be unsuitable "surrogates". Among the proposed markers of oxidant status, the blood level of total cholesterol is a good marker of cardiovascular risk but it is not necessarily associated to oxidative stress: in an apparently paradoxical way some subjects having normal blood total cholesterol can exhibit an increased level of free radicals. Indeed the "good" cholesterol becomes "bad" cholesterol when it is oxidized by circulating oxidizing agents (e. g. alkoxyl and hydroperoxyl radicals from the hydroperoxides breakdown). Therefore, the classical distinction between "good cholesterol" (i. e. the cholesterol associated to High Density Lipoproteins, HDL) and "bad cholesterol" (i. e. the cholesterol associated to Low Density Lipoproteins, LDL) seems to have no sense. In fact, either HDL or LDL may undergo oxidative processes thus transforming both into "bad cholesterol", i. e. the oxidized cholesterol, the truly responsible agent of the atherogenic process. Both Erythrocyte Sedimentation Rate/Velocity (ESR/ESV) and C-Reactive Protein (CRP) are reliable markers of inflammation, a classical condition associated to increased rates of free radicals. However, normal values of ESR/ESV and/or CRP don't exclude a current condition of oxidative stress. Among the proposed markers of

antioxidant status, the uric acid, although recognized as a powerful antioxidant, cannot be believed solely as a reliable marker of the blood antioxidant defenses function, because the so-called plasma barrier to oxidation includes many agents (e. g. vitamin C, vitamin E, carotens, dietary polyphenols and bioflavonoids, and so on). The same concept is valid for serum albumin, although this protein exhibits an important function of the "shock-absorber" against free radicals generated in the blood. Therefore the above common laboratory tests appear inadequate and not sufficient to allow the clinician to make a diagnosis of oxidative stress. Of course such common tests become useful to the clinicians after the diagnosis of oxidative stress in order to identify either the cause or the mechanism mainly involved in the increased production of free radicals.

12. Can the widely diffused tests for food intolerance provide some information about the presence of a condition of oxidative stress?

The common tests for food intolerance – often devoid of any scientific basis – don't provide any valid information about the existence of a specific condition of oxidative stress.

13. What is the most specific and reliable method, as absolute, to demonstrate, in a living organism, the presence of free radicals and their amounts?

The elective method to measure free radicals in a living organism is the electron (spin) magnetic resonance spectrometry (EPR or ESR). Unfortunately, this method involves a very complex technique and some specific professions which are not available in common laboratories. Moreover the ESR is an expensive technique. Because of the above reasons, ESR is employed not for routine studies nor for screenings but rather for research purposes and, in particular, to validate other laboratory methods proposed for the oxidative stress evaluation (golden standard). Finally, the ESR, correctly performed, provides a direct information only on the free radical species whilst the oxidative stress is the consequence of a broken balance between pro- and anti-oxidant factors.

14. What is the general principle of the available tests, except the ESR, to evaluate specifically the oxidative stress?

Most specific tests for oxidative stress evaluation are based on the general principle according to which the imbalance between the production and the elimination of free radicals becomes evident in the body due to an increased concentration/activity of a number of compounds derived by the oxidant attack (e. g. hydroperoxides levels, glutathione peroxidase activity) and/or due to a decreased concen-



tration/activity of one or more components of the antioxidant system (vitamins, minerals, enzymes) in the tissues and/or extracellular fluids.

In particular, the otherwise routinely undetectable presence of free radicals in a living organism is evaluated by means of evidence (in the tissues and/or extracellular fluids) of molecular species which have been variously modified by the oxidative attack. Because peroxidation is the most common mechanism involved in free radical induced oxidative damage, the dosing of blood hydroperoxides (ROOH) provides very reliable information on the severity of the oxidative damage undergone by the body, i. e. the more or less faithful "fingerprint" of the oxidant component of the oxidative stress of every body.

On the other hand, the generation of the rust – the transition of the iron from the ferrous (Fe^{2+}) to the ferric (Fe^{3+}) state – represents one of the most common natural oxidative processes and iron is normally present in the body. Therefore, the ability of a plasma sample to lead again a solution of this transition metal from the ferric to the ferrous state can be clearly assumed as a measure of the antioxidant capacity of the biological sample to be tested.

15. What are the specific tests currently available on the market to evaluate oxidative stress?

On the basis of the above concepts, the presently available laboratory tests evaluate either the oxidant component (free radical production) or the antioxidant component (antioxidant activity) of the oxidative stress (*Table 2. 1*).

Table 2. 1. Common methods to evacuate the oxidative stress.

Pro-oxidant Status	Anti-oxidant Status
d-ROMs test	BAP test
TBAR test (MDA)	OXY-Adsorbent test
Liperoxide assay (LPO)	Total antioxidant status (TAS)
Isoprostanos dosing	-SHp test
Chemiluminescence	Dosing of single oxidants

Of course, not all the available tests have the same scientific valence. Indeed one should distinguish the so called "first-line" methods from the "second-line" methods and to select further, among antioxidant measurements, tests to evaluate intracellular changes (e. g. enzymatic assays, like for glutathione peroxidase) and tests to evaluate extracellular changes (e. g. vitamins dosing).

16. In order to provide a reliable assessment of oxidative stress, is it preferable to perform the tests on blood or on urine?

Generally, it is always preferable to perform the tests on blood because the by-products from cell oxidation accumulate primarily in this extracellular fluid and it is the location of the first antioxidant barrier. The passage of biochemical "markers" from blood to urine involves often further changes

which cannot be a direct consequence of the primary oxidative damage, so that such potential by-products in urine can lose at least partially the original significance of reliable oxidative stress biomarkers. Moreover, among the commercially available tests on urine, some methods are very rough (e. g. "visual" evaluation of the oxidant concentration by the color intensity as developed after adding of a reactant), some are at the moment not sufficiently specific (e. g. immune dosing of urinary isoprostans) while some are the expression of late damage (e. g. MDA-TBAR). Finally, the blood sampling is generally less problematic and more accepted by the patient compared to the urine pooling which involves the care of a whole day (dosing on a 24 hours-sample).

17. What are the main features of an "ideal test" to evaluate oxidative stress?

The "ideal" test to assess oxidative stress should be adequately validated by comparison with other reference methods which have been universally recognized by the scientific community. Among the main requisites, such "ideal test" should have sufficient levels of sensitivity, specificity and precision. It should be an expression of sufficiently stable markers, able i) to allow an accurate evaluation of the oxidative stress level; ii) to provide reliable information of an early stage of a disease; iii) to anticipate the progression or the worsening of the diseases during a systematic monitoring; iv) to change with an adequate sensitivity according to eventual specific treatments for the basic diseases or antioxidant supplementation. Finally, the ideal test should be based on minimally invasive procedure, well accepted by the patient, quick and easy to perform, with an optimal costs/benefits ratio.

18. What are the general criteria to be followed in the choice of the most adequate test – in the actual commercial panorama – for oxidative stress assessment?

In the choice of the most adequate test, it is proper that the assessment of oxidative stress be "global", i. e. able to evaluate both the pro-oxidant and the anti-oxidant component. Therefore one should select at least two different assays. The first one to measure the level of free radical production and the second one to measure the antioxidant capacity or potential.

19. What is the panel of tests which are showing particularly usefulness in the routine assessment of oxidative stress?

Among the currently available tests on the market, the International Observatory of Oxidative Stress, Free Radicals and Antioxidant Systems, after an accurate analysis of the scientific literature and research on the diffusion of the proposed methods in the clinical practice, has identified in the panel of the Italian Chemist Mauro Carratelli one of the most suitable and powerful diagnostic tools to quantify



free radicals and antioxidant capacity, which up until now has been developed for routine clinical use. The "Carratelli's panel" involves the photometric quantification of both reactive oxygen metabolites (d-ROMs test) and plasma antioxidant barrier (BAP test, OXY-Adsorbent test and -SHp test) on biological samples (when required, whole blood, blood plasma, blood serum, tissues/cells extracts). Such tests can be performed with a multiple analyzer (automated procedure) too.

20. What is the most innovative aspect of the d-ROMs and BAP tests?

The most interesting and innovative aspect of these tests is in the fact that can be performed by means of the easy-to-use dedicated instrument FRAS system (developed by H&D s.r.l., Parma, Italy) which has been invented by Dr. Fabrizio Callegari.



3. The assessment of oxidant status: the d-ROMs test.

The concept of "global evaluation of oxidative stress" implies firstly an accurate measurement of the level of free radicals produced in the body. As a matter of fact, free radicals are only a part of all the chemical species responsible of oxidative stress. Oxidative stress is induced by other agents indeed, like hydrogen peroxide or hypochlorous acid, which are not free radicals. Therefore all chemical species – radical and not radical species – which are responsible of oxidative stress have been grouped in a unique large family of "reactive or oxidant chemical species". On this basis, the first step of the "global assessment of oxidative stress" requires the measurement of the global level of oxidant chemical species – oxidant or pro-oxidant status – a goal that can be reached thanks to the d-ROMs test.

21. What is the d-ROMs test?

The d-ROMs test is a photometric test, e. g. a test which can be performed in a laboratory by means of an analytical instrument called "photometer". For ambulatory and routine measurements, the d-ROMs test is proposed with the FRAS system, which includes both the photometric device and an incorporated and thermostated mini-centrifuge, in order to allow safely the separation of plasma/serum from blood cells.

22. What does the d-ROMs test measure?

The d-ROMs test allows for measuring, substantially, the blood concentration of hydroperoxides (ROOH), a class of chemical oxidant species belonging to the wide family of Reactive Oxygen Metabolites (ROMs).

23. What is a hydroperoxide?

The hydroperoxides are relatively stable chemical species which are generated by the oxidation of a wide class of organic biologically relevant molecules (e. g. glucosides, lipids, amino acids, peptides, proteins, nucleotides, and so on). In turn the hydroperoxides, in some particular conditions (e. g. in the presence of free iron) can generate free radicals. Because of this behavior the hydroperoxides are considered not only as the "witnesses" of oxidative damage but also as specific "marker" of oxidative damage and of course as potential "amplifier" of tissue damage (due to their potential to generate again free radicals).

24. On which kind of biological sample can the d-ROMs test be performed?

The d-ROMs test can be performed on samples from whole blood (generally capillary blood, as driven by finger-puncture, or venous blood), on serum blood, on heparinated plasma blood and on some extracellular fluids.

25. Is the use of anticoagulants allowed during the drawing of the blood?

Heparin only is allowed. The use of chelating agents, including ethylene diamine tetraacetate (EDTA) or citrate, is forbidden because they interfere with the chemical reactions of the test thus leading to an underestimation of the results.

26. For how much time, after the drawing, can the fresh blood be stored before undergoing the d-ROMs test?

The time is approximately one-to-two hours, of course by using heparin as only anticoagulant, according to the "laboratory good practice" for whole blood samples. During this interval – which should be as short as possible – it is mandatory to store the sample to adequate and constant temperature and to avoid any trauma to the tube. In fact both the above factors can induce an haemolysis with consequent underestimation of the results.

27. Can the d-ROMs test be performed on previously frozen serum/plasma samples and, eventually, after repeated thawing cycles?

According to the published data, the d-ROMs test can be performed on previously frozen serum/plasma heparinised blood samples, even after repeated thawing cycles, without significant changes in the analytical suitability.

28. On what kind of biological samples CAN NOT the d-ROMs TEST BE PERFORMED?

The d-ROMs test CANNOT BE performed on urine.

29. What is the most common procedure for performing the d-ROMs test?

The most common procedure for performing the d-ROMs test involves i) the dilution of a small amount (20 microliters) of whole (capillary) blood (as driven by finger puncture) in an acidic buffered solution; ii) the adding of an oxidizable colorless aromatic ammine in water solution (chromogen mixture); iii) the centrifugation of the final solution (in order to allow the separation of serum/plasma blood from the (red) cells); iv) the measurement by a photometer of the absorbance change (as the expression of the change of the color of the chromogen from colorless to pink per minute).

A variant of this procedure involves the adding firstly of the acidic buffered solution; and secondly of the sample blood to a tube where the chromogen has been previously condensed at the bottom of the cuvette (d-ROMs test CON).

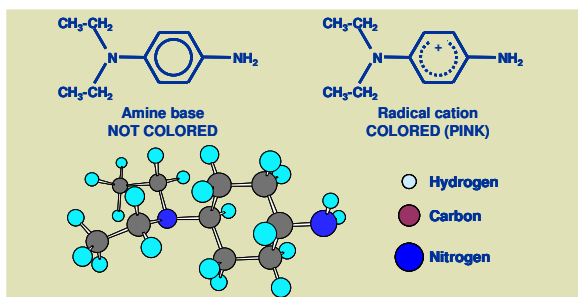


Figure 3. 1. The two forms of the d-ROMs test chromogen.

30. Does there exist some particular "stratagem" to avoid some common errors during the execution of the d-ROMs test?

The Producer makes available a specific document ("Hints for knowing and for avoiding the most common causes of error with FRAS") approved by The International Observatory of Oxidative Stress, Free Radicals and Antioxidant Systems, which depicts how to prevent and to correct the most common errors that the operator can make before (pre-analytical step), during (analytical step) and after (post-analytical step) the d-ROMs test execution.

31. What is the principle of the d-ROMs test?

The d-ROMs test is based on the application "in vitro" (in a tube) of a phenomenon that occurs "in vivo" (in the microcirculation) (Figure 3.1.). In fact, the dilution of the blood in an acidic buffered solution (R_2 reagent) 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 hydroperoxides (ROOH) breakdown with generation of free radicals (RO^\bullet , alkoxy, and ROO^\bullet , hydroperoxyl radicals). When the colorless oxidizable chromogenic mixture (R_1 reagent, the aromatic amine N,N-diethylparaphenyldiamine) is added to this solution, the highly unstable newly generated free radicals (RO^\bullet and ROO^\bullet) pull out an electron from the aromatic amine, which becomes a colored radical cation. This later one is relatively stable and can be detected and measured (Figure 3. 2).

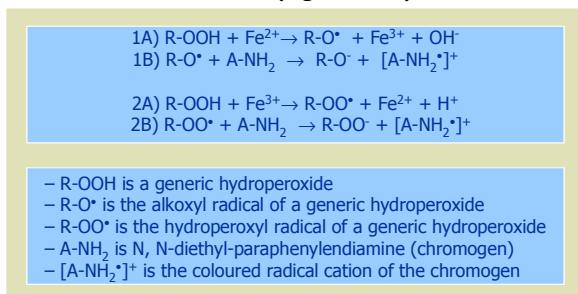


Figure 3. 2. The biochemical principle of d-ROMs test.

Because the chromogen is originally colorless and it becomes pink-to-red when it releases an electron, by the pink color intensity (proportional to the radical cation concentration) it is possible, by a photometer (calculation of the absorbance change

at 505 nm, Δ_{abs}/min), to evaluate the concentration of free radicals and hence the concentration of hydroperoxides initially present in the biological sample (of course by using an adequate biochemical standard, i. e. a control serum provided by the producer with known value).

32. What is the evidence of the above proposed mechanism of the d-ROMs test? In other words, with which analytical technique has the d-ROMs test been validated?

The evidence showing that the d-ROMs test is a reliable and suitable assay to effectively measure the blood hydroperoxides has been provided since 1997 by the Italian National Council of Research (CNR), by validating the test with the Electron Spin Resonance Spectrometry, which is the technique universally recognized as the "golden standard" technique to study "in vitro" the radical species, by Alberti and coworkers (http://www.isof.cnr.it/radpol/personale/alberti_ita.pdf).

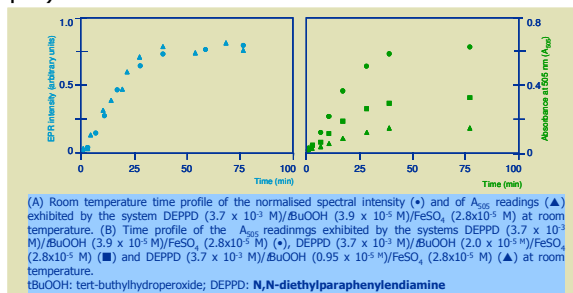


Figure 3. 3. Conclusive evidence of the d-ROMs test validation by means of the Electron Spin Resonance (ESR) Spectroscopy: the radical cation of N,N-diethylparaphenyldiamine, responsible for the ESR spectrum, is responsible also of the absorbance in the visible at 505 nm as detected by the photometrical way.

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 entirely overlapped to that one obtained by following, in parallel, the course of the same reaction by a photometer (Figure 3. 3).

33. Is it possible that the photometric signal (i. e. the change of absorbance at 505 nm) as developed during the d-ROMs test can be attributable to other oxidant agents/activities, besides alkoxy and hydroperoxyl radicals in turn generated by the iron-dependent hydroperoxides breakdown?

The pre-treatment of the serum sample with chelating agents, like ethylene diamine tetraacetate (EDTA), by making unusable for the catalysis, the iron, is followed by a significant reduction but not by the zeroing of the ESR/photometric signal. This experimental datum indicates that, at least a part of the absorbance change at 505 nm, as detected by performing the d-ROMs test, is not all due to hydroperoxides, but also to other oxidizing chemical

species and/or enzymatic activity. For instance, the so-called chloroamines, which are believed to be reliable markers of the hypochlorous induced oxidative damage on peptidic/proteic amine groups, can contribute to the absorbance change of d-ROMs test. Moreover, since the pre-treatment of serum sample 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-diethylparaphenyldiamine apparently due to the ceruloplasmin at low pH. By indicating the possibility that the d-ROMs test can measure more than one class of oxidants, in turn derived from different metabolic pathways, these findings reinforce the clinical significance of the d-ROMs test as a reliable method able to provide a global and suitable measure of the "total" serum oxidant status.

34. How are the results of the d-ROMs test expressed and which is their normal range?

The absorbance change per minute at 505 nm ($\Delta A_{505/min}$), as observed by performing the d-ROMs test on serum of a sample of about 5,000 apparently healthy subject, showed a Gauss-like distribution (Figure 3. 4).

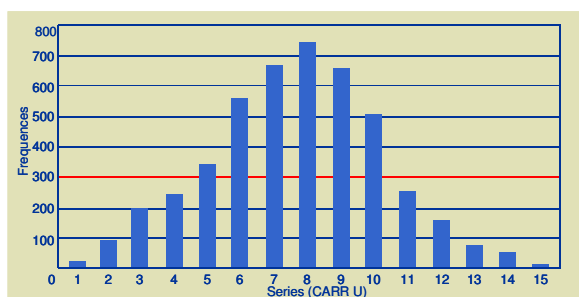


Figure 3. 4. The distribution of d-ROMs test values in a wide sample (n=4547) of apparently healthy peoples (Tuscany, Italy).

On this basis, values of absorbance ($\Delta A_{505/min}$) between 0.025 and 0.030 has been assumed as the reference interval of the test in the "normal" population (Table 3. 1).

Series	Intervals (CARR U)	Intervals (mg H ₂ O ₂ /dL)	Frequencies (n)	Cumulative (%)
-	(CARR U)	(mg H ₂ O ₂ /dL)	(n)	(%)
1	200-210	16.00-16.80	29	0.6
2	211-220	16.88-17.60	89	2.6
3	221-230	17.68-18.40	193	6.8
4	231-240	18.48-19.20	244	12.2
5	241-250	19.28-20.00	342	19.7
6	251-260	20.08-20.80	547	31.8
7	261-270	20.88-21.60	659	46.3
8	271-280	21.68-22.40	731	62.3
9	281-290	22.48-23.20	654	76.7
10	291-300	23.28-24.00	491	87.5
11	300-310	24.08-24.80	256	93.1
12	311-320	24.88-25.60	162	96.7
13	321-330	25.68-26.40	80	98.5
14	331-340	26.48-27.20	57	99.7
15	341-350	27.28-28.00	13	100.0
Total			4547	100.0

Table 3. 1. The d-ROMs test values in a sample of adult peoples clinically healthy.

Of course, in order to have an adequately wide range of measure, the $\Delta A_{505/min}$ value is automatically multiplied by the analyser for a correction factor (~ 10.000), thus generating the measure units of the test, which are expressed as CARR U, i. e. CARRATELLI UNITS.. This justifies the range of normality definitively established as between 250 and 300 CARR U.

35. What is the equivalent of ONE CARR U?

One CARR U is equivalent to 0.08 mg/dL of a hydrogen peroxide water solution.

36. What is the significance of the CARR U of the d-ROMs test?

The CARR U are substantially the "label" or the "brand" to recognise the d-ROMs test and, concomitantly, the highest assumption of responsibility by the inventor Mauro Carratelli toward all the users of d-ROMs test. In other words, due to the way it was conceived, the CARR U marks the d-ROMs test versus other tests, including the so-called FORT test, which is an evident and bad copy of the original test.

In this subject, it is important to underline that the CARR U are original non-conventional units, having a precise scientific fundament, which can be converted in a moment to chemical units (it is sufficient to multiply their value for 0.08 to obtain the equivalent results as conventional units e. g. mg/dL of hydrogen peroxide). Other tests tried to copy the principle of CARR U thus generating, for instance, the so-called FORT UNITS, which, however, are neither original nor having any biochemical-clinical correspondence.

37. On the basis of the concept and the definition of "CARR U", a normal serum (300 CARR U) should have 24 mg/dL hydrogen peroxide. But this concentration is not compatible with the life. How this can be explained?

Many Authors and clinical users of the commercially available kits of d-ROMs test, by associating the test to a peroxide measure, found it more convenient to directly indicate the results of the d-ROMs test as the serum hydroperoxides concentration, thus making a conceptual mistake. However, just in order to avoid such a mistake, the producer clearly indicates the results of d-ROMs test as "CARR U" and he specifies that in experiments of calibration 1 CARR U is "equivalent" to 0.08 mg/dL of hydrogen peroxide. Of course this "equivalence" doesn't mean that a normal serum (300 CARR U) really contains or 0.24 mg/dL (7054 $\mu\text{mol/L}$) hydrogen peroxide, a level of peroxides not compatible with the life. On the other hand, the expression of d-ROMs test results as CARR U (which correspondence with chemical units has been established correctly) is potentially useful in clinical practice, at least in comparison with its expression as $\Delta A_{505/min}$



or t-butyl-hydroperoxide concentration, which are an unusual form of common chemical analysis (e. g. blood glucose or cholesterol).

38. Do different d-ROMs test values correspond to different levels of oxidative stress?

It was established that the oxidative stress can exhibit different degrees of severity, according to the results of the d-ROMs test (Table 3. 2.)

Table 3. 2. Severity of oxidative stress depending on the d-ROMs test results

ROM level		Oxidative stress
(CARR U)	(mg H ₂ O ₂ /dL)	(Severity)
300-320	24.08-25.60	Border-line
321-340	25.68-27.20	Low oxidative stress
341-400	27.28-32.00	Middle oxidative stress
401-500	32.08-40.00	High oxidative stress
>500	>40.00	Very high oxidative stress
Normal range: 250-300 CARR U		
1 CARR U is equivalent to 0.08 mg H ₂ O ₂ /dL		

39. Which are the analytical performances of the d-ROMs test?

The results of many studies, even very recent, indicate that the d-ROMs test is a reliable, precise, repeatable, with acceptable within-run and between run coefficients of variation (CV), even with manual procedure (1-3%) (Table 3.3). The lowest limit of sensitivity is estimated to 17 CARR U. The maximal linearity is within the range of 50 to 500 CARR U. The test is not subjected to analytic interferences by most common serum analytes, including triglycerides (up to 28.2 mmol/L), haemoglobin (up to 0.068 mmol/L), bilirubin (up to 171 mmol/L), and so on.

Tested parameters	Low ROMs level sera	High ROMs level sera
Media (mAbs/min)	21.0 ÷ 21.5	28.9 ÷ 29.7
Within run imprecision (%)	1.00 ÷ 1.30	0.73 ÷ 1.75
Between run imprecision (%)	0.67 ÷ 1.28	1.27 ÷ 1.60
Total CV (%)	1.46 ÷ 1.63	1.76 ÷ 2.09

Table 3. 3. Analytical performance of the d-ROMs test in a study as available in the scientific literature.

40. Do the results of the d-ROMs test change depending on the kind of drawing?

The results of the d-ROMs test don't change. Indeed up to now no statistically significant difference has been show among capillary, arterial and venous blood drawing.

41. What is the volume of the blood sample generally required to perform the d-ROMs test?

In the case of whole blood, the d-ROMs test needs only 20 microliters. For serum or plasma, according to the different procedures, also a smaller volume (up to 3 microliter) of sample may be required.

42. Do the results of the d-ROMs test change depending on the age, the gender, the race, or other physiological or physiological-like conditions?

In the absence of a disease, the results of the d-ROMs test don't change depending on the age, with the only exception of the neonatal age; indeed in the first week after the delivery the value of the d-ROMs test in newborns is almost one half of that one of adults (e. g. ~130 CARR U); vice versa, eventual higher value in senescent peoples compared to the adult may reflect the associated morbidities rather than a "physiological" higher oxidant status due to the age. Moreover, the results of the d-ROMs test don't change depending on the gender, although some researchers found higher values in the women compared to the men in some wide series. This is only an apparent difference, because in these series the operators performed the d-ROMs test on a sample of whole (capillary) blood and this led to an overestimation of the results in women, due to the fact that females show a trend to a lower haematocrit and therefore a higher percent of plasma with an apparent higher oxidant status compared to males as measured by means of the d-ROMs test. However, it is real and expected the significant difference found between non-pregnant and pregnant women, with the latter having the highest values of d-ROMs test; this phenomenon is typical not only of Humans but also of other animal species and it was shown that the values of d-ROMs test increase progressively during the pregnancy thus reaching the highest value (up to 900 CARR U) at the moment of the delivery and decreasing thereafter. Some racial differences has been found also, with Afro Americans having higher values and the Oriental people having the lower values compared to Caucasians. Therefore it is always valid the principle that every user should assess his own range of normality in the study population. Finally a "physiological" or "quasi-physiological" increase of d-ROMs test is expected respectively immediately after a physical effort or immediately after alcohol intake.

43. Do the results of the d-ROMs test change during the same day or in the medium-long term?

In the absence of a disease and/or other physiological or para-physiological events able to induce a significant oxidative stress, the results of the d-ROMs test don't change significantly during one day or one week or some months, if the operator follows carefully all the instructions of the producer. Therefore, we can deduce that every individual shows a specific "basal value" or "reference value" which, although variable, is in the range of "normality" (250 to 300 CARR U), with possible "cues" over or under this interval, due to the unimodal Gauss-like distribution of the d-ROMs test



values in the apparently healthy peoples. Taken together these findings lead to the practical conclusion that every person should undergo the d-ROMs test in an apparently healthy condition and to refer to this value in the occasion of further check-up, every time a condition able to induce an oxidative stress has been encountered. In such conditions, the clinician may consider not the absolute value but the relative increase of the d-ROMs test results compared to the previous measurement; for instance if in an athlete the d-ROMs test values increase from 220 CARR U (first determination) to 300 CARR U (second determination) the clinician cannot consider that the second value is in the "normal range" but he should consider that the change (+80 CARR U, about +35% compared the basal value) is significant to suspect a condition of oxidative stress.

44. Does the d-ROMs test have to be performed before meals?

It is preferable to perform the d-ROMs test before meals or at least an adequate interval of time after a copious meal, a massive intake of alcohol or antioxidant vitamins by intravenous route, and a massive physical effort.

45. Besides Humans, does any data exist regarding the "normal range" of the d-ROMs test in other Animal species?

Yes, a large amount of studies allowed to establish the range of normality in several Animal species, including Fishes, Reptiles, Birds and Mammals (Rodents, Cats, Dogs, Swine, Cows, Ovines, Horses and so on). For instance, Dogs show normally one third of the d-ROMs test value of Humans, maybe because Dogs instead of Humans conserved the enzymatic pathway needed to produce the antioxidant ascorbic acid (vitamin C) from glucose. According to the available literature, up to now the highest reported values of d-ROMs test were in Pigs with the lowest in Fishes.

46. What are the general principles the clinician should follow in the interpretation of the results of d-ROMs test?

The general principles to interpret and to manage the results of d-ROMs test are the object of specific Guide-Lines that the International Observatory of Oxidative Stress proposes periodically on the basis of the scientific literature and the clinical practice. Such Guide Lines follow an algorithm that allows the operators i) to interpret easily the results of the d-ROMs test, ii) to reach when possible the goal of a etiological diagnosis and iii) finally to prescribe the right treatment. All the Guide Lines take into account the severity of the degree of oxidative stress (*Table 3.2*). Values of d-ROMs test under 250 CARR U, in this case, can be considered as "normal" in a subject following a good lifestyle or belonging to the oriental race or in well-trained athletes. However, besides these cases, in all the remaining

conditions the clinician should interpret carefully low results of d-ROMs test, which can be the consequence of an antioxidant abuse, a not reported cortisone treatment, an immune deficit (see Diabetes Mellitus Type I), an hypothyroidism and so on. On the other hand, pathological conditions, which most frequently are responsible for increased values of d-ROMs test, are inflammatory processes, the impairment of cell respiration, the oscillations of oxygen partial pressure (pO_2) and all the toxicities.

47. To which biochemical test currently available in the routine use can the d-ROMs test be conceptually compared?

The d-ROMs test is an original test which allows for the evaluation of oxidant status, i. e. the global capacity of plasma to induce an oxidation, as detected by the color change of an aromatic amine-derived oxidizable substrate (the chromogen). Therefore, the d-ROMs test is a specific test, useful to evaluate the oxidative stress, which has not any equivalent assay, even conceptually, with any common test proposed for routine clinical chemistry. Moreover the d-ROMs test is not comparable to the biochemical tests often used to evaluate the cardiovascular risk, including C-Reactive Protein (CRP), homocysteine and cholesterol. Regarding the CRP, it was shown that in inflammatory conditions, like the rheumatic disease, the CRP levels go back to the normal values before the "normalization" of the d-ROMs test values, which remains alone to witness the existence of actual damage, i. e. the oxidative damage. Regarding the homocysteine, the d-ROMs test can correlate with this marker of cardiovascular risk because this toxic amino acid is an oxidant. Regarding the cholesterol, paradigmatic are the results of some studies which showed an elevated d-ROMs test in subjects having normal or slightly increased levels of total cholesterol. To further demonstrate that the real risk factor is not only the increased total cholesterol or the increased "bad" cholesterol (LDL-cholesterol) or the decreased "good" cholesterol (HDL-cholesterol), but the "oxidized" cholesterol, which is "bad" independently on the lipoproteic carrier (LDL or HDL). Therefore, the clinician should be familiar with the d-ROMs test as a specific test which provides some very precious indications, otherwise not obtainable, on an independent health risk factor, i. e. the oxidative stress.

48. What is the main information that the d-ROMs test provides to the clinician?

The d-ROMs test, through an accurate measurement of the oxidant status, provides the clinician for information, otherwise not acquirable by any of all the available biochemical tests, on the general wellness status of the body, a status which widely depends only on the rhythm of the biological oxidations. The values of the d-ROMs test, therefore, are a faithful "mirror" of either the endogenous (cell



respiration) or the reactive (inflammation) oxidative processes, and hence they provide a reliable information on the rate of the physiological process of aging in a determined instance.

It's up to the clinician, referring to the specific GUIDE LINES, to interpret correctly and to manage adequately the obtained results. For instance, a condition of hypertension, regularly treated with anti-hypertensive drugs, but associated to high d-ROMs test values may be a not optimally controlled hypertension, which can suggest to the clinician the prescription of another anti-hypertensive drug, having per-sé intrinsic antioxidant activity (e. g. a calcium blocker like lercanidipine), or the combination with an antioxidant supplement with the aim to reduce the d-ROMs test values.

Of course, to have the d-ROMs test values in the normal range does not mean the absence of a disease, but only a very low risk of oxidative stress. In fact the diseases that can affect the body are much more numerous than those specifically associated to oxidative stress (about one hundred, *Table 1. 4*)

49. What is the place and the position of the d-ROMs test on the current market of the available methods to evaluate the oxidative stress?

The d-ROMs test surely is the only reliable test now available on the market to evaluate the oxidant status in the clinical practice. In fact, the d-ROMs test is variously diffused in more than 30 different Countries, where it is performed in prestigious health institutions (e. g. universities, research centers, clinics, hospitals and so on) as well as in common ambulatories, with the aim to perform a research or to obtain from it very useful information in the medical diagnosis. Other tests, although methodologically reliable, remain yet in the limits of pure research.

50. Are there some correlations between the results of the d-ROMs test and those of other tests proposed to evaluate the oxidant status?

Among all the available tests, the d-ROMs test is one of the very few assays whose results showed a close relationship with the results provided by the electron spin resonance, that is considered the "golden standard" technique to evaluate the free radicals in living organisms. Moreover, the d-ROMs test shows a good correlation with other tests for oxidant status, including the malonyldialdehyde (MDA) and the isoprostanes assays, whose scientific validity has been widely reported in hundreds and hundreds of academic papers. Compared to MDA assay, however, the d-ROMs test provides earlier and more reliable results and it appears more specific than isoprostane assay, at least when the comparison is performed with the immune method.

51. What are the "indications" i. e. which kind of subjects should undergo the d-ROMs test according to the indication of the clinician?

According to the available scientific literature, either the candidates to the tests of the Carratelli panel or the aims of the measurement are now clearly identifiable.

It is clear that all healthy peoples should undergo the d-ROMs test, because all the individuals are potentially exposed to the risk of producing exaggerated amounts of free radicals. The primary aim of the test is indeed to identify and to prevent oxidative stress and its unwanted consequences (early aging, diseases).

The d-ROMs test should be even more systematically performed on all the clinically asymptomatic subjects, which are exposed for a number of reasons to factors able to increase the production of free radicals (e. g. radiations, pollutants, smoke) and/or to reduce the clearance of reactive species (e. g. unbalanced diet). In these cases also, the aim of d-ROMs test is to identify and to prevent oxidative stress and its unwanted consequences (early aging, diseases).

Moreover, the d-ROMs test should be carried out on all the patients with oxidative stress-related diseases (over one hundred), such as Alzheimer's disease, Parkinson's disease, stroke, infarction, Crohn's diseases, rheumatoid arthritis, AIDS, some cancers, etc. In all these cases, the aims of the d-ROMs test are to monitor oxidative stress and to prevent its consequences, to monitor the efficacy of specific therapy on the current disease, and, noticeably, to monitor the efficacy of specific therapy, in combination with an eventual antioxidant integration, on oxidative stress associated with the current disease. On this subject, it must be stressed that, in many of the above mentioned diseases, almost all having a chronic course, oxidative stress tends to raise the role of an additional health risk factor. Therefore, it must be controlled in order to optimize results of the therapy. In other words, the evidence, by means of the d-ROMs test, of a condition of oxidative stress is an index of incomplete control of the current disease. Therefore, it suggests to the clinician an integrated therapeutic approach, in which should take place not only conventional, medical and/or surgical, treatments, but also the correction of life-style and, eventually, the intake of antioxidants.

Finally, ideal candidates for the d-ROMs test are all the subjects who undergo pharmacotherapy (e. g. with chemo-therapeutics, hormone replacement therapy, contraceptive pill, etc.) surgical interventions (e. g. organ transplantation, by-pass, etc.), including dialysis, because those conditions could favor oxidative stress. In all these conditions, the aims are to identify and to prevent oxidative

stress and its consequences, and, particularly, to monitor the efficacy of eventual current measures whose finality is to prevent oxidative tissue damage.

52. Can the d-ROMs test be considered as a "predictive" test of diseases?

Yes. This has been shown in cardiovascular diseases. A recent study performed by the researchers of the Italian National Council of Research (Pisa, Italy) demonstrated unequivocally that patients having higher d-ROMs test values, as monitored for a follow-up period of 24 months, showed higher cardiovascular morbidity and mortality over the time compared to the subjects having the d-ROMs test results in the normal range (Figure 3. 5). Moreover, the d-ROMs test was proven a precious predictive marker in the treatment of the hepatitis C virus infection. On this basis we cannot exclude that the d-ROMs test can be a predictive test for other diseases which, as well as cardiovascular diseases and hepatitis, are closely related to the oxidative stress.

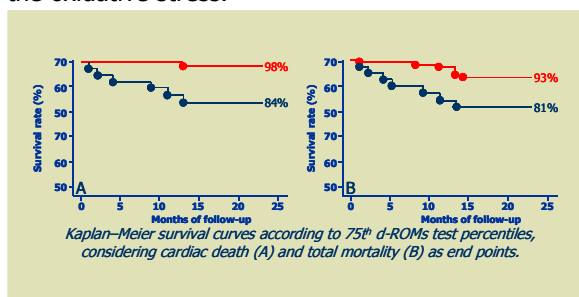


Figure 3. 5. The predictive significance of d-ROMs test: reduced survival rate by vascular and all causes of death in subjects with higher values of the d-ROMs test compared to subject having the d-ROMs test values within the normal range.

53. In which clinical conditions or diseases has the d-ROMs test proven to be very useful?

On the basis of the available scientific literature (about three hundred academic papers), the d-ROMs test has proven to be useful, in the context of its finalities, in almost all the fields of Human Medicine.

The usefulness of the d-ROMs test in aesthetic medicine was shown evident in the monitoring of treatments aimed to slow down skin aging and to reduce the severity of the "cellulites". In the so-called alternative medicine, d-ROMs test was successfully applied, according to three trials, in order to assess the efficacy of some specific therapies, like ozone-therapy, hyperbaric therapy and transcutaneous ginkgo biloba administration.

In the field of human andrology and fertility, the d-ROMs test was shown very promising in the assessment of semen hydroperoxides and, in particular, in the monitoring of the effectiveness of antioxidant supplements as proposed for male infertili-

ty. Chronic obstructive pulmonary diseases (COPD) and other respiratory diseases are a very interesting fields of application of the d-ROMs test. The same consideration is valid for cardiovascular diseases.

The d-ROMs test was shown very useful indeed in the monitoring of oxidative stress associated to hypertension, carotid stenosis, carotid endarterectomy, coronary angioplasty, peripheral vascular diseases, venous insufficiency, and other diseases, according to the findings of several clinical trials and reports. According to the authoritative magazine "Circulation", the d-ROMs test is among the "emerging plasma biomarkers predictive of first atherothrombotic event".

More recently the d-ROMs test was shown to be a reliable test to demonstrate a condition of latent oxidative stress in patients with clinical remission suffering from Chron's disease, a very serious and relatively common chronic bowel diseases. As expected, the d-ROMs test is useful in monitoring oxidative stress and antioxidant therapy in aging. Myelodysplastic syndromes and thrombophilic conditions are the most studied fields of haematology where oxidative stress has been assessed by means of the d-ROMs test. Liver diseases-associated oxidative stress has been successfully evaluated by means of the d-ROMs test. Recently, d-ROMs test was successfully used also in order to assess the efficacy of a homeopathic drug on primary lymphoedema of low extremities. Significant were also the data from patients suffering from HIV infection, a well-known condition related to oxidative stress.

Neonatology and paediatrics are very promising fields for the use of the d-ROMs test. Newborns, independently of the gender, were found to have indeed significantly lower levels of ROMs than those of adults; moreover, an imbalance between pro-oxidant (high d-ROMs test) and antioxidant status (low OXY-adsorbent and -SHp test) was shown in Down's children, while phototherapy proved efficacy in reducing both bilirubin levels and d-ROMs test values in newborns with jaundice. The d-ROMs test has been successfully performed also in order to assess oxidative stress that can be associated to kidney diseases, particularly in chronic renal failure and its treatment, i. e. dialysis and kidney transplantation. In the field of neurology and psychiatry, a case-control trial demonstrated, by means of the d-ROMs test, that antioxidant therapy significantly lowers oxidative stress levels in patients with senile dementia. Another placebo-controlled trial demonstrated that chelant therapy with D-penicillamine is able to reduce serum levels of ROMs in Alzheimer's disease. More recently, patients with amyotrophic lateral sclerosis were shown to have higher d-ROMs test values compared to healthy controls, thus suggesting that free radicals can play an important role in the pathogenesis of neuronal loss of this disease. Several studies showed the practical usefulness of

d-ROMs test in the monitoring of oxidative stress in the fields of nutrition and metabolism, in particular, in heavy drinkers, the obese, diabetics, and dyslipidemic subjects, as well as in the assessment of antioxidant formulas clinical efficacy or in the correlation between oxidative stress and hyperhomocysteinemia (Figure 3. 6).

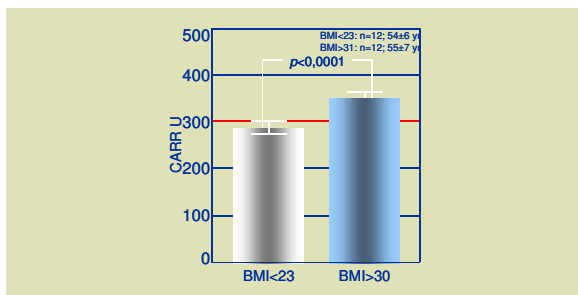


Figure 3. 6. Significantly higher d-ROMs test values in the obese compared to normal-weighted subjects (BMI, Body Mass Index).

In the oncology field, a marked and significant increase in d-ROMs test values was found after chemo- or radio-therapy, compared to the values measured before therapy in patients with some kinds of cancer. However some antioxidant treatments are able to reduce oxidative stress levels – as measured by means of d-ROMs test – in such patients. Both age-related maculopathy and Ménière’s syndrome were shown associated to high levels of d-ROMs test, thus demonstrating the usefulness of this test both in ophthalmology and otolaryngology, respectively. Patients with rheumatoid arthritis showed higher levels of d-ROMs test compared to healthy control subjects. Finally, several sports, which implicate a noticeable muscular engagement, including football, due to the intensity and/or the duration of effort, were shown generally associated to an increase of d-ROMs test values after the performance (Figure 3. 7); moreover, d-ROMs test has proven to be useful in monitoring antioxidant treatments efficacy in exercising peoples.

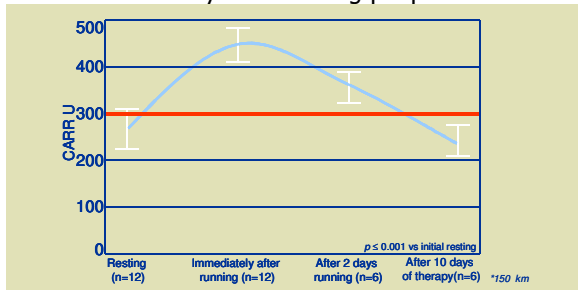


Figure 3. 7. Time-course of d-ROMs test values in a cyclic race (treatment: AR₀ Stenovit).

54. In summary, what are the main points of the d-ROMs test?

After a deep analysis of the available scientific literature and according to the clinical experience of the last ten years, we believe that the d-ROMs test should and must enter into the “outfit” of the clinician with the aim of helping the clinician to ameliorate the quality and possibly the duration of the life of their patients. The d-ROMs test is the only and unique test available on the market that:

- allows for the evaluation of the level of hydroperoxides, markers and amplifiers of oxidative damage produced by the oxidant action of free radicals on a wide range of biological molecules (not only lipids but also glucosides, amino acids, peptides, proteins, nucleotides, and so on);
- has been validated by means of the Electron Spin Resonance Spectrometry, the “golden standard” technique to analyze free radicals in vivo;
- shows excellent analytical performances in terms of precision, accuracy, sensitivity, specificity, repeatability;
- expresses its results in specific and original measure of unit, the CARR U, universally recognized by the international scientific community and accepted by the clinicians due to its high practical usefulness;
- can be easily performed, requires only a few minutes and a very simple instrumentation (only a photometer);
- is based on a very solid scientific background, as documented by more than 300 articles, most in peer-reviewed journals;
- is diffused in more than 30 different Countries, often in prestigious health institutions (in Italy, it is currently used in the Universities of Milan, Siena, Rome, Naples, Catania and so on, in the National Health Institute, in the National Council of Research and so on);
- it is already in use in the clinical practice in many health structures even in ambulatory and private clinics and hospitals in Italy and in many in other Countries;
- received many appreciations by the Scientific Community, among which the insertion into the list of the “emerging plasma biomarkers of first atherothrombotic event” by American Health Association (Circulation), the election of a reference test to study oxidative stress by International Union of Angiology and the international patent for many Countries, including Europe and United States
- offers to the clinician the possibility to manage concretely its use by means of the Guide Lines and, more recently, a specific software (WIN OS MANAGER[®]);
- shows a very favorable cost/benefit ratio.



4. The assessment of antioxidant status: the BAP test.

Blood plasma of living organisms contains several compounds which are able, taken together, to oppose the oxidant potential of the oxidant reactive chemical species. Any compound, either "endogenous" (i. e. albumin, transferrin, ceruloplasmin, bilirubin, uric acid, reduced glutathione, etc.) or "exogenous" (i. e. tocopherols, carotens, ubiquinol, ascorbate, methionine, flavonoids, polyphenols, etc.), if it is able to give electrons, is able to block the potential damage of free radicals also. The reactivity of a free radical is indeed the result of a lack of electrons. Each compound of the above listed – antioxidants – has its own antioxidant power or capacity, i. e. it is able to oppose more or less effectively, depending on the reduction oxidation potential, the "oxidant" action of free radicals. Such a power is due to the property of the components of the barrier to give "reducing equivalents" (e. g. electrons or hydrogen atoms) to the oxidant species, thus avoiding that such reactive species subtract them from essential biochemical components and hence the prevention of the triggering of dangerous chain reactions. Of course, any injury to such "plasma barrier to oxidation" – e. g. exposure either to radiations, or xenobiotics or infectious agents – can result in oxidative tissue damage and, therefore, in early aging and in the so-called oxidative stress related diseases (e. g. atherosclerosis, arterial hypertension, stroke, myocardial infarction, diabetes, arthritis, dementia, colitis, pancreatitis, respiratory diseases, cancer, infections, etc.) (Table 1. 4). From the methodological point of view, starting from the concept that the simplest oxidation existing in Nature is the change of iron from ferrous to ferric form, as it happens in the generation of the rust, one can consider as antioxidant a solution, such as the blood plasma, which is able to bring back the iron from its ferric form to the ferrous form. On this basis Mauro Carratelli developed the BAP test.

55. What is the BAP test?

The BAP test, i. e. the test to measure the biological antioxidant potential, is a photometric test, e. g. a test which can be performed in a laboratory by means of an analytical instrument called "photometer". For ambulatory and routine measurements, the BAP test is performed with the FRAS4 system, which includes both the photometric device and an incorporated and thermostated mini-centrifuge, in order to allow safely the separation of plasma/serum from blood cells.

56. What does the BAP test measure?

The BAP test allows for the substantial measuring of the blood concentration of antioxidants as agents able to reduce the iron from its ferric (Fe^{3+}) to ferrous form (Fe^{2+}). At the moment

there is not any data available on each antioxidant detected and quantified by the BAP test, for comparing to other previous tests measuring the ability of serum/plasma to reduce transition metals (e. g. FRAP and CuRAP assays). The BAP test provides a global measurement of many antioxidants, including uric acid, ascorbic acid, proteins, α -tocopherol, bilirubin and so on. In other words, as well as it happens for other tests for the measurement of antioxidant status (e. g. Total Antioxidant Status by Randox, or ORAC), the BAP test has not been designed to provide any information about the concentration of a single antioxidant, just because this information, alone, is of very scarce clinical value.

57. On what kind of biological sample can the BAP test be performed?

The BAP test can be performed on serum or plasma samples, with or without heparin, even if obtained from whole capillary blood (as drawn by finger-puncture).

58. During the drawing of the blood is the use of anticoagulants allowed?

Only heparin is allowed. The use of chelating agents, including ethylene diamine tetraacetate (EDTA) or citrate, is forbidden because they interfere with the chemical reactions of the test thus leading to an underestimation of the results.

59. For how much time after the drawing, can the fresh blood be stored before undergoing the BAP test?

The time is approximately one-to-two hours, of course by using heparin as the only anticoagulant, according to the "laboratory good practice" for whole blood samples. To reach this goal the producer makes available in each kit disposable heparinised microtubes. During the above interval – which should be as short as possible – it is mandatory to store the sample to the adequate and constant temperature and to avoid any trauma to the tube. In fact both the above factors can induce haemolysis with the consequent underestimation of the results.

60. Can the BAP test be performed on previously frozen serum/plasma samples and, eventually, after repeated thawing cycles?

Although no data is available about this topic, due to easy oxidizability of some components of the antioxidant plasma barrier, we suggest the performing of the BAP test on fresh blood samples to be processed in the same day, after separating the fluid component from the blood cells.

61. On what kind of biological samples the BAP TEST CANNOT BE PERFORMED?

The BAP test CANNOT BE performed on urine.

62. What is the most common procedure for performing the BAP test?

The most common procedure for performing the BAP test involves initially the dilution of a small amount of plasma (as obtained by the centrifugation of a sample of whole blood) in a colored solution, as prepared immediately before performing the test by mixing two reagents in liquid phase, a ferric salt and a thiocyanate derivative. The adding of the blood plasma induces a decolorization less or more intense depending on the biological antioxidant potential of the sample, which is measured photometrically by monitoring the absorbance at 505 nm and comparing it with the colored solution alone.

63. Does there exist some particular "stratagem" to avoid common errors during the execution of the BAP test?

The Producer makes available a specific document ("Hints for knowing and for avoiding the most common causes of error with FRAS") approved by The International Observatory of Oxidative Stress, Free Radicals and Antioxidant Systems, which details how to prevent and to correct the most common errors that the operator can make before (pre-analytical step), during (analytical step) and after (post-analytical step) the BAP test execution.

64. What is the principle of the BAP test?

The BAP test is based on the application of what we observe in Nature, i. e. the formation of the rust. As it is well known, the oxidation of iron induces the change of this metal from its ferrous (Fe^{2+}) to its ferric (Fe^{3+}) form. Since iron is also a well represented transition metal of the body, it has been chosen as a redox indicator in the BAP test. Therefore, after measuring the absorbance of a colored solution, as prepared immediately before the testing by mixing the R_1 reagent (the chromogen, a thiocyanate derivative) with the R_2 reagent (ferric chloride, FeCl_3), the entity of decoloration – as detected photometrically as absorbance change at 505 nm ($\Delta\text{Abs}_{505\text{nm}/\text{min}}$) – after the adding of the sample, will be directly proportional to the concentration of all the agents able to bring back the iron to its ferrous form, i. e. to the biological antioxidant potential of the plasma sample.

65. What is the evidence of the above proposed mechanism of the BAP test? In other words with which analytical technique was the BAP test validated?

The BAP test provides an evaluation of the whole antioxidant capacity of blood plasma, measured as reducing potential against the ferric iron. Several compounds may contribute to this "biological antioxidant potential", some of which exhibit a "scavenger" activity against free radicals, i. e. they have the ability to directly neutralize free radicals. On these basis, a recent study performed at the Tokyo University (Japan) showed, by means of the

Electron Spin Resonance Spectrometry (ESR/EPR) that the bilirubin – a "natural" by-product of the haemoglobin catabolism, normally present in the plasma – exhibits "in vitro" direct scavenging activity against either the hydroxyl radical (HO^{\bullet}), the most potentially dangerous oxygen free radicals in living organisms, or the 1,1-diphenylpicrylhydrazyl radical already at physiological conditions. In experiments performed in parallel with the BAP test it was shown that the results of both assay (ESR/EPR and photometry) were superimposable (*Figure 4. 1*).

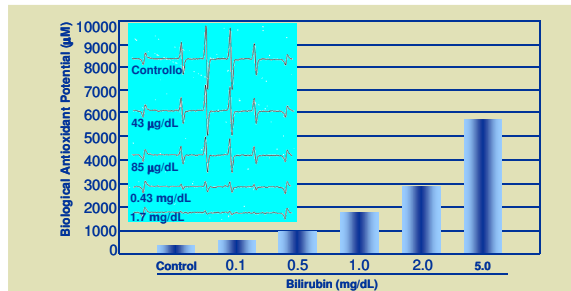


Figure 4. 1. Experimental BAP test validation by means of the Electron Spin Resonance spectrometry (ESR/EPR): with the increase of the concentration of bilirubin, both the BAP value, as photometrically measured (histograms), and the scavenger activity of bilirubin against the hydroxyl radical, as measured by ESR/EPR (box), increases.

By considering that ESR/EPR is the "golden standard" technique to study the free radicals, the BAP test must be considered, due to this authoritative validation, a test really able to detect and quantify in a specific and suitable manner scavenging/antioxidant activities, as confirmed by the above proposed principle of its mechanism.

On the other hand, the BAP test is substantially a simplified variant of the well known and widespread FRAP assay, i. e. the determination of the ferric reducing activity of plasma. Experimental trials showed that the BAP results satisfactorily correlate with those of FRAP, which is considered the comparison method most similar to BAP test, in the actual commercial panorama of the assays proposed to evaluate the plasma antioxidant capacity.

66. How are the results of the BAP test expressed and what is their normal range?

The results of BAP test are expressed as micromoles of reduced iron per Liter of biological sample (blood or serum plasma). The absorbance change ($\Delta A_{505/\text{min}}$) shown after performing the BAP test on the plasma of a wide sample of apparently healthy peoples demonstrated that the great part of recruited individual shows values over 2200 micromoles/L; hence this value has been individuated as the cut-off. Therefore, values under this limit indicate a pathological reduction of the "biologically active" components of plasma barrier.



67. Does different BAP test values correspond to different levels of oxidative stress?

It was established that the impairment of the antioxidant barrier can exhibit different degrees of severity, according to the results of the BAP test (Table 4. 1.).

Table 4. 1. Severity of antioxidant status impairment depending on the results as provided by the BAP test.

µmoles of reduced iron per L of plasma blood sample	Impairment degree of the plasma antioxidant barrier
2,200 – 2,000	Border line condition
2,000 – 1,800	Slight reduction
1,800 – 1,600	Moderate reduction
1,600 – 1,400	Strong reduction
< 1,400	Very strong reduction
Optimal value: >2200 µmoles/L	

68. What are the analytical performances of the BAP test?

On the basis of the data published until now, the BAP test has proven reliable, precise and repeatable, having an acceptable within-run and between-run, even when performed manually (CV<5%). The lowest quantification limit is 418 micromoles/L. The linearity shows maximal between 800 and 10.000 micromoles/L. The only analytical interference seems to be related to the lipid concentration, as for almost all photometric tests. Indeed a hyperlipemic plasma can underestimate the results.

69. Do the results of the BAP test change depending on the kind of drawing?

No, the results of the BAP test don't change. Indeed up to now no statistically significant difference has been show among capillary, arterial and venous blood drawing.

70. What is the volume of the plasma/serum blood sample generally required for performing the BAP test?

Depending on the different procedures and applications, only 20 to 5 microliters are required.

71. Do the results of the BAP test change depending on the age, the gender, the race, or other physiological or physiological-like conditions?

In the absence of a disease, the results of the BAP test don't change depending on age, gender, race and other physiological/ physiological-like condition, with the only exception of elderly. Indeed the aged people show a trend to significantly lower BAP test values as compared to the middle aged.

72. Does the BAP test have to be performed before the meals?

It is mandatory to perform the BAP test before the meals or at least a conspicuous interval of time after a copious meal or a massive intake of alcohol or antioxidant vitamins.

73. Besides Humans, does there exist any data regarding the "normal range" of the BAP test in other Animal species?

Yes, some recent studies have allowed for the establishing of the range of normality in several Animal species, including some Fishes, Dogs, Cats and Bovines.

74. What general principles should the clinician follow in the interpretation of the results of the BAP test?

The general principles for the interpretation and management of the results of BAP test are the object of specific Guide-Lines that the International Observatory of Oxidative Stress proposes periodically on the basis of the scientific literature and the clinical practice. Such Guide Lines follow an algorithm that allows the operators i) to interpret easily the results of BAP test, ii) to reach when possible the goal of a etiological diagnosis and iii) finally to prescribe the right treatment. All the Guide Lines take into account the severity degree of the antioxidant plasma barrier (Table 4.1).

75. To which biochemical test currently available in the routine use can the BAP test be, even conceptually, compared?

The BAP test allows for the quantifying of the biological antioxidant potential, i. e. the set of all the biologically active substances able to reduce the iron from the ferric to the ferrous form. Therefore, the BAP test is a specific test useful for the evaluation of the oxidative stress, which doesn't have any equivalent assay or even conceptual similarities with other common tests proposed for routine clinical chemistry, like the isolated dosing of albumin or uric acid, sometimes proposed as "surrogates" to evaluate plasma antioxidant capacity. As a matter of fact, either the albumin, non-specifically, or the uric acid, specifically, may contribute to constitute the antioxidant plasma barrier, but their dosing (mg/dL) is not coherent with the evaluation of an antioxidant capacity (reducing activity). In other words, the contribution of both albumin and uric acid to the whole antioxidant barrier cannot be extrapolated and this makes unreliable the dosing of albumin or uric acid alone in the evaluation of antioxidant capacity of plasma.

76. What is the main information that the BAP test provides to the clinician?

The BAP test, through an accurate measurement of the antioxidant status, provides to the clinician with information not acquirable by any of the available biochemical tests, on the general wellness status of the body, a status which widely depends on the effectiveness of the physiological antioxidant systems.

It's up to the clinician to interpret and to manage correctly the results of BAP test, referring to the specific Guide Lines. On this subject, the BAP test must be considered as the exact complemen-



tary test of d-ROMs test. Thus both tests provide to the clinician a precise picture of the oxidative balance of each person, which is the right starting point for any eventual preventive/curative strategy. Any antioxidant treatment cannot in fact leave out of consideration the preliminary execution of the BAP test, according to the same principle by which one should take an hypocholesterolemic drug only after a specific biochemical assay demonstrates an increased level of blood cholesterol.

On this subject the BAP test has proven very useful in the monitoring of antioxidant treatments, because it is very sensitive to the intake of antioxidants. Of course as well as for the d-ROMs test, having the BAP test values in the normal range does not mean the absence of a disease but only a very low risk of oxidative stress. The diseases that can affect the body are in fact much more numerous than those specifically associated to the oxidative stress (about one hundred, *Table 1. 4*)

77. What is the place and the position of the BAP test on the current market of the available methods to evaluate the oxidative stress?

After a phase of experimental use, the BAP test is rapidly gaining a respectable place among the tests aimed to evaluate the antioxidant activity or capacity in the routine clinical practice.

In fact, some tests, such as the known Total Antioxidant Status (TAS), although valid by the methodological point of view, remain yet confined in a research context and were not validated by "golden standard" techniques, like the Electron Spin Resonance Spectrometry (ESR/EPR).

The BAP test evaluates the capacity of the plasma to reduce the iron from the ferric state to the ferrous state. The BAP test gives information similar to the ones of the FRAP test, only that the BAP test is simpler to carry out. The TAS test evaluates the capacity of the plasma to oppose the action of an oxidant solution.

From a conceptual point of view TAS and BAP measure the antioxidant capacity of the plasma in two different ways, hence these two tests have different indications. The BAP test measures the "dynamic" or biologically active component of the antioxidant barrier of the plasma and because of this reason it finds a great use in the evaluation of the efficacy of antioxidant compounds with a lower molecular weight like ascorbic acid, bilirubin and so on. The TAS test gives a picture of the "structural" component of the antioxidant barrier (proteins, mucopolisaccarides and so on), which is fairly slow to reconstruct. For this reason it gives a reliable picture of all the antioxidant. The advantages for the clinical doctor in using the BAP test is the capability to see the effects and efficacy of his possible therapies, because these therapies will influence first the "dynamic" components of the antioxidant system

and only much later the structural part. In this way he can dose correctly within short time the correct therapy for each patient (personalization of the treatment).

Using a visual explanation for the differences of the two types of tests, if the free radicals would be the attacking party of a fortress, the TAS test measures the defense capacity of the fortress walls, while the BAP test quantifies the defending troops ready on the windows and top of the walls.

78. Are there some correlations between the results of the BAP test and that of other tests proposed to evaluate the antioxidant status?

The BAP test was shown to correlate positively and significantly with the FRAP assay.

79. What are the "indications" i. e. which kind of subjects should undergo the BAP test according to the indication of the clinician?

According to the available scientific literature, the main indication of the BAP test is the evaluation of the antioxidant status, which can be impaired by an absolute or relative deficiency of antioxidants. Due to its peculiarity, the BAP test integrates well with the results of the d-ROMs test and it can be considered as its "complementary" test.

80. In which clinical conditions or diseases has the BAP test proven to be very useful?

In patients with neurotrauma, the BAP test was shown as a reliable method to demonstrate the scavenging activities and antioxidant potency of bilirubin even at physiological concentrations. This finding was in agreement with parallel experimental results obtained with Electron Spin Resonance Spectroscopy.

The clinical usefulness of the BAP test in Humans was firstly demonstrated in studies on undersea and hyperbaric medicine and more recently in chronically ill patients, in subjects undergoing acupuncture, and in athletes, where the BAP test was able to demonstrate a strong relationship between *in vitro* and *in vivo* antioxidant properties of a commercially available supplement. Similar results – increased values of BAP test after an antioxidant supplementation – were also obtained in Labrador dogs.

The current apparent discrepancy in the number of academic papers between the BAP test and the d-ROMs test may be due only to the fact that the BAP test entered only a few years ago in the clinical practice compared to the d-ROMs test. Indeed, there is a growing amount of convincing reports that this test will open new ways in the oxidative stress evaluation in Human and Veterinary Medicine.



81. In summary, what are the main points of the BAP test, according to the International Observatory of Oxidative Stress, Free Radicals and Antioxidant Systems?

Although the BAP test entered in the clinical practice only few years ago, the International Observatory of Oxidative Stress, Free Radicals and Antioxidant Systems, on the basis of the growing

available data from literature and clinical reports, suggests the justifiable insertion of the BAP test as a complementary test of d-ROMs test in the routine oxidative stress assessment. The BAP test not only shows good analytical performances but it is also easy to perform, quick, very useful in the monitoring of antioxidant treatments and of favorable cost/benefit ratio.

5. The dedicated instrumentation: the FRAS 4 system.



FRAS4, an integrated system invented by Dr Fabrizio Callegari, is now available for medical doctors and other health professionals. It allows for the global assessment of oxidative stress, by means of two tests, the d-ROMs and the BAP, both of which were invented and developed by the Italian Chemist Mauro Carratelli.

82. What is FRAS4?

FRAS 4 is a new integrated analytical system consisting of a dedicated photometer with an incorporated centrifuge, designed to allow for the global assessment of oxidative stress, by means of two tests, i. e. d-ROMs and BAP tests, on a small capillary blood sample, obtained by a finger puncture. The initials FRAS stand for Free Radical Analytical System, i. e. system for the analytical study of free radicals.

83. What are the main features of FRAS4?

The main features of FRAS4 are reported in the Table 5. 1.

Table 5. 1. Technical features of FRAS 4

General characteristics	
Dimensions	39 x 26 x 12 cm
Weight	Approx 3,9 kg
Power supply	100 - 240 VAC, 50 - 60 Hz
Power consumption	50 W
Photometric system	
Spectral region	505 nm obtained with interferential filter
Measuring principle	Absorbance. Lambert Beer law.
Reading cell	37°C real time displayed
Centrifuge	
Speed	6000 r. p. m. \pm 5%
Temperature	37°C real time displayed
Software	
Program	Resident on FLASH memory
Interface	RS 232 with 9 poles for PC connection
Display	Back lighted alphanumeric LCD
Printer	
Type	Graphic, thermic, with 192 dots per line
Results	Automatic printout
Autodiagnosis	
	Automatic error display
Working conditions	
Temperature	Room temperature, 15 - 35°C
Relative humidity	Up to 90%
Safety	DIRECTIVE 73/23/CEE ⁽⁵⁾
Electromag. compat.	DIRECTIVE 89/336/CEE
⁽⁵⁾ REGULATIONS: CEI-EN 61010-1, CLASS I; INSTAL. CATEG.II	

84. What is the most innovative technological feature of FRAS4?

The most innovative technological feature is the integration of the centrifuge in the analytical module. FRAS4 enables the operator to perform not

only the centrifugation but also the photometric analysis. The mini-centrifuge, in particular, is thermostated, thus ameliorating the analytical performances of the instrument.

85. How does FRAS4 manage all the steps of the analytical procedures?

Although procedures of the two tests are very simple, FRAS 4 possesses a self-instructing display that provides not only the photometer and centrifuge temperatures but also multilingual operating messages. All these functions are controlled by a particular software which manages the whole instrumentation. Moreover, this software can be updated by means of a personal computer.

86. Does FRAS4 require operations of calibration to maintain adequate standards of precision and repeatability?

FRAS4 is marketed already "calibrated". The operator, at the time of opening of a new diagnostic kit, must put into the instrument the so-called "K factor", which is printed on the packaging of the kit. This very simple procedure allows for the adaptation of the photometric readings to the specific features of the chromogen to be used. However, a calibrator (a serum with known titre) is available.

87. How does FRAS4 manage the presentation/output of the results?

FRAS 4 allows, by means of a mini-printer, the emission of a ticket with a personalized heading, by means of an optional serial port/USB (RS232), the data recording in a personal computer.

88. Do scientific studies performed with FRAS4 exist?

Yes. Some of these studies have been successfully published on prestigious Journals in the field of oxidative stress, such as Free Radical Research in Biology and Medicine.

89. In summary, what are the main points of FRAS4, according to the International Observatory of Oxidative Stress, Free Radicals and Antioxidant Systems?

The International Observatory of Oxidative Stress, Free Radicals and Antioxidant Systems, on the basis of the available scientific literature and clinical reports, suggests the use of FRAS4 in the clinical practice, because this analytical system, due to its reliability and precision together with its very easy management, allows for an objective and real-time evaluation of the oxidative balance, thus laying the foundations for effective strategies of prevention or treatment personalised for each individual. The recent development by dr. Eugenio Luigi Iorio of the WIN OS MANAGER® software will further ameliorate the performance of this very useful tool.

6. The management of the oxidative stress in the clinical practice. The Guide Lines and the *WIN OS MANAGER®* software



It is now possible to start to transfer into the clinical practice and, in general, to all the fields of applied sciences, including Human and Veterinary Medicine, Botanic and Ecology, the enormous potential of the results of studies that the basic biochemical research cumulated over the last 50 years on the role of free radicals and antioxidants. Such an itinerary is studded, like all the roads of Science, by errors and incomprehensions, but also rewarded by unique successes – for instance a Nobel prize due to the discovery of biological properties of the free radical nitric oxide (NO). The journey is now finally consolidating into trends to "exploit" the basic research to "produce" innovative technologies able to ameliorate the quality and/or the duration of life.

In the field of oxidative stress particular recognition is due to two pioneers, both of whom marked with their engagement this fashionable course during the last two decades and for this reasons were each awarded a special prize by the International Observatory of Oxidative Stress, Free Radicals and Antioxidant Systems. They are the American Kenneth H. Cooper, the promoter of the "antioxidant revolution" and the Italian Mauro Carratelli, the inventor of the d-ROMs test and the BAP test. (IN THE PHOTO ABOVE: Mr Kenneth H. Cooper is the first from the left and Mr Mauro Carratelli is the second from the right).

*More recently the Observatory produced some Guide Lines and developed a specific software (*WIN OS MANAGER®*) with the aim of helping the clinician manage in daily practice the patient at risk for oxidative stress, from the moment of the possible diagnosis till the proposal of a treatment.*

90. What rational elements are at the base of the oxidative stress evaluation?

Free radicals show a very short half-life, as low as a nanosecond for some oxidant species, so that the commonly available laboratory techniques are not able to identify and to quantify them. How-

ever, when their concentration increases up to cause a possible damage, some by-products, derived from the oxidised molecules, may accumulate in the involved tissues and particularly into the biological fluids, such as the blood. Such by-products are much more stable than the original free radicals and they can be more adequately identified and quantified by photometric techniques. Therefore, the detection, by means of the d-ROMs test, of an abnormally increased blood concentration of hydroperoxides is an unequivocal sign that the body has undergone and will further undergo oxidative damage. In such a specific case this is due to the fact that hydroperoxides are not only markers but also potential amplifiers of the oxidative damage. Similarly, a barrier which opposes the attack of free radicals is also available in the plasma. Therefore, the evidence, by means of the BAP test, of a reduced antioxidant biological potential indicates unequivocally whether the antioxidant "defenses" are deficient or not (*Figure 6. 1*).

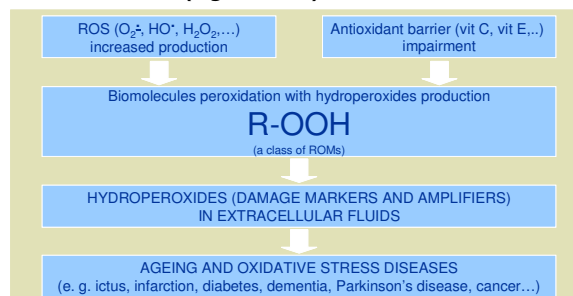


Figure 6. 1. Rationale to evaluate the oxidative stress.

By means of the concomitant execution of the d-ROMs test and of the BAP test, now made possible due to the analytical FRAS4 system, the clinician has on hand at the clinic a very suitable instrument to face not empirically nor roughly, but rationally and scientifically the problem of oxidative stress.

91. What is the routine the clinician must follow – there being the possibility of performing on the patient both the d-ROMs test and the BAP test – to pass from a mere hypothesis to the diagnosis of oxidative stress?

The clinician must follow the same routine as the one proposed for any common disease - clinical suspect, clinical history, visit and biochemical evaluation – but with the "aggravation" that oxidative stress, unlike any common disease, doesn't exhibit specific clinical symptoms... and it is systematically excluded from all the conventional Handbooks of Medicine!

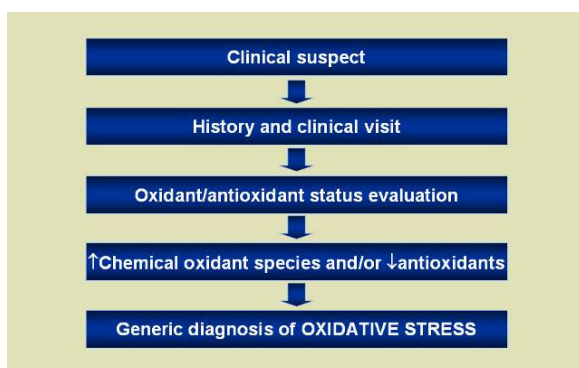


Figure 6. 2. The diagnostic itinerary of oxidative stress (Part I).

The starting point is always the clinical suspicion that is generated, in turn, by the knowledge of the problem. If the clinician doesn't know about oxidative stress he will not be able to formulate the correct questions aimed to evidence it! From this simple concept it becomes obvious the importance of the clinical history that will lead to a search for the existence of risk factors for oxidative stress, including age, physiological status (pregnancy, lactation, menopause), overweight/obesity, abnormal caloric intake, minerals and vitamins deficiency in the diet, alcohol abuse, cigarette smoke, inadequate exercise, psycho-emotional stress, significant exposure to UV radiations, significant exposure to electromagnetic radiations, significant exposure to environment pollutants, current intake of oestrogen-progesterone combination (especially as contraceptive pill), current chemotherapy, current radiotherapy, current dialysis, current cortisone treatments and so on.

The task of the clinician will be easier where the patient suffers from a known disease. In fact the clinician will have to search only the current disease among the known diseases associated to the oxidative stress (Table 1. 4). On this subject, all the following conditions are generally associated to an oxidative imbalance: recent trauma, recent viral infection, recent bacterial infection, infectious disease from other agents, recent inflammatory non infective disease, thyroid hyper-function, arterial hypertension, clinical signs of atherosclerosis, dislipidemia, complicated diabetes mellitus, liver dysfunction, neoplasms, malabsorption diseases, and so on. In each of the above cases a careful clinical visit will confirm the suspicion of any eventual disregarded but hypothesised disease on the basis of the clinical history.

The first step of the clinical routine will end with the biochemical analysis of the oxidative stress by means of the d-ROMs test and the BAP test. On the basis of the results the clinician will examine 6 possible combinations.

92. Combination 1: the results of both the tests, d-ROMs and BAP, are under the normal limit. What is the possible interpretation?

This condition is called "absolute hyporeactivity". Practically, the d-ROMs test values under the normal limits suggest an oxidant capacity of the serum lower than that expected while the values of BAP test under the optimal value indicate a reduced antioxidant potential. Therefore it may be that the whole ability of the body to react and to adapt its own oxidative balance to endogenous and/or exogenous stressors is not optimal.

93. Combination 2: the result of d-ROMs test is under the normal limit, while the result of BAP test is optimal. What is the possible interpretation?

This condition is called "relative hyporeactivity". Practically, in spite of the optimal value of BAP test, which indicates an adequate biological antioxidant potential, the value of d-ROMs test under the normal limits suggests a serum oxidant capacity reduced compared to that expected. Therefore, it is possible that the ability of the body to react and to adapt its own oxidative balance to endogenous and/or exogenous stressors is not optimal.

94. Combination 3: the results of both the tests, d-ROMs test and BAP test, are within the normal range. What is the possible interpretation?

The oxidative balance is optimal. Practically, values of the d-ROMs test within the normal range suggests a serum oxidant capacity within the expected limits, while optimal values of BAP test indicate an adequate antioxidant potential. Such elements are generally sufficient to exclude a condition of oxidative stress in progress. However, having the results of both the tests "within the normal range", doesn't exclude the existence of a disease in progress, but it indicates only a blood level of oxidative stress biomarkers within the mean range as detected in the clinically asymptomatic and apparently healthy population.

95. Combination 4: the d-ROMs test result is within the range, while that of BAP test is under the optimal value. What is the possible interpretation?

This combination is referred to a condition of "relative oxidative stress". Practically, d-ROMs test values within the normal range suggest a serum oxidant capacity in the normal limits of that expected, while BAP test values under the optimal value indicate a reduced antioxidant potential. Such elements should be interpreted as a condition of risk. In other words, the subject, although doesn't exhibit any positive biochemical marker of a current oxidative tissue damage (normal d-ROMs test results), is predisposed to the oxidative stress because his reduced defenses (low BAP test results) may be not able to allow to face optimally an eventual radical attack. Therefore, every endogenous or exogenous factor, potentially able to increase the serum oxidant capacity, will be more able to cause

a fee radical tissue damage compared to a normal oxidative balance.

96. Combination 5: the results of d-ROMs test is over the normal limit, while the result of BAP test is optimal. What is the possible interpretation?

This combination is referred to a condition of "potential oxidative stress". Practically, in spite of the optimal value of BAP test, that indicates an adequate biological antioxidant potential, the value of d-ROMs test over the normal limit suggests a serum oxidant capacity higher than expected. Such elements should be interpreted as a condition of risk, i. e. a kind of "compensated" oxidative balance. In other words, it could be that the cause responsible of the increased free radical production (e. g. cigarette smoke, arterial non-diagnosed hypertension, overweight and so on) are still able to control the oxidative damage in progress "engaging" the antioxidant defences. If the cause responsible of the increased production of free radicals is not rapidly removed (e. g. by stopping the cigarette smoking, by improving the life style, by taking some specific drugs and so on), the same cause will lead, in a variable interval of time, to the progressive reduction of antioxidant defenses, and hence to the clear picture of oxidative stress (evident imbalance between the production and the elimination of free radicals).

97. Combination 6: the result of d-ROMs test is above the normal range, while the BAP test is below the optimal value. What is the possible interpretation?

This condition is that of absolute oxidative stress. Practically, the values of d-ROMs test higher than the normal range suggest a serum oxidant capacity higher than that expected, while values of the BAP test under the optimal limit indicate a reduced antioxidant potential. This is the classical picture of the oxidative stress, where the production of free radicals is too high to be overcome by the defensive capacities of the antioxidant system of the body. The risk of oxidative lesions in the tissues is variably high depending on the degree of increased d-ROMs test/decreased BAP test values.

98. How can the clinician manage each of the above depicted conditions?

The answer is in the Guide Lines. Generally, the strategy to be followed should take into account the relative "burden" of the risk factors, the eventual diseases in progress and the results of both the tests (oxidation severity and level of impairment of antioxidant defences).

99. What general strategy should the clinician follow when the patient suffers from an evident condition of oxidative stress?

In the evident case of oxidative stress (increased d-ROMs test and/or decreased BAP test), on the model of a specific original algorithm, the

clinician will try to identify the possible cause(s) and the relative mechanism(s) responsible for the impaired oxidative balance (Figure 6. 3).

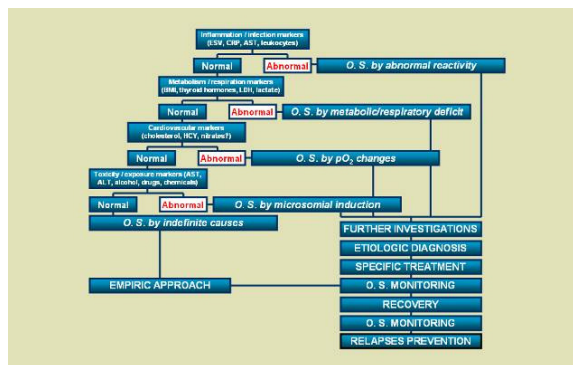


Figure 6. 3. The diagnostic itinerary of oxidative stress (Part II).

Therefore the clinician should try to establish, with the aid of adequate laboratory/instrumental analyses (leukocytes count, ESV, CRP, AST, BMI, fat mass/muscle mass ratio, thyroid biomarkers, serum lipid pattern, homocysteine, tumour markers and so on) whether the main mechanism responsible is one or more of those proposed (inflammation, impairment of mitochondrial respiratory function, ischemia-reperfusion damage and pharmaco-metabolic induction). On the basis of the prevalent mechanism, the clinician will be able to prescribe, in the single clinical case, a specific treatment able to reduce the increased oxidant capacity (causative or etiological therapy) and/or to strengthen the antioxidant defenses (supplementation).

100. What are the actual trends in the prevention and in the treatment of oxidative stress?

The prevention and/or the treatment of the diseases associated with the oxidative stress requires, besides specific options depending on the prevalent involved mechanism, an integrated approach that Kenneth Cooper (Dallas, Texas, US) defined some years ago as the "antioxidant revolution". In such a context it is very important, after undergoing the tests, to ameliorate the life style, to adopt the "Mediterranean alimentary model" and to undertake regular physical activity.

101. It is often difficult for the clinician to design "a diet" which is able to take into account not only the distribution of the nutrient and the caloric intake, but also the antioxidant requirements. Are there some Guide Lines for Food Intake to help him?

The American Guide Lines for Food Intake, some of which are followed by Oncologists for the prevention of tumours, clearly suggest to take everyday from 5 to 8 portions of fruits and vegetables, preferably fresh and in season. However, some Researchers prefer to this "empiric" suggestion a more objective criteria, like the one based on the ORAC



score. This system is able to quantify the "in vitro" antioxidant capacity of all common fruits and vegetables in "Oxygen Radical Adsorbent Capacity" units (Table 6. 1). For instance, 100 g of dried prunes allows an intake of 5770 ORAC UNITS.

Table 6. 1. Antioxidant contribution for some common food in ORAC units per 100 g

FRUITS	ORAC UNITS	VEGETABLES	ORAC UNITS
Dried prunes	5770	Cabbage	1770
Raisins	2830	Spinach	1260
Bilberry	2400	<i>Brussels sprouts</i>	980
Mulberry	2036	<i>Alfa alfa sprouts</i>	930
Strawberry	1540	<i>Broccoli</i>	890
Raspberry	1220	Beetroot	840
Plum	949	Red peppers	710
Orange	750	Onion	450
Black grapes	739	Wheat	400
Cherry	670	Egg-plant	390

By deriving the data from the ORAC TABLES, also classified on the basis of fruits and vegetables colour, the clinician can suggest a daily cocktail of fruits and vegetables of different colours taking into account the different seasons, until covering, as indicated by the above chart, the need of natural antioxidants detected on the basis of the BAP test results. Generally a moderate deficit of BAP requires fruits and vegetable able to reach the score of 3000 to 5000 ORAC UNITS.

Alternatively, the clinician can exploit the nutritional requirement found in RDA tables (recommended dietary allowances) and LARN tables (minimal levels of recommended nutrients), which vary depending on the geographic area, the age and the gender (Table 6. 2).

Table 6.2. LARN of common antioxidants

Vitamin A	1 mg/day
β-caroten	5 mg/ day*
Vitamin C	60 mg/day
Coenzyme Q ₁₀	5 mg/day
Selenium	50 – 200 µg/day*
Manganese	5 mg/day
Copper	2-3 mg/day
Zinc	55 mg/day

*No from LARN tables

102. Is it still valid the ancient aphorism "An apple a day keeps the doctor away"?

Theoretically, this old aphorism remains valid even today. However, we cannot exclude that the level of food nutrients, as expected on the basis of the above tables, is exactly the real level of the same nutrients we take when we "physically" eat a fruit or a vegetable. Indeed, the impoverishment of the soil (due to abnormal exploitation of the soil itself, acidic rains, increasing desertification, pollution and so on), the often uncontrolled use of pesticides, the processes of refinement of vegetables, the processes of transformation, storage, and even the

cooking of foods can variably affect the original, as described in the above tables, antioxidant content of fruits and vegetables. Therefore, as a precaution, many nutritionists today suggest the indiscriminate use of antioxidants. However, according to the Guide Lines of the International Observatory of Oxidative Stress, Free radicals and Antioxidant systems, the use of antioxidant supplements should be limited only to the documented cases of oxidative stress, as biochemically detected as instanced by means of the d-ROMs and the BAP test.

103. What are the fundamental criteria for the choice of the correct supplementation after a biochemical diagnosis of oxidative stress, according to the results of d-ROMs test and BAP test?

Before suggesting any supplementation, every clinician should try to identify and to remove the possible cause responsible for the increased production of free radicals. In particular, reduced values of BAP suggest the real need of an antioxidant supplement and the clinicians should follow some general criteria, which take into account the chemical characteristics and the amount of the micronutrients to be proposed, the possible onset of unwanted side effects, the route of administration, the clinical conditions of the patient, the concomitant administration of other drugs and so on.

Generally speaking, the wide variety of oxidants responsible for oxidative stress and their ubiquitous distribution into the body implies the necessity to have a formula with a wide and complete spectrum of actions. Unfortunately a unique formula able to fit the above criteria is not available. In fact it is well known that vitamin E is a powerful antioxidant, but its activity is reduced when the partial oxygen pressure is reduced, like in ischemic conditions where, on the contrary, β-carotens are most effective. On the other hand, vitamin C is particularly able to recycle oxidised vitamin E but, instead of vitamin E, it is not liposoluble and therefore it cannot reach fatty tissues whilst to the contrary the coenzyme Q₁₀ diffusion is not problematic. Therefore, since a unique antioxidant is only partially effective, it is indispensable that the clinician considers a cocktail of antioxidants, e. g. a formula containing multiple antioxidants with a wide range of activity. In the above examples, it is evident how vitamin E, β-carotens, vitamin C and coenzyme Q₁₀ can be complementary, thus justifying a combined intake.

After stating that the combined antioxidants are more effective than one antioxidant alone, the main problem to be solved is the relative dosing. Unfortunately, the opinions of researchers diverge one from another according to two main trends. The first one is the American opinion, according to which we should use a very large amount of antioxidants to prevent and to treat the oxidative

stress. The second one, prevalent in Europe and conceptually linked to the homeopathy, suggests the use of low doses of supplements. For instance, the antioxidant formula developed by Kenneth H. Cooper, relative to a daily intake of 1000 mg vitamin C, 5000 UI β -caroten, 400 IU vitamin E, 800 μ g folic acid, 25 mg vitamin B₆, 400 μ g vitamin B₁₂ showed the ability in a controlled study to reduce the oxidation degree of LDL (-14.6%), the homocysteine concentration (-17.2%) and the level of CRP (-24.5%). On the other hand, a formula containing similar antioxidants but at a much lower amount (*Table 6. 3*) was shown to reduce oxidative stress level as measured by means of the d-ROMs test in patients suffering from peripheral vascular diseases (claudicatio intermittens).

Table 6.3. An example of antioxidant non-official formula (AR₀ Stenovit).

Active principle	Quantity
Vitamin A	0.4 mg
β -caroten	0.05 mg
Vitamin C	30 mg
Vitamin E	5 mg
Coenzyme Q 10	20 mg
Selenium	48 mcg
Zinc	5 mg
Vitamin B6	1 mg
L-cysteine	10 mg
Flavonoids (Citrus)	30 mg

More recently many official formulas have been proposed with the twofold advantage of obtaining a more adequate personalisation of the treatment and of a better compliance to the supplement, compared to conventional formulas (*Table 6. 4*).

Table 6. 4. An example of antioxidant official formula

L- cisteine mg 100	Nicotinic acid (Vit. PP) mg 6,75
Alpha-lipoic acid mg 100	Piridoxine (Vit. B ₆) mg 0,3
Magnesium mg 37,5	Riboflavin (VitB ₂) mg 0,2
Selenium mg 0,01375	Tiamin (Vit B ₁) mg 0,21

After dosing has been established, the next major problem is the pharmaceutical formula. On this subject it has been established, also by means of the d-ROMs test, that a fluid formula is more effective than a "solid" formula (e. g. tablets, powder and so on).

A specific role is also played by the route of administration: for instance many active principles taken by oral route can be neutralised or affected during transit to the bowel, where variable amounts are "sequestered" by the liver, so that the "bio-availability" of the original supplement for other tissues/organs is reduced. This is the case of the reduced glutathione. On the other hand some clinical conditions, such a celiac disease, by involving the small intestine can affect the absorption of micronutrients. In these cases the clinician should consider the parenteral route (e. g. intravenous or intra-

muscular route). More recently spray oral formulas for sublingual absorption have been developed (Cellfood[®] multivitamins 100% RDA, Cellfood[®] Vitamin C plus). These spray formulas theoretically warrant a quick and easy gain of the circulating blood by the active principle, avoiding also transmission through the liver. In the remaining cases, when the intravenous route is not accepted or contra-indicated, the clinician should consider the administration of metabolic precursors of the antioxidant. For instance, the reduced glutathione is rapidly oxidised in the plasma and cannot be administered for intravenous route; in this case the clinician can consider the opportunity to administer some cysteine-enriched peptides able to reconstitute the glutathione into the cells (Prother[®]). Independently of the effectiveness of the antioxidant formula, a crucial aspect to be considered is the eventual toxicity. Indeed some antioxidants, including the vitamin C, can exhibit oxidant properties while other supplements such as β -carotens can increase the risk of accumulation into the fat deposits, due to their lipidic-affinity. Finally, when the patient presents some co-morbidities which require specific drugs, the clinician should consider the possible risk of the interaction between such drugs and the antioxidant supplements. This is the case of vitamin E, which can bind itself to the plasma protein and release anticoagulant in the blood, thus increasing the risk of hemorrhagic syndromes in a patient with thrombophilic conditions.

104. Does there exist, as in modern diet-therapy, a specific software able to help the clinician in the management of the patient suffering from oxidative stress?

The only actually available software is WIN OS MANAGER[®], an original software, invented by Eugenio Luigi Iorio, with the specific objective of helping health professionals in managing oxidative stress in clinical practice.

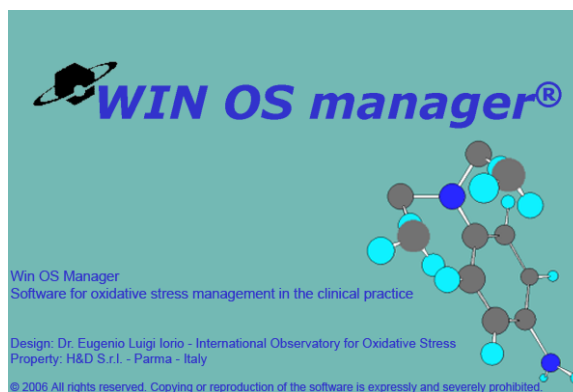


Figure 6. 4. Initial full screen window of the software WIN OS MANAGER[®], specifically designed to manage the oxidative stress in the clinical practice.



105. What is the “core” of WIN OS MANAGER®?

The “core” of WIN OS MANAGER® is a specific algorithm that allows the operator, on the basis of the inserted data (private data, clinical history, clinical visit, results of d-ROMs test and BAP test), to obtain reports (distinct for the clinician and the patient), where for each individual undergoing the evaluation is reported the following information: the global judgment on the current situation as a function of the oxidative stress (and the relative explanation); a series of general suggestions (as a function of physiological conditions, life style, potential exogenous and/or exogenous stressors, the iatrogenic factors and so on); indications and dosing of any eventual pharmacological or antioxidant treatment; any eventual instrumental/laboratory investigation (in order to identify the causes responsible for the oxidative stress); and the day of the next check-up.

106. Does WIN OS MANAGER® possess some particular utilities?

Yes. To complete the software, WIN OS MANAGER® shows some “utilities” which offer to the operator the opportunity to consult (and eventually to print) previous data and some subsidiary documents, including a specific diagnostic algorithm, the need of vitamins and antioxidants, the ORAC score of the most common fruits and vegetables, and so on.

107. Can WIN OS MANAGER® be installed on every kind of personal computer without any particular requirements?

Generally speaking YES. After the positive issue of the notification procedure by the web site of the International Observatory of Oxidative Stress, Free Radicals and Antioxidant Systems (www.oxidativestressobservatory.org) the operator can install the software into a computer, hence personalise the use and license with his own data and, finally, protect every data by means of ID and PASSWORD.

108. If, although all the shrewdness, an antioxidant treatment seems not capable of reducing high oxidative stress levels, what are the indications for the clinician?

It can happen that, after a therapy apparently carried out without errors, one or both of the tests, previously pathological, do not return in the “normal range” as expected. In such cases, before concluding that the therapy has been not effective and, therefore, needs to be changed with a more adequate treatment, a number of hypotheses should be verified.

The first foreseeable hypothesis is that the operator, during analysis, has made a mistake (e. g. use of reagents not correctly stored or after their expiration date, no chromogen adding, etc.). In these cases the advice is to check the procedure

and, eventually, to repeat the test(s) initially carried out incorrectly.

Second eventuality is that the antioxidant did not reach the needed plasma concentration able to carry out its “pharmacological” effects, e. g. due to a dosage lower than that prescribed. In this case the correct dosage must be reached. If the dosage is correct, the assessment of plasma level of the antioxidant (i. e. ascorbate, vitamin E) should be performed. Indeed, if plasma level of the antioxidant is lower than expected values, a condition able to reduce the absorption should be suspected (e. g. celiac disease). In this case, all the eventual causes of the event should be eliminated (i. e. by treating the intestinal disease that reduces the absorption of nutrients, e. g. by a gluten free diet). In the remaining cases, where indicated, the dosing of the supplements should be increased.

The third eventuality is that an accessory event has happened and that such event has been able to increase, without any clear evidence, the production of free radicals initially attributed to the basic condition. For example, in an athlete suffering from oxidative stress related to a strenuous exercise (primary event) can take place a hidden bacterial infection (accessory event). In such cases the advice is to identify and to treat also the accessory event and, if indicated, to increase the dosing of the supplement.

Fourth eventuality is that a pro-oxidation phenomenon occurred. Such event is not rare, for example, after the intake of generous amounts of ascorbate. In these cases generally it is sufficient to reduce the dosing of the formula.

If, after any reasonable attempt, the level of oxidative stress and particularly the d-ROMs test value remains yet difficult to control, generally speaking it is indicated to continue the search for the causes. Meanwhile, it is always appropriate to warrant to the patient an adequate antioxidant covering, at least corresponding to the recommended dietary allowances, and suggesting to him that he maintain under control all the risk factors associated to the oxidative stress, by means of an adequate life style.

If the clinician suspects a reactive condition (inflammation with or without infection), not yet evident by the common biochemical analyses, a short low-dosing treatment with cortisone – according to the experience of some Authors – should be indicated until the normalization of the d-ROMs test results. Of course any eventual cortisone-therapy must be performed according to the general guidelines of an increasing-decreasing protocol, after having excluded all the contra-indications and under the close medical control.

In the search for a possible cause of oxidative stress, we cannot exclude cases of apparently healthy individuals – which we also have observed –



with persistently increased values of the d-ROMs test. Such individuals should be considered into the extremities (tails) of the Gauss-like curve (*Figure 3.4*) and are candidates for three-six monthly check-up.

Unfortunately, it is probable that the Clinician will meet cases not responding adequately to the treatment and does not find any possible explanation among the above considered causes. It is likely, in these cases, that the treatment was inefficient, while it is to be excluded that the biochemical evaluation was inadequate. This possibility opens a new chapter with blank pages. Indeed, up to now there doesn't exist a solution that is valid for everybody, although it may be effective for most. This concept leads directly to the concept of personalization of the treatment, today entrusted to the clinician but in the near future probably directed to pharmacogenomics. Thus, with a simple salivary

specimen it will be possible to extract the DNA of the subject and to establish whether he will be sensitive to the antioxidant treatment. While waiting the validation of this novel approach, it is an engagement of the Clinician, on the basis of his clinical experience, according to science and conscience, to find the most adequate solution, as predicted, and on the other hand, also by our Guide Lines (*undefined oxidative stress*) (*Figure 6. 3*). The Clinician should try to individuate among the proposed treatments the most adequate for the patient (empirical measures) (*Figure 6. 3*). It is not important whether this "search" will require more time and some "adjustments" of the initial therapeutic protocol, with substitutions or additions of active principles or change in the dosing of antioxidants.



7. Concluding remarks

I It is now available for medical doctors and other health professionals, the innovative Carratelli panel that allows for the global assessment of oxidative stress, by means of two tests, the d-ROMs test and the BAP test.

The d-ROMs test allows for the determination of the blood concentration of reactive oxygen metabolites (ROMs) and, particularly, that of hydroperoxides, which are markers and amplifiers of free radicals-induced oxidative damage.

The BAP test allows for the determination of the effectiveness of plasma antioxidant barrier, as iron-reducing activity.

By means of these tests, today performable even in the ambulatory/clinic by means of the FRAS4 system, it is now possible to formulate a more precise and reliable laboratory diagnosis of oxidative stress, where the two distinct and opposite components, either pro- or anti-oxidant can be assessed in a distinguished manner. In other words, it is now possible to establish in "real time" whether oxidative stress depends on an increased produc-

tion of free radicals and/or on a decreased efficacy of inactivating them. Therefore, antioxidant therapy monitoring can now lean on more solid bases, thus going out of its actual "empirical phase".

Everybody should undergo the oxidative stress assessment, even if they are healthy, and even more if one is continuously exposed to pro-oxidant factors (i. e. incorrect life-style, pollutants in work environment, etc.) or is suffering from chronic degenerative diseases (i. e. diabetes, atherosclerosis, dementia, Parkinson's disease, Chron's disease, Down's disease, rheumatoid arthritis, cancers, etc.) or, finally, is undergoing some treatments (i.e. dialysis, by-pass, grafting, pill, radiotherapy, chemotherapy, etc.).

Only by means of this global assessment will it be possible to optimize specific therapies and to monitor the real effectiveness of antioxidant formulas, which intake frequently occurs without a preliminary test capable to demonstrate its necessity.

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