European **Rev**iew for **Med**ical and **Pharmacol**ogical **Sci**ences 2007; 11: 309-342

Oxidative stress tests: overview on reliability and use

Part I

B. PALMIERI, V. SBLENDORIO

Department of General Surgery and Surgical Specialties, University of Modena and Reggio Emilia Medical School, Surgical Clinic, Modena, Italy

Abstract. – Monitoring oxidative stress in humans is achieved by assaying products of oxidative damage or by investigating the potential of an organism, tissue or body fluids to withstand further oxidation. Unfortunately, there is little consensus concerning the selection of parameters of oxidative stress or antioxidant state to be determined in defined patients or diseases. This is not only due to the uncertainty wheter or not a certain parameter is playing a causative role. Moreover, the methods of determination described in the literature represent very different levels of analytical practicability, costs, and quality. Generally accepted reference ranges and interpretations of pathological situations are lacking as well as control materials. At present, the situation is changing dramatically and sophisticated methods like HPLC (High Performance Liquid Chromatography) and immunochemical determinations have become more and more common standard.

Key Words:

Oxidative stress, Free radicals, Antioxidants, Reactive oxygen species, Lipid peroxidation.

Introduction

The role of free radicals is gaining increasing worldwide attention since so many physiological and pathophysiological phenomena are related to redox status cell modification.

A free radical is, by definition, a chemical species containing unpaired electrons and is therefore paramagnetic[1]. Most of the oxygen derived free radicals relevant to cell biology are unstable, short-lived and highly reactive[2] (Table I). For these reasons, reactive oygen species (ROS) can initiate cellular tissue damage by modifying lipids, proteins and DNA, which can seriously compromise cell health and viability or induce a variety of cellular responses through generation of secondary reactive species, leading, at last, to cell death by necrosis or apoptosis. Oxidative damage of any of these biomolecules, if unchecked, is probably responsible of disease development. However, definitive evidence for this association is often lacking because of recognized shortcomings with methods available to assess oxidative stress status *in vivo* in humans[3].

There are some exogenous sources of free radicals such as UV-photolysis, radiation, ozone, pollution, pharmacological agents, smoking, alcohol, iron-overload, pesticides and mycotoxins[4]. Imbalance between production and elimination of free radicals may cause oxidative stress.

Free radicals can be scavenged by several metalloenzymes (e.g., glutathione peroxidase, catalase, superoxide dismutase) as well as by the non-enzymatic antioxidant defence system (e.g., tocopherol, β-carotene, ubiquinol, vitamin C, glutathione, lipoic acid, uric acid, metallothionein, bilirubin) which quench their activity. Therefore, much attention of nutritionists is now focused on the possible role of the enhancement of the defences against ROS[5] (Table II).

Despite the harmful cellular damaging effects, free radical reactions are also involved into beneficial physiological response when produced in high levels mediating cytotoxicity of polymorphonuclear leukocytes, macrophages and monocytes during the respiratory-burst[6].

Moreover, low levels of ROS are involved in the regulation of the tone of smooth muscle cells[7] and have been demonstrated to upregulate the redox sensitive transcription factors such as nuclear factor-κB and activator protein-1[8-10].

Corresponding Author: Beniamino Palmieri, MD; e-mail: palmieri@unimo.it

Table I. Free radicals.

Molecules	Symbol	Half-lives(s) at 37°C
Molecular oxygen	O_2	$> 10^2$
Lipid peroxide	ROOH	$> 10^2$
Semiquinone radical	$Q^{\bullet -}$	$> 10^2$
Hydrogen peroxide	H_2O_2	10
Peroxyl radical	$ROO^{\bullet}$	1×10^{-2}
Superoxide radical	$O_2^{\bullet -}$	1×10^{-6}
Singlet oxygen	$^1O_2$	1×10^{-6}
Alkoxyl radical	$RO^{\bullet}$	1×10^{-6}
Hydroxyl radical	$OH^{\bullet}$	1×10^{-9}

Highly specific analytical techniques are required to monitor the biological significance of free radicals.

Increased oxidative/nitrosative stress generally describes a condition in which cellular antioxidant defences are unable to completely inactivate the ROS[11] and reactive nitrogen species (RNS) generated because of excessive production of ROS/RNS, loss of antioxidant defences, or both.

The localization and effects of oxidative stress, as well as information regarding the nature of the ROS/RNS, may be revealed from the analysis of discrete biomarkers of oxidative/nitrosative stress/damage isolated from tissues and biological fluids. Biomarkers are quali-quantitative indicators of normal and pathological biochemical processes or of drug–induced effect in therapeutic protocols. Several *in vitro* markers of oxidative/nitrosative stress are available, including ROS/RNS themselves, but most are of limited value *in vivo* because they lack sensitivity and/or specificity or require invasive methods. Although some ROS/RNS have been directly detected *in vitro* by electron spin resonance with or without spin trapping reagents or by chemiluminescence, these methods are not yet applicable in clinical practice because of the instability of many reactive species and the need for expensive

Table II. Antioxidant defence system.

Preventive antioxidants:

a) *Non-radical decomposition of hydroperoxides and hydrogen peroxide*:

Catalase:	Decomposition of hydrogen peroxide: $2H_2O_2 \rightarrow 2H_2O + O_2$
Glutathione peroxidase (cellular)	Decomposition of hydrogen peroxide and free fatty acid hydroperoxides: $H_2O_2 + 2\ GSH \rightarrow 2\ H_2O + GSSG$ $LOOH + 2GS \rightarrow LOH + H_2O + GSSG$
Glutathione peroxidase (plasma)	Decomposition of hydrogen peroxide and phospholipid hydroperoxides
Phospholipid hydroperoxide	$PLOOH + 2GSH \rightarrow PLOH + H_2O$
Glutathione peroxidase	GSSG
Glutathione-S-transferase	Decomposition of lipid hydroperoxides
Thioredoxin	Reduction of peroxides

b) *Sequestration of metals by chelation*

Transferrin, lactoferrin:	Iron
Haptoglobin	Haemoglobin
Hemopexin	Stabilisation of heme
Ceruloplasmin, albumin	Copper

c) *Quenching of active oxygens*

Superoxide dismutase (SOD)	Disproportionation of superoxide: $2O_2^{\bullet -} + 2H + \rightarrow H_2O_2 + O_2$
Carotenoids, vitamin E	Quenching of singlet oxygen

Radicals-scavenging antioxidants: scavenge radicals to inhibit chain initiation and break chain propagation

Lipophilic:	Vitamin E, ubiquinol, carotenoids
Hydrophilic:	Vitamin C, uric acid, bilirubin, albumin

Repair and de novo enzymes: repair the damage and reconstitute membranes:

Lipase, protease, DNA repair enzymes, transferase

Adaptation: generate appropriate antioxidant enzymes and transfer them to appropriate site at the right time and concentration

equipment. Furthermore, ROS/RNS are usually too reactive and/or have a half-life too short (even much shorter than seconds) to allow direct measurements in cells/tissues or body fluids. Because molecular products formed from the reaction of ROS/RNS with biomolecules are usually considered more stable than ROS/RNS themselves, most commonly ROS/RNS have been tracked by measuring stable metabolites (e.g., nitrate/nitrite) and/or concentrations of their oxidation target products, including lipid peroxidation end products and oxidized proteins[12-16]. Techniques for quantification of oxidative damage markers are often called fingerprinting methods by which specific end products deriving from the interaction of the ROS with biomolecules, such as DNA, proteins, lipid and LMWA (low-molecular-weight antioxidant) are measured. The presence of these end products serves as proof of the prior existence of ROS that left their footprints in the cell. To function as suitable biomarkers of oxidative modifications in relation to disease, it is critical that such oxidation products are stable, can accumulate to detectable concentrations, reflect specific oxidation pathways and correlate with disease gravity, so that they can be utilised as diagnostic tools.

To demonstrate a role of ROS in a particular type of tissue injury, evidence should be presented that:

1. ROS are detectable locally and the time-course of their formation is such that they could play a role;
2. the chemical production of ROS produces similar lesions;
3. compounds able to remove ROS protect from the injury.

Measuring Free Radicals in Vivo

The increasing interest in the role of free radicals in the pathogenesis of human disease has led to widespread attempts to develop techniques suitable to measure free radicals and their reactions *in vivo*, specifically, in clinical pathology. The first major problem to be faced is the quick reactivity of free radicals reaction close to their biochemical source. Consequently, free radicals are not amenable to direct assay and free radical activity is usually assessed by indirect methods such as measurement of the various end products of reactions with lipids, proteins and DNA[17,18]. However, many of these products are themselves reactive, albeit orders of magnitude less than the free radicals that begat them. The second major problem is that the most commonly available biological fluid to be screened are blood, urine and expired breath. Clinical biochemistry detects usually abnormal metabolic products, recovered from these sources, which are related to specific diseases. On the contrary, reactive free radicals as end products of intracellular metabolism from different tissues have a microseconds-measurable half life and they are not detectable in the blood stream. In a very few special cases, the actual site of free radical generation may be the blood and direct (or semi-direct) detection of free radical species may be possible, but generally speaking only secondary free radical products are detectable in a body fluid. A wide array of analytical techniques has been developed to measure these end products though not all of them are suitable to detect clinical conditions sampling blood, urine and expired breath. Lipid peroxidation is the most intensively studied process and provides a number of possibilities for assays. Protein and nucleic acid oxidation are presently very appealing. The currently available techniques, however, are limited to semi-quantitative assays of damage to broad classes of biomolecules and there is an urgent need for more specific and informative methods.

Electron Spin Resonance and Radical Trapping

The only analytical technique that directly measures free radicals is electron spin resonance (ESR) spectrometry. However, since it is relatively insensitive and requires steady-state concentrations of free radicals in the micromolar range it's of very limited value for use *in vivo*. Whole-body ESR, analogous to whole-body NMR, has been investigated but not yet fully developed. Nevertheless, ESR has been used to detect free radicals in human tissue obtained *ex vivo*: an example is the detection of a signal believed to be that of lipid peroxyl radical in human uterine cervix[19]. ESR spectrometry can usually be applied to analysis of samples *in vivo* only through the technique of spin trapping. This involves the addition to samples of a compound known as spin-trap, which reacts rapidly with the free radicals to form radical-adducts that are very much more stable and longer-lived than the original species and can therefore build up to steady-

state concentrations in the detactable range. Spin-traps have been used in experimental animals to demonstrate the generation of free radicals *in vivo*, but as no effective spin traps presently exist that can be administered to humans, the technique is currently limited to samples of blood mixed with the spin trap as soon as possible after taking them. Despite the obvious shortcomings of this approach, valuable data has been obtained, for example, relating to free radical production during angioplasty[20].

Other trapping procedure allow a radical to react with a detector molecule to yield a stable product that can be evaluated using a variety of techniques, such as hydroxylation of salicyclic acid[21], the deoxyribose assay[22, 23], the cytochrome c reduction assay for detection of superoxide radicals[24], and detection of nitric oxide radicals by colored end-product compounds[25].

Thus, the attack of hydroxyl radicals on salicylic acid produces 2,3-dihydroxybenzoate (DHB) and on phenylalanine produces *o*-and *m*-tyrosines. These products are not produced enzymatically in humans. Thus, the method can be used *in vivo* and detection of 2,3-DHB or the tyrosines in body fluids can be taken as evidence of hydroxyl radical generation[26]. As the trapping compound has to complete with all other biomolecules for reaction with the radicals, this technique, like ESR-spin-trapping, is unlikely to provide more than semi-quantitative data.

Spin trapping is a powerful method that facilitates the visualization of free radicals, including those formed in complex biological systems. The spin trap is a diamagnetic compound that reacts with a reactive free radical to form a more stable radical adduct. Although detection through ESR spectroscopy offers some distinct advantages in its high sensitivity, and in some cases its specificity toward some radical species, there are also several drawbacks to using this technique.

The technique was developed in the lates 1960s by several laboratories[27]. Two groups of compounds are commonly utilized as spin-trapping agents: nitroso and nitrone compounds. The nitrogen atom of the nitroso spin trap reacts directly with the free radical species, giving distinctive spectral features. Two nitroso compounds are currently used in biological investigation: 2-methyl-2-nitroso propane (MNP) and 3,5-dibromo-4-nitrosobenzenesulfonate (DBNBS).

The nitrone spin traps each have a radical added to the carbon; the radical will be β with respect to the nitroxide radical center. The lack of spectral information about the trapped radical is the major drawback of this class of spin trap. Three commonly used spin traps will be discussed: phenyl-t-butyl nitrone (PBN), α(4-pyridyl-1-oxide)-N-t-butyl nitrone (POBN), and 5,5-dimethyl-1-pyrroline N-oxide (DMPO).

The technique has been associated with various cases of incorrect interpretations; these generally can be attributed to:

1. changes in the spin trap (nonradical, chemical, photochemical, enzymatic reactions);
2. perturbation of the biological system by the probe;
3. artifactual reporting associated with intrinsic properties of the probe.

Spin trapping is a good example of the complex interaction between the model system and the artificial addition of a probe, with a high possibility of recording "artifactual" results.

The first that has proposed the term spin trapping has been Janzen[27]. Spin trapping in biology is covered by various reviews[28,29]. An extensive literature survey has been carried out by Dodd[30]. Specifically devoted to examining the problems associated with the spin trapping of oxygen-centered free radicals are the reviews of Finkelstein et al[1], Rosen and Rauckman[31], Rosen et al[32], and Pou and Rosen[33].

Invaluable help in disentangling the number of spectra and attributions is given in the database for spin-trapping by Li and Chignell[34], which has been made feely available to all those interested in the field.

Electron Paramagnetic Resonance (EPR)

Another technique for the measurement of the oxidative stress status in biological systems is based on the X-band EPR (electron paramagnetic resonance) detection of a persistent nitroxide generated under physiological or pseudo-physiological conditions by oxidation of a highly lipophylic hydroxylamine probe. The probe employed is bis(1-hydroxy-2,2,6,6-tetramethyl-4-piperidinyl)-decandioate which is administrated as hydrochloride salt. This way of making OS status detectable involves the use of exogenous nitroxides as probe of the redox balance in a given enviroment. This probe is able to give a fast

reaction with the most of radical species involved in the oxidative stress. The rate at which the nitroxide is reduced to the diamagnetic hydroxylamine, which can be evaluated by EPR, is related to the reducing capacity of the organism and hence to its oxidative status[35]. Furthermore, it crosses cell membranes and distributes in a biological environment without the need to alter or destroy compartmentation. The method is therefore suitable for quantitative measurements of ROS and can be applied to human tissues in real clinical settings[36]. It has been successfully employed in systems of growing complexity and interest, ranging from subcellular fractions to whole animals and human liver. Liver disease was chosen as the prototype of a patology in which the involvement of inflammatory processes has a relevant role in the evaluation of the disease[37]. Thirty-two subjects, including 10 healthy controls, were enrolled after giving informed consent. Ten of the 22 patients had hepatitis C, 3 had hepatitis B, while the remainder had a variety of diseases characterized by an autoimmune nature which, for statistical purpose, were clustered in a group called nonviral liver diseases (NVLD). The method developed by Valgimigli et al[38] was enough simple and only moderately invasive: 2-3 mg of liver biopsy (obtained by the fine needle technique) were weighted and incubated for 5 minutes at 37°C with a physiological solution of the hydroxylamine I (1 mM) containing a metal chelating agent. After incubation, the sample was quickly frozen in liquid nitrogen to denaturate enzymes and stop any reaction, and subsequently warmed at room temperature prior to the EPR measurement. For practical reasons, these researchers monitored the maximum concentration of nitroxide instead of the full time evolution. Diseased tissue provided a more oxidizing enviroment than healthy liver. Furthermore, the nature of the disease affected the oxidative status.

The effect of the various experimental conditions on the final result, including lenght of incubation, time from tissue extraction to addition of the probe and time from incubation to EPR measurement, were sistematically investigated in order to set the optimal standardized experimental conditions. Interestingly, these results revealed that omogenization of the tissue is unnecessary since the signal measured immediately after omogenization in the presence of the probe was very close to that obtained after 5 minutes incubation with the whole biopsy. After calibration of the spectrometer response it was possible to obtain quantitative values for the oxidative stress. These results indicate that the OS level in diseased liver is several orders of magnitude higher than in healthy controls and the differences were highly significant.

An endogenous molecule might also function as a trap, although it can be argued that measuring specific end products of the trapping of RS (reactive species) by endogenous molecules is the same as measuring "biomarkers". Ascorbate reaction with free radicals is one example; another is urate, which is readily oxidized by a range of RS[39], including proxynitrite[40]. Several groups have used urate as a "selective" scavenger of $ONOO^-$ in animal studies, neglecting the fact that it reacts with many species[41]. One of urate's oxidation products, allantoin, can be measured in human body fluids and its plasma levels are elevated in conditions associated with oxidative stress, such as chronic inflammation, diabetes, premature birth, iron overload, chronic hearth failure and exercise[42-45]. Allantoin can also be measured in urine[46] and cerebral microdialysis fluid[47]. Levels of allantoin rise in the human muscle during exhaustive exercise, presumibly due to oxidation of urate by RS generated during exercise[48]. Allantoin measurement may be one of the more promising techniques for human use, since human urate levels in vivo are high and urate reacts with a wide range of RS[3].

Nuclear Magnetic Resonance (Nmr) Based "Metabolomics/Metabonomics" Analysis of Biofluids

Metabolomics (also called metabonomics) is defined as "the quantitative measurement of the dynamic multiparametric metabolic response of living systems to pathophysiological stimuli or genetic modification"[49]. High resolution nuclear ^{1}H-magnetic resonance (NMR) spectroscopic analysis of biofluids allows simultaneous detection of hundreds of low molecular weight species within a sample of body fluid, resulting in the generation of a metabolic profile or NMR "fingerprint" that is altered characteristically in response to physiological status[50]. Once NMR spectra are obtained, the highly complex spectra are analyzed using pattern recognition and multivariate statistical methods to produce models for samples classification[51]. This technology has

been widely applied to toxicology studies with a range of biological fluids, such as urine and plasma, in both experimental animals and humans[52,53]. Statistical analysis of urine samples has been shown to result in inherent clustering behaviour for drugs and toxins acting on different organs, such as liver or kidney, or having different toxic mechanisms. These cluster analyses are similar to those currently being developed for gene array expression analysis and proteomics, and have been demonstrated to classify toxins in test samples correctly. Metabolomic analysis, therefore, seems particularly suited to the analysis of biofluids from clinical/laboratory studies with the potential to measure simultaneously a range of oxidative stress products and other inflammatory markers. Analysis of such samples may lead to the identification of novel single biomarkers of interest for wider study in patient populations. Brindle and colleagues[54] studied serum metabolome obtained from coronary heart disease and healthy individuals. Moreover, application of metabolomics allows the simultaneous analysis of multiple end products and it may be that these "fingerprints" characteristic of disease are a more powerful and robust means by which to stratify disease severity, progression and to assess drug efficacy than the analysis of any single marker over a patient population.

Online Measurements of Oxidative Stress Biomarkers

Infrared laser spectroscopy is a promising method for free radical research, enabling online measurement of oxidative stress biomarkers, such as lipid peroxidation products, with high sensitivity and efficiency. Murtz et al[55] have developed real-time analysis of volatile ethane fractions in exhaled breath (gaseous molecular species) using laser absorption spectroscopy. The group monitored the ethane fraction exhaled by a smoker after smoking a cigarette every 30 min over a period of 4 h, and observed a strong increase and subsequent decay of the ethane fraction after smoking. This method is unique, with very sensitivity and specificity for rapid and precise breath testing. The detection limit is 300 volume parts per trillion ethane in exhaled breath with an integration time of 5 s. Another major advantage of this method is that it allows the analysis of biomarkers without pre-concentration or pre-treatment of exhaled breath. The development and introduction of this biosensor technique for immediate analysis of EBC (exhaled breath condensate) has potential for undertaking real-time EBC monitoring of oxidative stress in animal research and clinical practice. Newer techniques, such as online masurements using sensitive biosensors, are being developed for more reproducible measurement of hydrogen peroxide. For example, it is possible to detect hydrogen peroxide online (real-time) using a silver electrode or polymer with horseradish peroxidase[56,57]. A similar enzyme detector system also may be developed for real-time monitoring of 8-isoprostane.

Lipid Peroxidation

Lipid peroxidation is a complex process whereby polyunsaturated fatty acids (PUFAs) in the phospholipids of cellular membranes undergo reaction with oxygen to yield lipid hydroperoxides (LOOH). The reaction occurs through a free radical chain mechanism initiated by the abstraction of a hydrogen atom from a PUFA by a reactive free radical, followed by a complex sequence of propagative reactions.

The LOOH and conjugated dienes that are formed can decompose to form numerous other products including alkanals, alkenals, hydroxyalkenals, malondialdehyde (MDA) and volatile hydrocarbons[58]. Lipid peroxidation is often the first parameter to which researchers turn when they wish to prove the involvement of free radicals in cell damage. There are several reasons for this. First, lipid peroxidation is an extremely likely consequence if a reactive free radical is formed in a biological tissue where PUFAs are generally abundant. Second, lipid peroxidation is a very important process in free radical pathology as it's so damaging to cells. Finally, a vast array of analytical techniques has been developed to measure lipid peroxidation, though not all of them are applicable to the situation in vivo[59].

For all assays it's important that artifactual changes in lipid peroxidation products are minimised both during and after sampling. Radical-scavenging antioxidants and metal-chelating agents are added to prevent the further formation of lipid hydroperoxides and the breakdown of existing lipid hydroperoxides. Enzymic reactions

that may affect levels of products are inhibited by mixing the sample with acid or organic solvents. It is generally advisable to assay samples as quickly as possible after taking them, since a tendency to increased lipid peroxidation on storage has been reported[60,61]. Conversely, lipid hydroperoxides can deteriorate on storage[62].

The lipid peroxidation's reaction in biological membranes causes impairment of membrane functioning[63,64], decreases fluibility, inactivation of membrane-bound receptors and enzymes and increases non-specific permeability to ions such as Ca^{2+}. Additionally, lipid hydroperoxides decompose upon exposure to iron or copper ions, simple chelates of these metal ions (e.g. with phosphate esters), haem, and some iron proteins, including haemoglobin and myoglobin. Products of these complex decomposition reactions include hydrocarbon gases (such as ethane and pentane), radicals that can abstract further hydrogen atoms from fatty acid side chains and cytotoxic carbonyl molecules, of which the most harmful are the unsaturated aldehydes such as 4-hydroxy-2-trans-nonenal. Indeed, a major contributor to extracellular antioxidant defence in mammals is the existence in body fluids of proteins that bind copper ions (caeruloplasmin and albumin), iron ions (transferrin), haem (haemopexin) or haem proteins (haptoglobins) and stop them from accelerating lipid peroxidation and other free radical reactions[65,66].

Biomedical Lipid Peroxidation

The measurement of putative "elevated end products of lipid peroxidation" in human samples is probably the evidence most frequently quoted in support of the involvement of free radical reactions in tissue damage by disease or toxins. Studies beginning in the 1950s provided good evidence that several halogenated hydrocarbons exert some, or all, of their toxic effects by stimulating lipid peroxidation *in vivo*. This is particularly true of carbon tetrachloride and probably true of bromobenzene.

This early choice of halogenated hydrocarbons for study was both casual (in that it gave early emphasis to the important biological role of free radical reactions) but also unfortunate, since later studies have shown that most toxins stimulating oxidative damage to cells do not appear to act by accelerating the bulk peroxidation of cell membrane lipids[67]:

$$\text{toxin} \rightarrow \text{lipid peroxidation} \rightarrow \text{cell damage}$$

Rises in intracellular "free" Ca^{2+}, with consequent activation of proteases and nucleases and formation of "membrane blebs", oxidation of critical –SH groups and DNA damage are often more relevant toxic events than is the bulk peroxidation of membrane lipids[68].

Lipid peroxidation is often (but by no means always) a late event, accompanying rather than causing final cell death[69]. Indeed, cell and tissue destruction (whether mediated by radicals or otherwise) can often lead to more lipid peroxidation because antioxidants are diluted out and transition metal ions that can stimulate the peroxidation process are released from disrupted cells.

This stimulation of lipid peroxidation as a consequence of tissue injury can sometimes make a relevant contribution to worsening the injury. For example, in atherosclerosis there is good evidence that lipid peroxidation occurs within the atherosclerotic lesion and leads to foam cell generation and hence lesion growth[70]. In traumatic injury to the brain and spinal cord, good evidence again exists that iron ion release into the surrounding area, and consequent iron-stimulated free radical reactions, worsen the injury[71].

It is equally likely that in some other diseases, the increased rates of free radical reactions induced as a result of tissue injury make no significant contribution to the disease pathology. Each proposal that free radicals in general, or lipid peroxidation in particular, are important contributors to the pathology of a given disease must be carefully evaluated on its merits. This obviously requires accurate methodology for measuring these processes in cells, tissues and whole organisms.

Detection and Measurement of Lipid Peroxidation: General Principles

Oxidation of lipids can be measured at different stages, including:

1. losses of unsaturated fatty acids;
2. measurement of primary peroxidation products;
3. measurement of secondary carbonyls and hydrocarbon gases.

Between phases 1, 2 and 3 it is possible detect carbon-and oxygen-centred radicals (by ESR combined with the use of "spin traps") and identify these radicals by their ESR spectra[72].

It should be noted that the chemical composition of the end products of peroxidation will depend on the fatty acid composition of the lipid substrate used and upon what metal ions (if any) are present. Thus, copper and iron ions give different end-product distributions and so the selection of only a single test to monitor peroxidation can give misleading results. Copper salts efficiently decompose peroxides, leading to low concentrations of detectable peroxides but high amounts of some carbonyl molecules containing amino groups to form fluorescent products. The most accurate assays of lipid peroxidation are the most chemically sophisticated ones. They also require the most sample preparation and great care (e.g. by working under nitrogen) has to be taken to ensure that peroxidation does not occur during the handling of lipid material.

Measurement of Lipid Hydroperoxides

LOOH are the major initial molecular products of lipid peroxidation and can be measured in plasma by a lot of techniques. A sensitive and specific assay is based on the capacity of LOOH to initiate the cyclooxygenase reaction catalysed by activation of prostaglandin endoperoxide synthase and uses an oxygen electrode[73]. Another sensitive, even if expensive, method to measure plasma LOOH uses gas chromatography-mass spectrometry (GC/MS) and involves reduction of LOOH to the hydroxy acids with triphenylphosphine[74].

Plasma LOOH can be measured using commercially-available assay kits. One such kit relies upon the reaction of LOOH with a haem compound, concomitantly oxidising a precursor to produce methylene blue which is measured spectrophotometrically. Although very simple and quick, only total hydroperoxide concentrations are measured and results do not relate well with some other measures of lipid peroxidation[75].

Some methods have been developed that can distinguish specific or different classes of LOOH. These are based on separation according to lipid class of the various hydroperoxides in a Folch lipid extract of plasma by high performance liquid chromatography (HPLC) and measurement of the chemiluminescence produced during their breakdown in the presence of either luminol[76] or isoluminol[77].

The pathogenic role of lipid peroxidation in the reperfusion injury of the liver is still controversial. Caraceni et al[78] wanted to determine whether the damage caused by oxygen free radicals during reoxygenation in perfused rat hepatocytes is related to lipid peroxidation. Superoxide anion was detected by lucigenin-enhanced chemiluminescence. Lipid peroxidation and cell injury were assessed by the release of malondialdehyde and lactic dehydrogenase. Upon reoxygenation following 2.5 h of anoxia, isolated hepatocytes generated considerable amount of O_2^-. Following O_2^- formation, a significant increase in malondialdehyde release was measured. Cell injury was temporally delayed relative to O_2^- generation, but preceded the occurrence of a significant lipid peroxidation. Treatment with Vitamin E abolished lipid peroxidation but had no effect upon superoxide anion formation and cell injury. These results suggest that in perfused rat hepatocytes non-peroxidative mechanisms are more important than peroxidative mechanisms in the pathogenesis of the early phases of reoxygenation injury.

Gasbarrini et al[79] wanted to determine whether the formation of oxygen free radicals occurs in murine osteoblast-like cells (MC3T3-E1) exposed to anoxia and reoxygenation and to explore its relation to the reoxygenation injury. Cells were cast in agarose and perfused with oxygenated Krebs-Henseleit bicarbonate buffer. Anoxia was obtained by shifting the gas phase of the media to 95% N_2-5% CO_2. Oxygen free radicals were detected by enhanced chemiluminescence: anion superoxide or hydrogen peroxide was measured by adding lucigenin or luminol plus horseradish peroxidase to the media, respectively. Cell injury was assessed by the rate of lactate dehydrogenase release. During the control period, lucigenin and luminol plus horseradish chemiluminescences were 15 ± 1 nA per chamber and 20 ± 2 nA per chamber, respectively. and lactate dehydrogenase release was 10 ± 1 mU per minute. During anoxia, both chemiluminescences dropped to background levels, although lactate dehydrogenase release increased progressively to 38 ± 7 mU per minute. During reoxygenation, O_2 formation increased sharply to 45 ± 6 nA and decreased to control levels; H_2O_2 production increased slowly, reaching 42 ± 7 nA at the end of the reoxygenation period; lactate dehydrogenase declined progressively to control values. These data show that osteoblastlike cells produce measurable amounts of superoxide and hydrogen peroxide radicals during reoxygenation. Because lactate dehydrogenase release did not appear to relate to chemiluminescence, oxyradical flux may serve as a signal for other events that eventually lead to cell injury.

Ojetti et al[80] evaluated the combined use of chemiluminescence and gastroendoscopy techniques and to assess the real-time production of free radicals during ischemic damage of the gastric wall in an animal model. For the experiment, an optical junction was set up between a fibroendoscope and a luminograph apparatus. Three pigs were submitted to gastrofibroendoscopy before, during and after 30 min of clamping of the coeliac artery. Under basal conditions, at the end of the ischemic phase and at the beginning of reperfusion, 1 mM of lucigenin, a specific superoxide enhancer, was injected in the left gastric artery of the animal. The endoscopic live images and chemiluminescence emission were recorded and successively superimposed to measure rate and spatial distribution of photon emission (photons/s). Free radical production was not observed under basal conditions or during the ischemic phase, but significantly increased during reperfusion reaching a maximum peak after 15 min (0.6 ± 0.2 photons × 10(5)/s) and decreased progressively thereafter. The superimposition of live and chemiluminescence images allowed the determination of the regional production rate and distribution of photons.

The LOOH are identified by comparison with authentic standards: although hydroperoxides of free fatty acids are commercially available, those of others lipids (e.g. phospholipids, cholesterol esters) must be synthesised, which is both time-consuming and inconvenient. The assays have picomolar sensitivity and additionally, LOOH is achieved by monitoring conjugated dienes either at 234 nm or by measuring the complete UV absorption spectrum of the sample with a diode-array detector. Alternatively, treatment with the reducing agent sodium borohydride will eliminate the chemiluminescent signal. The assay is relatively specific for hydroperoxides although ubiquinols in human plasma produce a positive response.

Accurate measurement of LOOH is difficult due to their rapid degradation *in vitro*. It is extremely important to minimise this by the addition of antioxidants and quick processing of samples at 4°C which is often not possible in a clinical situation. Also, these HPLC methods, although specific and sensitive, are time-consuming in their analysis and preparation of standards and are best used only when information on individual hydroperoxides is required. Free fatty acid- and cholesterol-hydroperoxides have been detected in patients with adult respiratory distress syndrome[81] or undergoing angioplasty.

Measurement of Conjugated Dienes

LOOH possess a conjugated diene structure having a characteristic UV absorption around 234 nm. Measurement of this absorbance has been extremely useful as an index of peroxidation in pure lipid systems and in tissue preparations from experimental animals. There are, however, difficulties in measuring conjugated dienes in biological materials because many of the other substances present (e.g. haem proteins) absorb strongly in the UV and create a high background. This is partly eliminated by extraction of conjugated dienes into an organic solvent such as chloroform/metahnol. However, PUFA, themselves, and carbonyl compounds produced from the breakdown of LOOH absorb UV light strongly at about 210 nm so that the conjugated diene absorbance appears as a shoulder on the PUFA absorbance spectrum[82].

Measurement is also complicated by the relatively low levels of conjugated dienes normally present in human plasma. A second derivative spectroscopy method[83] allows greater sensitivity, since the conjugated diene shoulder that appears in the ordinary spectrum translates into a sharp minimum peak that is more easily measurable. The increased resolution of this technique may allow discrimination between the different conjugated diene structures present. However, most (90%) of the conugated diene in human plasma is a non-oxygen-containing isomer of linoleic acid (9, 11-octadecadienoic acid) that can be assayed specifically by HPLC[84]. This product is not found in the plasma of animals subjected to oxidative stress and may be of dietary origin or produced by the metabolism of gut bacteria. Application of conjugated diene methods to human body fluids is thus probably not measuring lipid peroxidation and is not recommended for human studies.

Measurement of Thiobarbituric Acid Reacting Substances (TBARS) and Malondialdehyde (MDA)

The thiobarbituric acid (TBA) assay is the most common and easiest method used as an indicator of lipid peroxidation and free radical activity in biological samples. The assay is based upon the reaction of two molecules of TBA with one of MDA, a physiologic ketoaldehyde produced by peroxidative decomposition of unsaturated lipids as a byproduct of arachidonate metabolism. The excess MDA produced as a result

of tissue injury can combine with free amino groups of proteins (MDA reacts mainly with Lys residues by Michael addition), producing MDA-modified protein adducts. Modification of proteins by MDA could conceivably alter their biological properties. Moreover, MDA-modified proteins are immunogenic, and autoantibodies against MDA-modified Lys residues have been detected in the sera of rabbits and humans. Some studies have reported that the titer of these autoantibodies is related to the burden of, and may predict progression of, atherosclerosis and myocardial infarction. Higher titers of autoantibodies have also been correlated to coronary artery disease[11].

There are a lot of variations[85] but basically the sample is heated with TBA under acidic conditions and the amount of pink-coloured MDA-TBA adduct produced is measured at 532 nm. For increased sensitivity, the complex can be extracted into an organic solvent such as butanol and measured fluorometrically[86]. In a few experimental systems the TBA test has been demonstrated actually to be measuring MDA itself. In uncharacterised systems it is usual to refer to the assay of TBA-reactive substances (TBARS) as the test is not specific for MDA.

The test itself is very simple and quick but its application to biological samples can be problematic. The exact conditions of the test are very important. Biological samples normally contain only a small amount of free MDA and in tests where the unseparated sample is incubated for a prolonged time the majority of the MDA measured is formed by the decomposition of LOOH and further peroxidation during the heating stage of the assay itself. The widely used "Yagi test" that utilise TBA[87], are probably assays of lipid hydroperoxides. Various biological compounds react with TBA and the fluorescence method may be more selective than spectrophotometry. Other factors that can markedly affect the apparent concentration of TBARS in plasma include the iron content of reagents used in the analysis and the storage of samples at –70°C, although the addition of EDTA to chelate iron may reduce variability. To further minimise the problems related with the TBA test, the MDA-TBA adduct may be measured by HPLC and GC although this is time-consuming, involving complex sample preparation to remove contaminants or sample extraction into organic solvents to improve sensitivity and peak separation.

Direct assessment of free MDA is most reliably done by HPLC but the technique requires very careful handling of the sample. However, MDA is a minor product of lipid peroxidation and is readily metabolised; it is therefore not a promising subject for the analysis of lipid peroxidation *in vivo*.

Plasma MDA concentrations are increased in diabetes mellitus and MDA can be found in the atherosclerotic plaques promoted by diabetes[88]. Increasead MDA concentrations have been found in samples from women with preeclampsia[89], in plasma and breath condensates from asthmatics[90] and in the brains of patients suffering from Parkinson disease (PD), whereas increased TBARS have been observed in plasma of patients with amyotrophic lateral sclerosis (ALS) as well as in Alzheimer's patients[91].

Measurement of Aldehydes Other Than MDA

A great number of various aldehydes are produced during lipid peroxidation and they differ greatly in their biological activity and capacity to cause further damage. The different classes of aldehydic peroxidation products in biological samples can be quantified by a method developed by Esterbauer and Cheeseman[86]. The aldehydes are derivatised with dinitrophenylhydrazine (DNPH), the various classes of different polarity (e.g. alkanals, hydroxyalkenals, alkenals) separated by TLC (Thin Layer Chromatography) and the individual aldehydes then resolved by HPLC with UV detection. An alternative procedure involves HPLC separation of the fluorescent cyclohexanedione (CHD) derivatives of the aldehydes[92]. These techniques are generally extremely time-consuming and quite expensive and are unlikely at present to be used as routine measures of lipid peroxidation. They are only likely to be used where it is necessary to know the full range of aldehydes produced in a particular condition.

Hydroalkenals, such as 4-hydroxynonenal (HNE), are probably the most important end products of the lipid peroxidation process in terms of cytotoxicity. HNE is a major and toxic aldehyde produced by free radical attack on ω-6 polyunsaturated fatty acids (arachidonic, linoleic and linolenic acids)[93] and is considered a second toxic messenger of oxygen free radicals[94,95]. HNE undergoes many reactions with proteins, peptides, phospholipides and nucleic acids; it therefore has a high biological activity and exhibits

various cytotoxic, mutagenic, genotoxic and signal effects, including inhibition of protein and DNA synthesis, inactivation of enzymes, stimulation of phospholipase C, reduction of gap-junction communication, stimulation of neutrophil chemotaxis, modulation of platelet aggregation and modulation of the expression of some genes[96]. Additionally, HNE may be an important mediator of oxidative stress-induced apoptosis, cellular proliferation and signaling pathways[97]. HNE is permanently formed at basal concentrations under physiologic conditions, but its production is greatly enhanced in pathologic conditions associated to lipid peroxidation. Under physiologic conditions, the cellular concentrations of HNE ranges from 0.1 to 3 μmol/L. Under conditions of oxidative stress, HNE concentrations are significantly increased in plasma, some organs and cell types[98]. During heavy stress, e.g., in patients with severe rheumatologic diseases such as rheumatoid arthritis, systemic sclerosis, lupus erythematosus, chronic lymphedema or chronic renal failure, serum HNE is increased to concentrations up to 3- to 10-fold higher than physiologic concentrations[99]. HNE and acrolein, compound present in some environmental sources like cigarette smoke, are highly reactive toward proteins (particularly, HNE is much more reactive to proteins than to DNA), forming stable covalent adducts with His, Lys and Cys residues through Michael addition; these adducts are known as advanced lipoxidation end products (ALEs)[100,101]. This process introduces carbonyl groups into proteins.

Furthermore, concentrations of acrolein- and HNE-protein adducts are increased in cardiovascular disease[102]. Acrolein reacts with Lys residues of apolipoprotein A-I (apoA-I), the major protein of HDL, which plays a relevant role in mobilizing cholesterol from artery wall macrophages. Acrolein adducts colocalize with apoA-I in human atherosclerotic lesions. Moreover, the capacity of acrolein-modified apoA-I to remove cholesterol from cultured cells is impaired, suggesting that carbonylation might interfere with the normal function of apoA-I in promoting cholesterol removal from artery wall cells, thus playing a critical role in atherogenesis[103].

Increased concentrations of HNE-protein adducts have been reported in the lungs of smokers with and without chronic obstructive pulmonary disease (COPD). Notably, HNE concentrations in the pulmonary epithelium, airway endothelium and, particularly, neutrophils of COPD patients were found to be inversely related to lung function[104]. COPD patients also had higher diaphragm concentrations of both protein carbonyls and HNE-protein adducts. Furthermore, a negative correlation was found between carbonyl groups and airway obstruction (i.e., concentrations of reactive carbonyls related to disease severity) and between HNE-protein adducts and respiratory muscle strength (i.e., HNE-protein adduct formation associated to respiratory muscle function)[105].

Because HNE is such an important, biologically active product it may be of interest to measure specifically. HNE can be measured by HPLC with UV detection or GC-MS which is more sensitive but expensive.

Isoprostanes

F_2-Isoprostanes (F_2-IsoPs), isoprostanes containing an F-type prostane ring, are a family of, theoretically, 64 prostaglandin $F_{2\alpha}$-like molecules produced *in vivo*, primarily *in situ*, by nonenzymatic free-radical-catalyzed peroxidation of esterified arachidonic acid and then cleaved and released into the circulation by phospholipases(s) before excretion in the urine as free isoprostanes. Reports have shown that F_2-IsoPs are authentic, reliable biomarkers of lipid peroxidation and are useful *in vivo* indicators of oxidative stress in various clinical conditions, such as acute and chronic inflammation, ischemia/reperfusion injury, diabetes and atherosclerosis[106-110]. F_2-IsoPs have also been used to assess *in vivo* oxidative response to some drugs, antioxidants or dietary interventions for their free-radical-scavenging properties. Various techniques for F2-IsoP quantification are available (GC-MS)[111]. Additionally, to being markers of oxidative stress and antagonists of the action of prostaglandins, they may also exert unique biological effects.

A tissue that does not contain isoprostanes is yet to be reported. Isoprostanes have also been found in measurable quantities in most of the biological fluids analyzed, including plasma, urine, synovial fluid, bronchoalveolar fluid, bile, lymph, microdialysis fluid from various organs, and amniotic, pericardial, and seminal fluid, even if plasma and urine are the sample types that are commonly analyzed, being the most convenient to obtain and the least invasive[112].

At present, measurement of F_2-IsoPs is regarded as one of the most reliable approaches for the assessment of oxidative status or free-radical-

mediated lipid peroxidation *in vivo*. Available data indicate that quantification of F_2-isoprostanes in either plasma or urine gives a highly precise and accurate index of oxidative stress[113]. Whereas the biological validity of F_2-IsoPs as biomarkers of oxidative status is well established, it is technically quite complicated to measure F_2-IsoPs and their metabolites in body fluids and some limits with respect to their measurement must be taken into account. F_2-IsoPs are chemically stable *in vivo* and *ex vivo*, but once they produced and released into the circulation, they are fastly metabolized (even if not as quickly or as extensively as prostaglandins) and eliminated. Their fast disappearance from plasma may prevent practical application. Current techniques: GC-MS, GC-tandem MS (GC-MS/MS), liquid chromatography (LC)-MS, LC-MS/MS, enzyme immunoassays and radioimmuno assays (RIAs), are able to detect the steady-state concentrations of F_2-IsoPs in many tissues and body fluids, even in the basal state, concentrations after any degree of oxidant stress or lipid peroxidation in vivo. Different internal standards (^{18}O- or ^{2}H-labeled analogs of specific isoprostane isomers) are available from commercial sources to quantify the isoprostanes by MS techniques.

Alternative methods have also been developed to quantify F_2-IsoPs by immunologic techniques (RIAs and enzyme immunoassays) and a few immunoassay reagent sets are commercially available. A potential drawback of these techniques is that limited information is currently available regarding their precision and accuracy. Moreover, few data exist comparing F_2-IsoP concentrations measured by immunoassays with MS results. Futhermore, the sensitivity and/or specificity of these assays may vary substantially among manufacturers. However, even if MS techniques of F_2-IsoP quantification are considered the "gold standard", immunoassays have expanded research in this area because of their low cost and relative ease of use. Additionally to commercial immunoassays, some researchers have generated polyclonal antibodies and have developed assays for F_2-IsoP[114]. It appears that there is good correlation between these techniques and MS.

Various analytical methods are available for the analysis of isoprostanes, the most sensitive, highly specific and reliable technique being GC with negative-ion chemical ionization (NICI) MS. For quantification of lipid peroxidation, measurements of F_2-IsoP have a clear advantage over currently available techniques such as assays for MDA, TBARS, lipid hydroperoxides or conjugated dienes, which are hampered by some methodologic limits.

F_2-IsoPs are very well suited as biomarkers of oxidative stress for the following reasons:

1. The *in vivo* formation of isoprostanes increases as a function of oxidative stress[115,116].
2. They can be measured accurately down to picomolar concentrations with analytical methods such as GC-MS, GC-MS/MS, LC-MS, LC-MS/MS or RIA. The first 4 methods can easily differentiate among the various types of isoprostanes, but they require extensive preparation of the material (e.g., phospholipid extraction and alkaline hydrolysis) and/or expensive instrumentation. RIAs are somewhat easier to perform and are widely available commercially. However, many of these are not able (or have not been shown to be able) to distinguish between the prostanoids and the isoprostanes, much less between the different types of isoprostanes.
3. They are stable in isolated samples of body fluids, including urine and exhaled breath condensates, providing an exceedingly noninvasive route for their measurement.
4. Their measured values do not exhibit diurnal variations and are not affected by lipid content in the diet[117,118]. However, they do vary markedly in clinical and experimental conditions characterized by oxidative stress and closely parallel disease severity. Some diurnal variation in urinary F2-IsoP excretion does occur within individual humans, even if this variation is not present when F_2-IsoPs are evaluated on a group level. Furthermore, even if pooled urine samples are likely preferable, F_2-IsoPs determined in urine collected in the morning or in several spot urine samples adequately represent the daily F2-IsoP excretion.
5. They are specific products of peroxidation.
6. They are present in detectable amounts in all healthy tissues and biological fluids, thus allowing definition of a reference interval.

Because of free-radical-catalyzed conversion of arachidonic acid to isoprostanes, precautions must be taken to avoid artifactual formation during sample storage and processing. Blood plasma samples contain considerable amounts of arachidonic acid, mainly esterified to membrane phospholipids. Storage of these samples at –80°C and addition of antioxidants (e.g., butylated hydroxy-

toluene and triphenylphosphine) during sample preparation is therefore recommended. Moreover, isoprostanes in blood samples may occur as free fatty acids or esterified to phospholipids or lipoproteins. Thus, one has to distinguish between the two fractions of isoprostanes in human blood, i.e., free and total (free plus esterified). Analysis of the esterified molecules requires hydrolysis to yield the free derivatives. Because urine samples have a very low lipid content, autooxidation is not a problem. Nevertheless, as a precaution, samples should be supplemented with EDTA and 4-hydroxy-2,2,6,6-tetramethylpiperidine 1-oyl (4-hydroxy-TEMPO) and stored at –20°C.

Different diseases and experimental conditions have been shown to be related to marked increases in urinary, plasma and tissue concentrations of F_2-IsoPs. However, it has been suggested they should be considered not just mere markers, but also "mediators" of disease, as they evoke important biological responses in virtually every cell type found within the lung. Infact, the isoprostanes may mediate many of the features of the disease states for which they are used as indicators. 8-iso-prostaglandin$F_{2\alpha}$, the biologically active component, is produced in great amount in otherwise "normal" individuals exposed to cigarette smoke, allergenes, ozone or hyperoxia and during ventilated ischemia. It is also markedly increased, serving as a biomarker, in the bronchoalveolar lavage (BAL) fluid, plasma, urine or exhaled breath condensate (a noninvasive technique for direct measurement of oxidative stress in the lungs) in some pulmonary diseases such as asthma, COPD, interstitial lung disease, cystic fibrosis, pulmonary hypertension, acute chest syndrome, sickle cell disease, acute lung injury (including acute respiratory distress syndrome, ARDS) and severe respiratory failure in infants as well as in healthy chronic smokers[119,120]. Systemic and synovial fluid concentrations of 8-iso-$PGF_{2\alpha}$ are higher in patients with rheumatoid arthritis, psoriatic arthritis, reactive arthritis and osteoarthritis than in healthy controls. Plasma concentrations are increased in patients with cardiovascular disease and it has been suggested that this may be a useful biomarker of risk[121]. Similarly, some cardiovascular conditions feature marked increases in F_2-IsoP concentrations, including during and after cardiopulmonary bypass, renal, cerebral and myocardial ischemia-reperfusion injury, unstable angina, heart failure, coronary heart disease, acute ischemic stroke, hypercholesterolemia and atherosclerosis. Urinary 8-iso-$PGF_{2\alpha}$, measured by GC-MS/MS, was found to be a novel, sensitive and independent risk marker in patients with coronary heart disease, additionally to know risk factor of this pathology, i.e., diabetes mellitus, hypercholesterolemia, hypertension, obesity and smoking[122]. Increased concentrations of 8-iso-$PGF_{2\alpha}$ have also been found in plasma or urine samples from patients with type 2 diabetes.

Lipid peroxidation in Human Material: Past and Future

Despite the problems that can occur with assays such as the TBA test, diene conjugation and light emission, they usually work adequately when applied to measurements of peroxidation in liposomes, microsomes or other isolated membrane fractions, provided that one is alert to the various artefacts that can arise[123]. Much more serious problems occur when these assays are applied to human body fluids or to tissue extracts.

By far the most misleading assay to use on human material, especially plasma, is the TBA test. Plasma contains many compounds that react in the TBA assay, including bile pigments, amino acids and carbohydrates. Some of these substances (e.g. bile salts) produce a different chromogen, and this interference can be overcome by separating out the authentic $(TBA)_2$-MDA adduct (e.g. by HPLC) before measurement. However, this solves only part of the problem because some molecules (especially amino acids and sugars) react in the assay to form an authentic $(TBA)_2$-MDA adduct. The lack of specificity of the TBA assay when applied to plasma is dramatically illustred by a simple experiment performed by Lands et al[124]. Using the cyclooxygenase assay these researchers measured the lipid peroxide amount of some human plasma samples as about 0.5 μM. Expressing the results of a TBA test on the same samples as "peroxide equivalents"gave a value of 38 μM. When specific chemical assays are used, the authors and others[125] find that human plasma, freshly taken from healthy subjects, has less than 0.1 μM lipid peroxide. This is perhaps not surprising, since even if peroxides do form *in vivo* and enter the circulation, they can be quickly cleared. For example, although lipid peroxidation is thought to be relevant in atherosclerosis, it seems to be peroxidation in the arterial wall that matters, not peroxidation in the bulk plasma. Thus, some of the earlier suggestions that circulating lipid peroxides

kill vascular endothelial cells and initiate atherosclerosis need to be re-evaluated.

In order to know as much as possible about the real occurrence of lipid peroxidation in human material, it is important to use methods that give specific chemical information about what is present. Indeed, food scientists have followed this principle for years. Thus various groups are separating the different peroxidation products before measuring them. This is often done by HPLC; for example, HPLC methods for measuring cytotoxic aldehydes are available[126]. However, conversion of material into volatile derivatives, separation by gas chromatography and identification by mass spectrometry is likely to give more precise chemical information when complex mixtures are being studied[127,128]. Thus, derivatization and mass spectrometry have been used to characterize peroxidized fatty acids and cholesterol oxidation products in human atherosclerotic lesions[129].

Specificity can also be achieved by the use of antibody techniques, particularly monoclonal antibodies. Thus, antibodies directed against low-density lipoprotein that has undergone peroxidation or has been treated with 4-hydroxynonenal bind to rabbit atherosclerotic lesions. Additionally, low-density lipoproteins eluted from such lesions can bind to antibody specific for MDA-treated low-density lipoproteins. Antibody-based methods can also be applied to plasma samples. Using such specific techniques, the exact role played by lipid peroxidation in cell injury and death mediated by toxins and in human disease should at last become clearer[130].

Measurement of Protein Damage

Reactive free radicals can modify amino acid residues of proteins and lead to cross-linking, changes in conformation and loss of function. Oxidatively damaged proteins are likely to be removed rapidly by proteases rather than accumulate to readily-detectable levels.

Glutathione and S-Glutathionylated Proteins

Because blood glutathione concentrations may reflect glutathione status in other, less accessible tissues, measurement of both reduced glutathione (GSH) and glutathione disulfide (GSSG) in blood has been considered relevant as an index of whole-body GSH status and a useful indicator of oxidative stress status in humans. Different techniques have been optimized to identify and quantify glutathione forms in human samples, including spectrophotometric, fluorometric and bioluminometric assays, often applied to HPLC analysis, as well as the more recently developed GC-MS and HPLC-electrospray ionization-MS techniques[131,132]. Futhermore, a wide variety of techniques have been introduced for the determination of GSH and GSSG in human blood, the measurement of which, particularly that of GSSG, could be overstimated if samples are not properly processed[133,134]. A specific warning has to be addressed to correct sample manipulation and prevention of artifactual GSH oxidation. The authors have shown that the main artifact results from sample acidification (for protein separation) without prevention of artificial oxidation of –SH groups by blocking with alkylating agents. Actually, many published articles reporting concentrations of GSH, GSSG and S-glutathionylated proteins in blood, both from healthy controls and patients affected by various pathologies, are not artifact free, which makes the conclusions reached in these articles meaningless. Consequently, the notion that some pathophysiologic conditions can alter and/or be influenced by the GSH/GSSG homeostasis of blood still needs to be confirmed.

It is well known that a decrease in GSH concentration may be associated with ageing[135] and the pathogenesis of many diseases, including rheumatoid arthritis, amyotrophic lateral sclerosis, acquired immune deficiency syndrome, Alzheimer's disease, alcoholic liver disease, cataract genesis, respiratory distress syndrome, cardiovascular disease and Werner syndrome. Furthermore, there is a drastic depletion in cytoplasmic concentrations of GSH within the substantia nigra of Parkinson's disease patients[136]. Depletion of total GSH (GSH + 2 GSSG + protein-bound glutathione) and a decreased GSH:GSSG ratio are indicators of oxidative/nitrosative stress in ischemic brain disease[137], cardiovascular diseases[138] and cancer[139], and decreased concentrations of GSH are consistently observed in both types of diabetes mellitus. Low GSH concentrations and a high GSSG:GSH ratio have been measured in blood of patients with various diseases, including breast and lung cancer, coronary heart surgery and preeclampsia[140]. The GSH system is also altered in lung inflammatory conditions. For example, GSH concentrations are increased in the epithelial lining fluid of

chronic smokers, whereas they decrease fastly in patients with mild asthma during an asthma exacerbation. Similarly, GSH concentrations in the epithelial lining fluid are decreased in idiopathic pulmonary fibrosis, asbestosis, acute respiratory distress syndrome and in HIV-positive patients[141]. Total GSH was markedly decreased in older patients with chronic diseases[142], the deficit being attributable to lower GSH concentrations and not to higher GSSG. These results suggested that the decrease in GSH might be used to monitor the severity and progress of the diseases. Conversely, total GSH concentrations are high in the blood of elderly persons who are in excellent physical and mental breath[143].

Tyrosine Oxidation, Nitration and Halogenation

The toxicity of NO is enhanced by its reaction with a superoxide to form $ONOO^{-}$[144]. It or secondary metabolites can cause tyrosine nitration in protein, creating nitrotyrosine, a footprint detectable *in vivo*.

Analysis of 3-nitrotyrosine (NO_2-Tyr), a stable marker for $NO^{-}\bullet$ derived oxidants, and halogenated Tyr products such as 3-chlorotyrosine (Cl-Tyr) or 3-bromotyrosine has been performed in some diseases and different techniques have been developed for such measurements[145-149]. The quantitative measurement of NO_2-Tyr is hindered by severe methodologic problems. The most of the data available on NO_2-Tyr in tissues and fluids have been derived from antibody-based techniques, which however, are often not rigorously validated. Therefore, such immunologic techniques should be considered semiquantitative and the results interpreted accordingly. HPLC with ultraviolet detection does not provide adequate sensitivity or specificity for biological materials. In contrast, HPLC with electrochemical detection, LC-MS/MS, electron capture-negative chemical ionization (EC-NCI) GC-MS and GC-MS/MS are able to quantify NO_2-Tyr in biological materials and human plasma[150-152].

At present, only MS/MS-based techniques, both GC-MS/MS and LC-MS, provide reliable values for circulating and excreted NO_2-Tyr, with LC-MS/MS being at present considerably less sensitive than GC-MS/MS and that the basal concentrations obtained by this analytical approach may serve as reference values.

Another methodologic problem is considerable interference by coeluting molecules, which can eliminated only by use of MS/MS.

Increased concentrations of stable halogenated Tyr residues have been detected in proteins isolated from atherosclerotic plaques as well as in plasma and airway secretions of patients with asthma, ARDS and cystic fibrosis, and halogenated Tyr residues are widely used as markers for damage mediated by hypohalous acids (HOCL and HOBr) in these diseases[153-156]. The major products are Cl-Tyr and 3-bromotyrosine, but dihalogenated compounds (3,5-dichlorotyrosine and 3,5-dibromotyrosine) are formed with high excesses of HOCL and HOBr. Dramatic selective enrichment in protein-bound NO_2-Tyr and Cl-Tyr amount within ApoA-I, the major protein constituent within HDL, recovered from human plasma and atherosclerotic lesions has been demonstrated by proteomic and MS methods. Analysis of serum also showed that protein-bound NO_2-Tyr and Cl-Tyr concentrations in ApoA-I are markedly higher in individuals with established coronary heart disease[157,158]. These results suggest that increased concentrations of Cl-Tyr and NO_2-Tyr in circulating HDL might represent specific markers for clinically significant atherosclerosis.

Increased concentrations of nitrated plasma proteins have been associated with predisposition to develop lung injury in premature infants as well as with unfavorable outcome on development of lung injury[159]. The clinical relevance of protein Tyr nitration has been emphasized by the observation of a strong association between protein bound NO_2-Tyr concentrations and coronary artery disease risk. Circulating concentrations of protein-bound NO_2-Tyr may serve as an independent biomarker to assess atherosclerosis risk, burden and incident cardiac events, as well as to monitor the vasculoprotective action of drugs such as statins (hydroxymethylglutaryl-CoA reductase inhibitors)[160].

Patients with lung cancer have significantly higher serum concentrations of nitrated proteins, supporting the presence of oxidative and nitrosative stress[161,162]. Specific locations and targets of Tyr nitration in lung cancer have, recently, been detailed[163]. Increased nitrotyrosine immunostaining is limited mainly to the tumor and not to surrounding healthy tissue or is weakly reactive in different regions of the lung from the same patients with cancer, suggesting a unique enviroment inside the tumor that may contribute to the disease process. This was noted in squamous cell carcinoma as in the well-differentiated adenocarcinoma. Using proteomic and genomic

approaches, authors have identified the protein targets. Most of the nitrated proteins fall into 4 categories: oxidant defense (such as manganese superoxide dismutase and carbonic anhydrase), energy production (many glycolitic enzymes), structure (such as α-actin, α- and β-tubulin and vimentin) and those involved in apoptosis (annexins).

Tyrosine nitration is one of the earliest markers found in brains from persons affected by Alzheimer's disease, in the plaques of brains from persons with multiple sclerosis and in degenerating upper and lower motor neurons in ALS patients[164]. Nitrated α-synuclein selectively accumulates in Lewy bodies and protein inclusions in many pathologies (Alzheimer's disease, Parkinson's disease, synucleinopathies and tauopathies). Nitrated proteins have been evidenced in some inflammatory disease, chronic renal failure, rheumatoid arthritis, type 1 and type 2 diabetes and cystic fybrosis. On the other hand, basal protein nitration has been detected under physiologic conditions in most tissues, including plasma and the human pituitary and some of the nitrated proteins have been identified. Two-dimensional Western blotting and LC-MS/MS analyses have been used to detect and characterize 4 nitrated proteins, including actin, in the healthy human pituitary, which partecipate in neurotransmission, cellular immunity, and cellular structure and motility[165]. These results are consistent with the emerging perspective that low-level Tyr nitration may be a physiologic regulator of a signaling pathway[166].

Carbonylated Proteins

Protein carbonyls may be produced by the oxidation of some amino acid side chains (e.g., in Lys, Arg, Pro and Thr); by the formation of Michael adducts between Lys, His and Cys residues and α,β-unsaturated aldehydes, forming ALEs (Advanced Lipooxidation End Products); and by glycation/glycoxidation of Lys amino groups, forming advanced glycation end AGE products[167-169]. The generation of carbonyl molecules is the most general and widely used marker of severe protein oxidation both *in vitro* and *in vivo*, with different assays developed for the quantification of these species (170). The chemical stability of protein carbonyls makes them suitable targets for laboratory measurements and is also useful for their storage: their stability during storage for 10 years at –80°C has been demonstrated.

Assays of general oxidative damage to proteins, while an important index of oxidative stress occuring *in vivo*, need to be replaced with assays of oxidative damage to specific proteins having relevance to the lesion under consideration.

As a marker of oxidative damage to proteins, carbonyls have been shown to accumulate during aging, ischemia/reperfusion injury, chronic inflammation, cystic fibrosis and many of age-related diseases in some organisms[171].

Specific carbonylated proteins have been detected in both the brain tissue and plasma of Alzheimer's disease patients[172]. The observation of carbonylated proteins in plasma suggests that these oxidized species may be useful as diagnostic biomarkers for (possibly early) Alzheimer's disease.

The carbonyl content in plasma proteins (mainly albumin and γ-globulins) from children with different forms of juvenile chronic arthritis was significantly higher than in healthy children, and more importantly, the carbonyls increased in parallel with the activity of the disease.

Correlation between the carbonyl concentration and the activity or the type of chronic juvenile arthritis indicates that plasma protein carbonyl groups are a good marker of inflammatory process activity and may allow the use of carbonyls as a clinical marker of antioxidant barrier impairment in this group of patients as well as for monitoring possible pharmacologic treatments[173].

Plasma concentrations of protein carbonyls, as well as free F_2-IsoPs and protein reduced thiols, differ significantly between chronic kidney disease patients and healthy people. Furthermore, such biomarkers of oxidative/nitrosative stress are significantly higher in patients with diabetes and hypercholesterolemia[174].

Winterbourn et al[175] determined that protein carbonyl concentrations were increased in both plasma and BAL (bronchoalveolar lavage) fluid of patients with severe sepsis or major trauma, which correlated well with measured concentrations of ALEs and with indices of neutrophilia and neutrophil activation. Moreover, patients with acute pancreatitis had significantly increased plasma concentrations of protein carbonyls, which were related to disease severity, thus confirming that this protein modification could be a useful plasma marker of oxidative damage.

Measurement of DNA Damage

Cellular DNA damage can be caused by ROS produced under several conditions and different methods have been developed to measure the oxidatively modified nucleobases in DNA[176,177]. Oxidative DNA damage seems to relate to an increased risk of cancer development later in life[178]. DNA subjected to attack by hydroxyl radical produces a wide range of base and sugar modification products. Amongst these, the major reaction product of ·OH with thymine is thymine glycol and with guanine, 8-hydroxy-guanine. These DNA products are eliminated by repair enzymes (excision enzyme and glycolases) and are excreted in the urine either as the free base products or as the nucleoside derivatives, thymidine glycol (Tg) and 8-hydroxydeoxyguanosine. The latter products can be used as an index of radical attack upon DNA *in vivo* and Cathcart[179] haved calculated from such measurements that oxidative damage to mammalian DNA may total about 10^5 oxidative "hits" per cell per day[179]. Measurement of urine samples is based on chromatographic pre-purification by normal, reverse-phase or immuno-affinity columns to prevent interference by many urinary compounds, followed by derivatization and analysis. Originally, HPLC combined with UV detection was used to measure Tg[31] but great concentrations of samples were required to obtain sufficient sensitivity. Most current procedures are based on HPLC, GC-MS, LC-MS and antibody-based methods[180]. The advantages of artifacts produced during measurement of 8OHdG are useful for visualization of damage, but they seem likely to be only semiquantitative.

DNA can also be damaged by RNS, undergoing mainly nitration and deamination of purines. Techniques for the measurement of DNA base nitration and deamination products have been developed but may need more refinement and validation before they can routinely applied to human materials.

None of the analytical techniques mentioned above identifies where the oxidative damage is located. Another problem in studying damage to DNA by ROS/RNS is the limited availability of human tissues from which to obtain DNA. Most studies are performed on DNA isolated from lymphocytes or total leukocytes from human blood and it is assumed (possibly erroneously) that changes here are reflected in other tissues.

8-hydroxydeoxyguanosine (8-OHdG), an oxidized form of guanine, is a major oxidative DNA-damage product that can produce mutation. This compound causes A:T to C:C or G:C to T:A transversion mutations because of its base pairing with adenine as well as cytosine. Measurement of 8-OHdG in urine has been used to assess "whole-body" oxidative DNA damage. This can be achieved by HPLC and MS methods. However, 8-OHdG can arise from degradation of oxidized dGTP in the DNA precursor pool, not just from removal of oxidized guanine residues from DNA by repair processes. Furthermore, there are many other products of oxidative DNA damage. Hence, urinary 8-OHdG is a partial measure of damage to guanine residues in DNA and its nucleotide precursor pool, and 8-OHdG concentrations may not truly reflect rates of oxidative damage to DNA.

Papa et al[181] wanted to assess the production of ROS and 8-OHdG in gastric mucosa, according to *H. pylori* status and cytotoxic associated gene product A (CagA) and to determine the relationship between ROS production and amount of 8-OHdG. Gastric biopsy specimens were obtained from 60 consecutive patients. ROS generation was measured by luminol enhanced chemiluminescence. 8-OHdG detection was performed by an immunoperoxidase method, using a specific anti 8-OHdG monoclonal antibody. 40/60 patients (67%) were *H. pylori*-positive. ROS generation was significantly higher in patients positive for H. pylori infection as compared to negative. 8-OHdG detection was performed in 30 patients in which CagA presence was also investigated. High expression of 8-OHdG was detected in 14/20 (70%) *H. pylori*-positive patients (13 $CagA^+$ and 1 $CagA^-$) and in 2/10 (20%) *H. pylori*-negative patients. A significant correlation was found between ROS production and 8-OHdG content.

However, the recently completed Biomarkers of Oxidative Stress Study (BOSS), using acute CCl_4 poisoning in rodents as a model for oxidative stress, has demonstrated that 8-OHdG in urine is a potential candidate general biomarker of oxidative stress, whereas neither leukocyte DNA-MDA adducts nor DNA-strand breaks resulted from CCl_4 treatment.

Immunohistochemical accumulation of high levels of 8-OHdG was reported to occur in various human tumors, like high-grade glioma, compared to adiacent, normal tissue or low-grade glioma[182]. These studies suggested that oxidative stress may play a role in tumor progression.

As with other indices of whole-body oxidative stress, the measurement of products of oxidative DNA damage is limited by some problems, including the obscurity of the tissue of origin of the products.

Another technique utilized to detect DNA adducts is the comet assay[183]. Other methods exist to determine single- and double-strand breaks[184]. Different oxidized adducts of DNA can be determined. Examples are DNA-aldehyde adducts, such as deoxyguanosine-malondialdehyde adducts[185], or the end product of the reaction between DNA and 4-hydroxynonenal, the aldehyde formed following exposure to ROS[186] to produce N_2-ethenodeoxyguanosine.

Measurement of Antioxidants

Different animal studies have shown that antioxidants delay or protect against the oxidative damage produced by free radical reactions. Radical-scavenging antioxidants are consumed during this process and antioxidant status is sometimes used indirectly to assess free radical activity. The commonly used TRAP assay (Total [peroxyl] Radical-trapping Antioxidant Parameter) is, in it basic form, an empirical measurement of antioxidant activity in plasma[187]. Assessment of the relative contribution of individual antioxidants (ascorbate, urate, α-tocopherol, protein sulphydryls) to the total antioxidant capacity requires separate specific assays. Measurements of either TRAP or the individual antioxidants are not likely to be useful indices of free radical generation as the latter would need to be extensive for the steady-state concentrations of the antioxidants to be disturbed *in vivo*. However, antioxidant amounts are interesting parameters in themselves, indicative of the propensity of the individual to oxidative stress.

Many methods exist for evaluating the activity and composition of the anti-oxidant enzymes, which, along with the LMWA, constitute the two major components of the anti-oxidative system. Some techniques that directly evaluate enzymatic activity utilize spectroscopic measurements or gel-activity procedures; other methods employ immunocytochemistry. Assays of anti-oxidant enzymes may indicate prior exposure of the cell to oxidative stress, even if these enzymes are under regulation, and one might detect an increase, rather than a decrease, in their activity. ROS may serve as a stimulating species for induction of antioxidant enzymes on the one hand and, on the other, may themselves damage the proteins; for example, O_2^- might inactivate catalase. Determination of the fate of LMWA may serve as a better indicator for ROS, because the adduct is specific to these molecules.

Determination of the ratio between oxidant and reductant (e.g., ascorbate/dehydroascorbic acid or GSH/GSSG) may therefore serve as indicator of oxidative damage. One of the approaches most commonly used is the measurement of the total anti-oxidant activity of a biological site. Depletion of one anti-oxidant molecules causes changes in the level of overall anti-oxidant molecules and may be evaluated using a variety of techniques including biochemical, immunohistochemical, spectroscopical and electrochemical[188].

The total-antioxidant-activity assay offers many advantages and is considered a useful tool for detecting oxidative stress phenomena in bodily fluids and tissues. It may serve as an appropriate tool for the evaluation of anti-oxidant therapy. Determinations of total LMWA rather than individual anti-oxidants are important, because LMWAs work in concert[189], and measurement of only one or a few compounds out of many present at a specific biological location might be misleading. Moreover, measurement of the total LMWA ensures a reliable picture of the physiological situation. Now it's don't know the concentration of a specific compound at a specific location at a given moment. Sometimes the researchers try to detect compounds that are not present in the site under investigation.

A few dozen LMWA exist, and usually only a few of them, such as vitamin E and ascorbic and uric acids, are revealed; thus, many compounds that can be present at the biological site are neglected. The measurement of the total LMWA is designed to overcome these problems. Numerous procedures allow measurement of the total LMWA activity. These include indirect and direct methods for measuring total anti-oxidant activity originating from the LMWA. Indirect methods are those that measure consequential factors of redox capacity, such as oxidation products formed or concentrations of major redox couples in the biological enviroment, by fluorescent or spectrophotometric techniques. In this approach one assumes that a biological redox buffer exists in the form of a redox couple

that is sensitive to changes in the redox enviroment. Thus, it reflects changes in the reducing power of the measured sample, which is in correlation with all of the LMWAs. Other indirect techniques are inibition methods that involve adding a radical species to the sample together with a scavenger that can be monitored with laboratory instruments. The LMWAs present in the sample under investigation can quench the radical and, therefore, interfere in its reaction with the added scavenger. Examples of indirect methods are:

1. measurement of electrochemical couples, such as GSH/GSSG (glutathione/oxidized glutathione[190]);
2. NADH/NAD^+ (reduced nicotinamide dinucleotide/nicotinamide dinucleotide), and ascorbic acid/ascorbate[191,192];
3. the Trolox equivalent-antioxidant capacity (TEAC) assay[193];
4. the total radical-trapping potential (TRAP), an assay to define, for example, the stage of atherosclerosis[194,195];
5. the chemiluminescence method for superoxide detection;
6. the oxygen-radical absorbance capacity (ORAC) methodology.

Direct methods for measuring total LMWA are those that utilize an external probe to measure the reducing or oxidizing capacity of a system. An example is an electrode, in which the current is proportional to the concentrations of the scavenger or the redox couple under investigation. These direct methods can be classified into 2 groups: chemical and electrochemical. The chemical methods measure a known redox active couple whose reduced and measured as a function of concentration. For example, the ferric-reducing antioxidant power (FRAP) assay is based on the reaction of the redox couple ferric/ferrous with anti-oxidants in the sample and results in the creation of a blue color that can be measured at 593 nm[196].

Several methods have been developed to assess the total antioxidant capacity (TAC); the molecules measured using these assays are reducants, able to reduce oxidant species and protect oxidizable compounds[197,198]. However, the number of different anti-oxidants in serum or other biological samples makes it difficult to measure each element separately. Additionally, the possible interaction among different anti-oxidants *in vivo* could make the measurement of any individual anti-oxidant unrepresentative of the overall anti-oxidant status. Moreover, because the measured TAC of a biological samples depends on which procedure is used in the measurement[199], the comparison of different analytical methods represents a crucial factor in helping researchers to choose and to understand the results obtained using a specific method, due to the different principles on which they are based[200]. For example, studies evaluating the single contribution of pure plasma component to the total anti-oxidant activities of blood samples, indicate that the main contribution in the FRAP assay, but not in others, is the acid uric. On the contrary, the anti-oxidant capacity of reduced glutathione is not detected by using the FRAP assay but significantly contributes to the anti-oxidant capacity measured by utilizing other tests. Recent data showed differences between the FRAP assay and other methods in TAC measured in the same sample from normal individuals. Thus, each method is sensitive to various anti-oxidants in a different manner and consequently may also evidence, as compared to another method, different levels of anti-oxidant capacity in the same sample. Moreover, the interaction between anti-oxidant components may complicate the evaluation of *in vivo* results.

Horoz et al[201] aimed to measure total anti-oxidant response (TAR) using a novel automated method in nonalcoholic steatohepatitis (NASH) subjects. As a reciprocal measure, they also aimed to determine total peroxide levels in the same plasma samples. The ratio percentage of the total plasma peroxide level to the plasma TAR value was regarded as oxidative stress index (OSI)[202]. Twenty-two subjects with biopsy proven NASH (19 male, 3 female; mean age 37.7 ± 8.8) and 22 healthy controls (17 male, 5 female; mean age 34.6 ± 9.3) were enrolled. The most important indications for liver biopsy in those 22 subjects were ultrasonographically diagnosed fatty liver and elevation in alanine aminotransferase (ALT). The total anti-oxidant status of the plasma was measured using a novel automated colorimetric measurement method for TAR developed by Erel[203]. In this method, the hydroxyl radical, the most potent biological radical, is produced by the Fenton reaction, and reacts with the colourless substrate O-dianisidine to produce

the dianisyl radical, which is brigth yellowish-brown in colour. Upon the addition of a plasma sample, the oxidative reactions initiated by the hydroxyl radicals present in the reaction mix are suppressed by the anti-oxidant components of the plasma, preventing the colour change and thereby providing an effective measure of the total anti-oxidant capacity of the plasma. The assay results are expressed as mmol Trolox eq./L, and the precision of this assay is excellent, being lower than 3%. The total plasma peroxide concentrations were determined using the FOX_2 method[80] with minor modifications. The FOX_2 test sistem is based on the oxidation of ferrous iron to ferric iron by the various types of peroxides contained in the plasma samples, in the presence of xylenol orange which produces a coloured ferric-xylenol orange complex whose absorbance can be measured. Total antioxidant response of subjects with NASH was significantly lower than controls ($p < 0.05$), while mean total peroxide level and mean oxidative stress index were higher (all $p < 0.05$). In subjects with NASH, fibrosis score was significantly correlated with total peroxide level, total antioxidant response and oxidative stress index ($p < 0.05$, r = 0.607; $p < 0.05$, r = –0.506; $p < 0.05$, r = 0.728, respectively). However, no correlation was observed between necroinflammatory grade and those oxidative status parameters (all $p > 0.05$). NASH is associated with increased oxidant capacity, especially in the presence of liver fibrosis. The novel automated assay is a reliable and easily applicable method for total plasma antioxidant response measurement in NASH.

Total anti-oxidant capacity was measured in whole and protein-free serum by an enhanced chemiluminescence technique[204]. Total glutathione in fresh whole blood, GSH and GSSG in plasma were determined using 5,5'-dithiobis(2-nitrobenzoic acid)[205]. Selenium was determined using a simple single-tube fluorimetric assay[206]. Vitamin A[207], vitamin C[208] and vitamin E[209] were determined by established laboratory techniques.

Hepatic fibrogenic activity was measured in serum using the Type III procollagen intact PIIINP radioimmunoassay (Orion Diagnostica, Espoo, Finland). C-reactive protein (CRP) was assayed using an in-house, antibody sandwich ELISA technique. Rabbit anti-human CRP antibodies (unlabelled and horse-radish peroxidase-labelled), calibrators and controls were obtained from Dakocytomation (Glostrop, Denmark) and o-phenylenediamine (Sigma-Aldrich, Poole, Dorset) was used to detect the amount of bound analyte. Serum aspartate transaminase (ASAT), alanine transaminase (ALAT), alkaline phosphatase (ALP), γ-glutamyl transferase (γ-GT), total protein, albumin, bilirubin and urate were determined by standard automated techniques.

Markers of lipid peroxidation, antioxidant status, hepatic fibrogenesis, inflammation and liver function were measured in blood and urine from 24 patients with established alcoholic cirrhosis and in 49 age- and sex-matched controls. In the ALD group, lipid peroxidation markers 8-isoprostane and malondialdehyde were significantly increased ($p < 0.001$), as was the ratio of oxidized to reduced glutathione ($p = 0.027$). The antioxidants selenium, glutathione (whole blood and plasma) and vitamins A, C and E were all significantly decreased ($p < 0.001$); median plasma glutathione levels were only 19% of control levels. PIIINP, a serum marker of hepatic fibrogenesis, and CRP were both increased ($p < 0.001$). Urinary 8-isoprostane correlated positively with PIIINP, CRP and markers of cholestasis (alkaline phosphatase and bilirubin) and negatively with glutathione (whole blood), vitamins A and E and albumin.

The electrochemical methods include a lot of techniques, such as potentiometry, electrochemical titration and voltammetry[210]. Measurement of the reducing power by voltammetric methods offers several advantages. Such measurements can be performed easily and and rapidly, allow the evaluation of numerous samples without sophisticated extraction and treatment, and thus are most suitable for screening a large number of samples. Information derived from these measurements cannot be obtained by other methods. The evaluations provide information about all LMWA of both lipophilic and hydrophilic nature and can be conducted in cells, biological fluids and tissues. A unique characteristic, the reducing-power profile can supply information concerning the type and concentration of LMWA. The profile is specific to the tissue and each biological site possesses its own characteristic set of data. Changes in the profile can immediately indicate the occurrence of oxidative stress to system.

Principles and Methodologies of Cyclic Voltammetry (CV) and Examples of Evaluation of Biological Reducing Power

Voltammetric measurements have been conducted for many years to measure electron transfer between molecules and evaluate oxidation/reduction potentials of various redox-active compounds[211]. These methodologies can provide information concerning thermodynamic, kinetic and analytical features of the tested compounds[212]. This technique is useful to the evaluation of the overall reducing power of a biological sample[213]. Following preparation of the sample for measurement, the sample is introduced into the tested well. A potentiostat with a 3-electrode system, required to conduct the measurement, consists of a working electrode (e.g., glassy carbon, mercury film or platinum electrode), a reference electrode (e.g., silver/silver chloride or calomel electrode) and an auxiliary electrode (e.g., a platinum wire). Following introduction of the sample, the voltage is linearly applied to the working electrode and changed from a start to stop potential and immediately swept back at the same swept rate to the start. This potential is aimed to oxidize or reduce a species present in the solution in the voltammetric cell. The resulting current vs potential is recorded to produce a cyclic voltammogram[214,215] that can supply thermodynamic, kinetic and analytical information concerning the electrochemical couple under investigation[216]. The position of the current wave (e.g., anodic wave) on the voltage axis (x-axis of the voltammogram) can be determined and is referred to as the potential at which the peak current occurs. This potential can be defined as the oxidation potential of a compound for a given set of conditions. Analitically, it is used to monitor concentration. When several compounds have the same or close oxidation potentials, the anodic wave obtained is composed of all of these compounds and the peak potential is evaluated for the whole group. In this case the anodic current describes the concentrations of these molecules. This pattern is usually seen in voltammetric measurements of biological samples. Although the voltammogram cannot provide specific information on the exact nature of the LMWA, it can supply data concerning the reducing power of the sample under investigation.

Examples of Evaluation of Total Reducing Power in Some Clinical and Pathological Cases

Since this methodology for quantification of the overall LMWA was first introduced[217], it has been used in a variety of clinical situations and pathological disorder, including diabetes[218,219] ulcerative colitis[220], brain degenerative diseases and head trauma[221], skin status and pathologies[222] and irradiation therapy as well as study of the aging process and stages of embryonic development[223]. Biological fluids[224] such as seminal fluid, cerebrospinal fluid, saliva, sweat, urine, plasma and gastric juice possess reducing power derived from their LMWA content.

Immunohistochemical Markers Used in Toxicology Pathology in Visualization of Oxidative-Stress Phenomena

Oxidative-stress markers have been divided into 3 categories. First, molecules modified by free radicals, such as 4-hydroxy-2-nonenal, malondialdehyde and 8-oxo-2'-deoxyguanosine (8-oxo-dG). The concentrations of these products are proportional to dose and they are detected at the sites where free-radical attacks occur. Second, antioxidant enzymes and molecules are associated with the metabolism of radicals, such as GSH and catalase. Finally, transcriptional factors are included, such as nuclear factor-κβ (NF-κβ) and c-myc which are modulated by these radicals. Because tissue collected during toxicity studies are fixed chiefly in formalin, researchers must focus on well-defined products that are stable in this fixative and unlikely to share homology with formalin-induced modifications[225]. Reviewing histochemical and immunohistochemical approaches to the study of oxidative stress, Raina et al[225] stated that "the importance of *in situ* methods over bulk analysis cannot be overstated when considering the structural and cellular complexity of tissues and the effects of diseases thereof. Indeed, *in situ* detection allows detection of specific cell types affected or specific localization such that a process affecting only a small fraction of the tissue or cells can be readily visualized".

Nuclear Factor-κB

Nuclear factor-κB (NF-κB) is a transcriptional factor implicated in inflammation and immune activation and activated by oxidants and cytokines[226]. This factor normally resides in an inactive form in the cytoplasm and has been shown to enhance iNOS gene expression in different types of cells, like macrophages[227].

Cyclooxygenase-2

Cyclooxygenase catalyzes the formation of prostaglandins and other eicosanoids from arachidonic acid. Cyclooxygenase-2 (COX-2) is induced at the site of inflammation following stimulation with pro-inflammatory agents, such as interleukin-1 (IL-1), tumor necrosis factor-α and lipopolysaccharides. Researchers have suggested that the release from inflammatory cells of NO, increases COX-2 activity[228]. COX-2 overexpression is involved in cellular proliferation and carcinogenesis in different organs[229,230] and COX-2-specific inhibitors prevent lung carcinogenesis[231].

Glutathione S-Transferase-pi

Drug-metabolizing enzymes, such as glutathione S-transferase (GST)s and antioxidant systems, such as glutathione, vitamins, catalase and superoxide dismutase function concertedly as the two most important inducible defense systems against electrophiles and xenobiotic toxicity[232]. The expression of these 2 systems occurs through a common regulatory region named the antioxidant responsive element (ARE)[233]. Nuclear factor 2 (Nrf2) has been show to be a key molecule that responds to reactive electrophiles by activating ARE-mediated gene expression. Glutathione S-transferase-pi (GST-pi), a member of this family of phase II detoxification enzymes, catalyzes intracellular detoxification reactions, including the inactivation of electrophilic carcinogens by catalyzing their conjugation with glutathione[234]. Additionally, GSTs have endogenous substrates, such as lipid and nucleic acid hydroperoxides and alkenals, which result from the decomposition of lipid hydroxyperoxides[235].

Inducible Nitric Oxide Synthase

Nitric oxide is synthesized in a variety of tissues via the catalytic activity of nitric oxide synthase (NOS). The inducible form, iNOS, is found mainly in mononuclear phagocytes where it may be incuced by endotoxins and/or cytokines; it is able of producing high levels of NO (nitric oxide)[236]. Although the role of NO in tumor biology remains controversial, most data indicate that it promotes tumor progression[237]. Increased iNOS expression may play a role in human tumorigenesis, as, for example, in the prostate where high-grade prostatic intraepithelial neoplasia (PIN) and carcinoma display more intense iNOS immunoreactivity than benign prostatic hyperplasia and low-grade PIN samples[238].

Extensive iNOS immunoexpression (average grade, severe) has been noted within infiltrating macrophages at sites of chronic active inflammation, the major lesion in rats exposed by inhalation for 3 months and 2 years to the lung carcinogen IP[239]. Lesion progression suggested that the foreign bodies (IP) introduced into the lungs attracted macrophages for their digestion and removal and induced severe inflammation, which further enhanced NO generation. The change in this and other markers analyzed in this research support the hypothesis that oxidative stress paly a relevant role in the development of lung cancer from IP inhalation.

Haem Oxygenase I

Haem oxygenase (HO)-1, a heat shock protein, is the inducible form of the rate-limiting enzyme of haem degradation[240]. It is induced by a lot of stimuli, including heat shock, hyperoxia and oxidative stress and represents a powerful endogenous protective mechanism against free radicals in a variety of pathological conditions. Liu et al[241], studying in frozen tissues the immunohistochemical localization of HO-1 in experimental autoimmune encephalomyelitis, which serves as a model for multiple sclerosis (MS) in human, noted a high expression in scattered macrophage-like and perivascular cells in inflammed lesions of the spinal cord. Repeated intraperitoneal injection of the HO-1 inducer, hemin, was associated with attenuation of spinal cord inflammation and reduced HO-1 immunoexpression. The latter was probably attributable to fewer macrophages, known to be the main source of ROS generation. These results suggest that pharmacological modulation of HO-1 expression may serve as a novel approach to therapeutic intervention in MS.

Uric Acid and Oxidative Stress

Uric acid (UA) is the final product of purine metabolism in humans. The final two reactions of its production catalyzing the conversion of hypoxanthine to xanthine and the latter to uric acid

are catalysed by the enzyme xanthine oxidoreductase (XOR), which may exist into two interconvertible forms, namely xanthine dehydrogenase or xanthine oxidase[242]. In most species, UA is metabolised to allantoin by the enzyme urate oxidase: allantoin is then conversed to allantoate and finally glyoxylate plus urea. All of these products are much more soluble than UA in water. Humans lack the enzyme urate oxidase due to a defective gene that is not transcribed[243] thus, the plasma levels of UA in humans are higher (200-400 μmol/l; 3.4-6.8 mg/dl) in comparison to most other mammals. Most of the serum UA is excreted in urine as long as renal function is not impaired, while low-sodium ducts have the effect of raising the net re-absorption of UA in the proximal tubule and thus, increase serum UA concentration[244,245]. UA is present intracellularly and in all body fluids but usually at lower levels than in plasma. At physiological pH, almost all UA is ionized to urate and has a single negative charge. Serum levels of UA are correlated with changes in the amounts of dietary purine consumed[246]. Due to urate's limited solubility in water, excess production *in vivo* can lead to its crystallization out of solution (e.g. in gout, where urate accumulates in joints causing arthritis). UA is also produced in conditions of ischaemia-reperfusion (I/R) during the oxidation of hypoxanthine dehydrogenase (XDH) and xanthine oxidase (XO). In physiological conditions, it is mainly found in the dehydrogenase form, with highest levels found in liver and intestine. XHD has greater affinity for oxidised nicotinamide adenine dinucleotide (NAD^+) compared to oxygen, as the electron acceptor, when catalysing the oxidation of hypoxanthine and xanthine to urate. Under ischaemic conditions, ATP is degraded to adenine and xanthine, while at the same time there is increased conversion of XDH to XO. Consequently, XO uses molecular oxygen instead of NAD^+ during reperfusion and leads to formation of the free radical superoxide anion (O_2^-)[247-249]. Superoxide anion can form hydrogen peroxide through superoxide dismutase activity and, in presence of iron, the extremely reactive hydroxyl radical by Fenton-type reactions[250,251]. The latter uses molecular oxygen as electron acceptor and generates superoxide anion and other reactive oxygen products.

The role of uric acid in conditions associated with oxidative stress is not completely defined. Evidence mainly based on epidemiological studies suggests that increased serum levels of uric acid are a risk factor for cardiovascular disease where oxidative stress plays an important pathophysiological role. Also, allopurinol, a xanthine oxidoreductase inhibitor that lowers serum levels of uric acid exerts protective effects in situations associated with oxidative stress (e.g. ischaemia-reperfusion injury, cardiovascular disease). However, there is increasing experimental and clinical evidence showing that uric acid has an important role *in vivo* as an antioxidant.

Factors such as hypoxia, cytokines and glucocorticoids lead also to the strong expression of XOR. Harrison[252] suggested that the XHD-NADH oxidase pathway can also lead to the production of the superoxide anion and contribute to I/R oxidative stress. Recent evidence suggests that XOR can produce NO under hypoxic conditions through the reduction of inorganic nitrate to NO[253]. Allopurinol is a hypoxanthine analogue that reacts with XOR to yield alloxanthine (oxypurinol), which binds to XOR and inhibits its action, therefore, its use for the managment of arthritis due to hyperuricaemia[254].

Antioxidant Properties of UA

It has been proposed that UA may represent one of the most important low-molecular-mass anti-oxidants in the human biological fluids[255-258]. Ames et al[255] proposed in the early eighties that UA can have biological significance as an antioxidant and showed, by *in vitro* experiments, that it is a powerful scavenger of peroxyl radical (RO_2), hydroxyl radicals ($\cdot OH$) and singlet oxygen. The researchers speculated that UA may contribute to increased life-span in humans by providing protection against oxidative stress-provoked ageing and cancer. UA is an oxidizable substrate from haem protein/H_2O_2 systems and is able to protect against oxidative damage by acting as an electron donor. Apart from its action as radical scavenger, UA can also chelate metal ions, like iron and copper, converting them to poorly reactive forms unable to catalyse free-radical reactions[259,260].

Upon reactions with ROS and other oxidizing agents, UA can be oxidized to allantoin and several other oxidation compounds. Thus, the determination of UA and/or allantoin is a useful tool in the assessment of the level of oxidative stress in humans.

Squadrito[261] suggests that UA is a specific inhibitor of radicals produced by the decomposition of peroxynitrite ($ONOO^\cdot$), the product of interaction of NO with the superoxide anion. Per-

oxynitrite is a strong oxidizing agent able to interact with almost all important cell components inducing cell injury[262]. It can also induced nitration of tyrosine residues in proteins influencing their structures and functions[263]. Squadrito et al[261] studied the kinetics of the reaction of UA with peroxynitrite using stopped-flow spectroscopy. They found that peroxynitrite reacts wiyh carbon dioxide (CO_2) in human blood plasma nearly 920 times faster than with UA. Thus, UA is not a direct scavenger of peroxynitrite since it cannot compete with CO_2. The researchers postulated that the therapeutic effects of UA may be related to the scavenging of the radicals such as CO_3^- and $NO_2^\cdot$ which are formed by the reaction of peroxynitrite with CO_2. It was also concluded that the trapping of secondary radicals which resulted from the fast reaction of peroxynitrite with CO_2 may represent a new and viable approach for ameliorating the side effects correlated with peroxynitrite in many pathologies. Evidence suggests that the extent to which UA may prevent several possible reactions of peroxynitrite depends on the concentration of dicarbonic. A nitrated derivate of UA, believed to result from scavenging peroxynitrite or its decomposition products, was found to induce endothelium-independent dilation of the aorta in rats.

Consistent with the antioxidant role of UA *in vivo* is the hypothesis that the loss of urate oxidase in humans (and the accompanying rise in serum levels of UA) improves antioxidant defence. Watanabe et al[264] hypothesised that higher UA concentrations in humans provide a survival advantage because hyperuricaemia maintains better blood pressure in the face of low dietary salt.

Conclusion

In conclusion, this review has been focused on different methods of evaluating the free radical activity in the human body, with a specific attention to reliability and clinical meaning of such techniques: as a matter of fact, the oxidant-antioxidant balance is a physiological condition involving so many biochemical reactions that an exhaustive monitoring of it would require widespread endpoints evaluations.

In order to get the most out of the presently-available techniques it's important that they be used intelligently. The choice of assay must be appropriate for the putative mechanism of damage: for example, if DNA damage is implicated, measurement of lipid peroxidation may be irrelevant. Assays of oxidative damage should be coupled with careful observations of the progress of the clinical symptoms and the effects of any antioxidant intervention therapies be studied from both aspects. Only in this way can the clinical symptoms be reliably correlated with the indices of free radical generation and an understanding of their association be reached.

References

1) Finkelstein E, Rosen GM, Rauckmann GJ. Spin trapping of superoxide and hydroxyl radical: practical aspects. Arch Biochem Biophys 1980; 200: 1-16.
2) Florence TM. The role of free radicals in cancer and aging. In: I. E. Dreosti (Ed): Trace Elements, Micronutrients and Free Radicals Humana Press, Totowa, New Jersey, 1991; pp. 171-198.
3) Halliwell B, Whiteman M. Measuring reactive species and oxidative damage in vivo and in cell culture: how should you do it and what the results mean? Br J Pharmacol 2004; 142: 231-255.
4) Machlin LJ, Bendich A. Free radical tissue damage: protective role of antioxidant nutrients. FASEB J 1987; 1: 441-445.
5) Sies H. Strategies of antioxidant defense. Eur J Biochem 1993; 215: 213-219.
6) Rice-Evans C, Burton R. Free-radical lipid interactions and their pathological consequences. Prog Lipid Res 1993; 32: 71-110.
7) Saran M, Michel C, Bors W. Reaction of NO with superoxide. Free Radic Res Commun 1990; 10: 221-226.
8) Scherck R, Zorbas H, Winnacker EL, Bauerle PA. The NF-kappa B transcription factor induces DNA bending which is modulated by its 65-kD subunit. Nucleic Acids Res 1990; 25: 6479-6502.
9) Sen CK, Packer L. Antioxidant and redox regulation of gene transcription. FASEB J 1996; 10: 709-729.
10) Packer L. Alpha-lipoic acid: a metabolic antioxidant which regulates NF-kappa B signal transduction and protects against oxidative injury. Drug Metab Rev 1998; 30: 245-275.
11) Stocker R, Keaney JF Jr. Role of oxidative modifications in atherosclerosis. Physiol Rev 2004; 84: 1381-1478.
12) Davies MJ, Fu S, Wang H, Dean RT. Stable markers of oxidant damage to proteins and their application in study of human disease. Free Radic Biol Med 1999; 27: 1151-1161.

13) Levine RL, Wehr N, Williams JA, Stadtman ER, Shacter E. Determination of carbonyl groups in oxidized proteins. Methods Mol Biol 2000; 99: 15-24.

14) Griffiths HR, Moller L, Bartosz G, Bast A, Bertoni-Freddari C, Collins A, Cooke M, Coolen S, Haenen G, Hoberg AM, Loft S, Lunec J, Olinski R, Parry J, Pompella A, Poulsen H, Verhagen H, Astley SB. Biomarkers. Mol Aspects Med 2002; 23: 101-208.

15) Tarpey MM, Wink DA, Grisham MB. Methods for detection of reactive metabolites of oxygen and nitrogen: in vitro and in vivo considerations. Am J Physiol Regul Integr Comp Physiol 2004; 286: 431-444.

16) Dalle-Donne I, Scaloni A, Giustarini D, Cavarra E, Tell G, Lungarella G, Colombo R, Rossi R, Milzani A. Proteins as biomarkers of oxidative stress in diseases: the contribution of redox proteomics. Mass Spectrom Rev 2005; 24: 55-99.

17) Pryor WA, Godber SS. Non-invasive measures of oxidative stress status in humans. Free Rad Biol Med 1991; 10: 177-184.

18) Slater TF. Overview of methods used for detecting lipid peroxidation. Methods Enzymol 1984; 105: 283-292.

19) Benedetto C, Bocci A, Dianzani MU, Ghiringhello B, Slater TF, Tomasi A, Vannini V. Electron spin resonance studies on normal human uterus and cervix and in benign and malignant uterine tumors. Cancer Res 1981; 41: 2936-2942.

20) Coghlan J, Flitter W, Holley A, Norell M, Mitchell AG, Ilsley CD, Slater TF. Detection of free radicals and lipid hydroperoxides in blood taken from the coronary sinus of man during percutaneous transluminal coronary angioplasty. Free Rad Res Commun 1991; 14: 409-417.

21) Halliwell B, Kaur H. Hydroxylation of salicylate and phenylalanine as assays for hydroxyl radicals: A cautionary note visited for the third time. Free Radic Res 1997; 27: 239-244.

22) Biaglow JE, Manevich Y, Uckum F, Held KD. Quantitation of hydroxyl radicals produced by radiation and copper-linked oxidation of ascorbate by 2-deoxy-D-ribose method. Free Radic Biol Med 1997; 22: 1129-1138.

23) Vittorio N, Oloff CM, Sellers DR. Automation of fluorometric method for the determination of 2-deoxy-D-glucose. Anal Biochem 1984; 141: 423-428.

24) Kutham H, Ullrich V, Estabrook RW. A quantitative test for superoxide radicals produced in biological systems. Biochem J 1982; 203: 551-558.

25) Amano F, Noda T. Improved detection of nitric oxide radical (NO) production in an activated macrophage culture with a radical scavanger, carboxy PTIO and Griess reagent. FEBS Lett 1995; 368: 425-428.

26) Grootveld M, Halliwell B. Aromatic hydroxylation as a potential means of measuring radical formation in vivo. Biochem J 1986; 273: 499-502.

27) Janzen EG. A critical review of spin trapping in biological systems. In: Free Radicals in Biology, 1980, Vol. IV (W. A. Pryor, ed.), Academic Press, N. Y, pp.115-153.

28) Mason RP. Spin trapping free radical metabolites of toxic chemicals. In: Spin Labeling in Pharmacology (J. L. Holtzman, ed.), Academic Press, New York, 1984, pp.87-129.

29) Mottley C, Mason RP. Nitroxide radical adducts in biology: chemistry, applications, and pitfalls. In: Biological Magnetic Resonance, 1989, Vol. 8 (L. J. Berliner and J. Reuben, eds.), Plenum Press, New York and London, pp. 489-546.

30) Dodd NJF. Free radical studies in biology and medicine. In: Electron Spin Resonance, 1990, Vol. 12A (M. C. R. symons, ed.) Royal Society of Chemistry, Cambridge, pp. 136-178.

31) Rosen GM, Rauckman EJ. Spin trapping of superoxide and hydroxyl radicals. Methods Enzymol 1984, 105: 198-209.

32) Rosen GM, Cohen MS, Britigan BE, Pou S. Application of spin traps to biological systems. Free Radic Res Commun 1990; 9: 187-195.

33) Pou S, Rosen GM. Spin-trapping of superoxide by 5,5-dimethyl-1-pyrroline N-oxide: application to isolated perfused organs. Anal Biochem 1990; 190: 321-325.

34) Li AS, Chignell CF. The NoH value in EPR spin trapping: a new parameter for the identification of 5,5-dimethyl-1-pyrroline-N-oxide spin adducts. J Biochem Biophys Methods 1991; 22: 83-87.

35) Miura Y, Utsumi H, Hamada A. Effects of inspired oxygen concentration on in vivo redox reaction of nitroxide radicals in whole mice. Biochem Biophys Res Commun 1992; 182: 1108-1114.

36) Valgimigli L, Valgimigli M, Gaiani S, Peduli GF, Bolondi L. Measurement of oxidative stress in human liver by EPR spin-probe technique. Free Radic Res 2000; 33: 167-178.

37) Akaike T, Suga M, Maeda H. Free radicals in viral pathogenesis: molecular mechanisms involving superoxide and NO. Proc Soc Exp Biol Med 1991; 198: 721-727.

38) Valgimigli L, Pedulli GF, Paolini M. Measurement of oxidative stress by EPR radical-probe technique. Free Radic Biol Med 2001; 31: 708-716.

39) Kaur H and Halliwell B. Action of biologically-relevant oxidizing species upon uric acid. Identification of uric acid oxidation products. Chem Biol Interact 1990; 73: 235-247.

40) Whiteman M, Ketsawatsakul U, Halliwell B. A reassessment of the peroxynitrite scavenging activity of uric acid. Ann NY Acad Sci 2002; 962: 242-259.

41) Grootveld M, Halliwell B. Measurement of allantoin and uric acid in human body fluids. A potential index of free-radical reactions in vivo? Biochem J 1987; 243: 803-808.

42) Moison RM, De Beaufort AJ, Haasnoot AA, Dubbelman TM, van Zoeren-Grobben D, Berger HM. Uric acid and ascorbic acid redox ratios in plasma and tracheal aspirate of preterm babies with acute and chronic lung disease. Free Radic Biol Med 1997; 23: 226-234.

43) Benzie IF, Chung W, Tomlinson B. Simultaneous measurement of allantoin and urate in plasma: analytical evaluation and potential clinical application in oxidant: antioxidant balance studies. Clin Chem 1999; 45: 901-904.

44) Mikami T, Kita K, Tomita S, Qu GJ, Tasaki Y, Ito A. Is allantoin in serum and urine a useful indicator of exercise-induced oxidative stress in humans? Free Radic Res 2000; 32: 235-244.

45) Doehner W, Schoene N, Rauchhaus M, leyva-Leon F, Pavitt DV, Reaveley DA, Schuler G, Coats AJ, Anker SD, Hambrecht R. Effects of xanthine oxidase inhibition with allopurinol on endothelial function and peripheral blood flow in hyperuricemic patients with chronic heart failure: results from 2-placebo-controlled studies. Circulation 2002; 105: 2619-2624.

46) Pavitt DV, de Fonseka S, Al-Khalaf N, Cam JM, Reaveley DA. Assay of serum allantoin in humans by gas chromatography-mass spectrometry. Clin Chim Acta 2002; 318: 63-70.

47) Yardim-Akaydin S, Sepici A, Ozkan Y, Torun M, Simsek B, Sepici V. Oxidation of uric acid in rheumatoid arthritis: is allantoin a marker of oxidative stress? Free Radic Res 2004; 38: 623-628.

48) Hellsten Y, Svensson M, Sjodin B, Smith S, Christensen A, Richter EA, Bangsbo J. Allantoin formation and urate and glutathione exchange in human muscle during submaximal exercise. Free Radic Biol Med 2001; 31: 1313-1322.

49) Nicholson JK, Lindon JC, Holmes E. Metabonomics: understanding the metabolic responses of living systems to pathophysiological stimuli via multivariate statistical analysis of biological NMR spectroscopic data. Xenobiotica 1999; 29: 1181-1189.

50) Nicholson JK, Wilson ID. High resolution proton NMR spectroscopy of biological fluids. Prog NMR Spectrosc 1989; 21: 449-501.

51) El-Deredy W. Pattern recognition approaches in biomedical and clinical magnetic resonance spectroscopy: a review. NMR Biomed 1997; 10: 99-124.

52) Holmes E, Foxall PJ, Nicholson JK, Neild GH, Brown SM, Beddell CR, Sweatman BC, Rahr E, Lindon JC, Spraul M, et al. Automatic data reduction and pattern recognition methods for analysis of 1H nuclear magnetic resonance spectra of human urine from normal and pathological states. Anal Biochem 1994; 220: 284-296.

53) Holmes E, Nicholls AW, Lindon JC. Chemometric models for toxicity classification on NMR spectra of biofluids. Chem Res Toxicol 2000; 13: 471-478.

54) Brindle JT, Antti H, Homes E, Tranter G, Nicholson JK, Bethell HW, Clarke S, Schofield PM, McKilligin E, Mosedale DE, Grainger DJ. Rapid and non-invasive diagnosis of the presence and severity of coronary heart disease using 1H-NMR-based metabonomics. Nat Med 2002; 8: 1439-1444.

55) Murtz M, Frech B, Urban W. High resolution cavity leak-out absorption spectroscopy in the 10 mm region. Appl Phys 1999; 68: 243-249.

56) Razola SS, Ruiz BL, Diez NM, Mark HB Jr, Kauffmann JM. Hydrogen peroxide sensitive amperometric biosensor based on horseradish peroxidase entrapped in a polypyrrole electrode. Biosens Bioelectron 2002; 17: 921-928.

57) Thanachasai S, Rokutanzono S, Yoshida S, Watanabe T. Novel hydrogen peroxide sensors based on peroxidase-carrying poly[pyrrole-co-[4-(3-pyrrolyl)butanesulfonate] copolymer films. Anal Sci 2002; 18: 773-777.

58) Halliwell B, Gutteridge JMC. Free Radicals in Biology and Medicine (2 edn), Oxford: Clarendon Press, 1989.

59) Cheeseman K. Methods of measuring lipid peroxidation in biological systems: an overview. In: Crastes De Paulet A, Doust-Blazy L, and Paoletti R eds. Free Radicals, Lipoproteins and Membrane Lipids. New York: Plenum Press, 1989, pp 407-421.

60) Young IS, Trimble ER. Measurement of malondialdehyde in plasma by high performance liquid chromatography with fluorimetric detection. Ann Clin Biochem 1991; 28: 504-508.

61) Duthie GG, Morrice PC, Ventresca PG, McLay JS. Effects of storage, iron and time of day on indices of lipid peroxidation in plasma from healthy volunteers. Clin Chim Acta 1992; 206: 207-213.

62) Holley A, Slater T. Measurement of lipid hydroperoxides in normal human blood plasma using HPLC-chemiluminescence linked to a diode array detector for measuring conjugated dienes. Free Rad Res Commun 1991; 15: 61-63.

63) Slater TF, Sawyer BC. The stimulatory effects of carbon tetrachloride and other halogenoalkanes on peroxidative reactions in rat liver fractions in vitro. General features of the systems used. Biochem J 1971; 123: 805-814.

64) Comporti M. Glutathione depleting agents and lipid peroxidation. Chem Phys Lipids 1987; 45: 143-169.

65) Kappus H. A survey of chemicals inducing lipid peroxidation in biological systems. Chem Phys Lipids 1987; 45: 105-115.

66) Halliwell B, Gutteridge JMC. Oxigen free radicals and iron in relation to biology and medicine: some problems and concepts. Arch Biochem Biophys 1986; 246: 501-514.

67) Gutteridge JMC, Smith A. Antioxidant protection by haemopexin of haem-stimulated lipid peroxidation. Biochem J 1988; 256: 861-866.

68) Orrenius S, McConkey DJ, Bellomo G, Nicotera P. Role of Ca2+ in toxic cell killing. Trends Pharmacol 1989; 10: 281-285.

69) Halliwell B. Oxidants and human disease: some new concepts. FASEB J 1987; 1: 358-364.

70) Halliwell B, Gutteridge JMC. Lipid peroxidation, oxygen radicals, cell damage and antioxidant therapy. Lancet 1984; 1: 1396-1397.

71) Steinberg D, Parthasarathy S, Carew TE, Khoo JC, Witztum JL. Beyond cholesterol. Modifications of low-density lipoprotein that increase its atherogenicity. N Engl J Med 1989; 320: 915-924.

72) Halliwell B. Oxidants and the central nervous system: some fundamental questions. Is oxidant damage relevant to Parkinson's disease, Alzheimer's disease, traumatic injury or stroke? Acta Neurol Scand Suppl 1989; 126: 23-33.

73) Davies MJ. Applications of electron spin resonance spectroscopy to the identification of radicals produced during lipid peroxidation. Chem Phys Lipids 1987; 44: 149-173.

74) Kulmacz R, Miller JJ, Pendleton R, Lands W. Cyclooxigenase initiation assay for hydroperoxides. Methods Enzymol 1990; 186: 431-438.

75) Hughs H, Smith C, Tsokos-Kuhn J, Mitchell J. Quantitation of lipid peroxidation by gas chromatography mass spectrometry. Anal Biochem 1986; 152: 107-112.

76) Tatsishi T, Yoshimine N, Kuzuya F. Serum lipid peroxide assayed by a new colorimetric method. Experimental Gerontol 1987; 22: 103-111.

77) Miyazawa T. Determination of phospholipid hydroperoxides in human blood plasma by a chemiluminescence-HPLC assay. Free Radic Biol Med 1989; 7: 209-217.

78) Caraceni P, Yao T, Degli Esposti S, Gasbarrini A, Bowie BT, Zern M, Borle AB, Van Thiel DH. Effect of vitamin E on reoxygenation injury experienced by isolated rat hepatocytes. Life Sci 1994; 55: 1427-1432.

79) Gasbarrini A, Grigolo B, Serra M, Baldini N, Scotlandi K, Gasbarrini A, Bernardi M, Facchini A. Generation of free radicals during anoxia and reoxygenation in perfused osteoblastlike cells. Clin Orthop Relat Res 1997; 338: 247-252.

80) Ojetti V, Di Campli C, Mutignani M, Migneco A, Tridente A, Silveri NG, Gasbarrini G, Gasbarrini A. Real time endoscopic imaging of oxyradical generation in pig stomach durino ischemia-reperfusion. Dig Liver Dis 2003; 35: 309-313.

81) Yamamoto Y, Frei B, Ames B. Assay of lipid hydroperoxides using high performance liquid chromatography with isoluminol chemiluminescence detection. Methods Enzymol 1990; 186: 371-380.

82) Kox W, Kox S, Fleck A, Holley A, Cheeseman K, Slater T. Aerobic and anaerobic lung oxygen consumption in patients with septic shock. Proc Physiol Soc C46, 1991.

83) Recknagel RO, Glende EA Jr. Spectrophotometric detection of liquid conjugated dienes. Methods Enzymol 1984; 105: 331-337.

84) Corongui F, Poli G, Dianzani M, Cheeseman K, Slater T. Lipid peroxidation and molecular damage to polyunsaturated fatty acids in rat liver. Recognition of two classes of hydroperoxides formed under conditions in vivo. Chem Biol Interact 1986; 59: 147-155.

85) Dormandy T, Wickens D. The experimental and clinical pathology of diene conjugation. Chem Phys Lipids 1987; 45: 353-364.

86) Esterbauer H, Cheeseman K. Determination of aldehydic lipid peroxidation products: malonaldehyde and 4-hydroxynonenal. Methods Enzymol 1990; 186: 407-421.

87) Yagi K. Assay for blood plasma or serum lipid peroxides. Methods Enzymol 1984; 105: 328-331.

88) Slatter DA, Bolton CH, Bailey AJ. The importance of lipid-derived malondialdehyde in diabetes mellitus. Diabetologia 2000; 43: 550-557.

89) Yoneyama Y, Sawa R, Suzuki S, Doi D, yoneyama K, Otsubo Y, Araki T. Relatioship between plasma malondialdehyde levels and adenosine deaminase activities in preeclampsia. Clin Chim Acta 2002; 322: 169-173.

90) Wood LG, Gibson PG, Garg ML. Biomarkers of lipid peroxidation, airway inflammation and asthma. Eur Respir J 2003; 21: 177-186.

91) Barnham KJ, Masters CL, Bush AI. Neurodegenerative diseases and oxidative stress. Nat Rev Drug Discov 2004; 3: 205-214.

92) Yoshino K, Matsuura T, Sano M, Saito S, Tomita I. Fluorimetric liquid chromatographic determination of aliphatic aldehydes arising from lipid peroxides. Chem Pharm Bull 1986; 34: 1694-1700.

93) Uchida K. 4-Hydroxy-2-nonenal: a product and mediator of oxidative stress. Prog Lipid Res 2003; 42: 318-343.

94) Esterbauer H, Schaur RJ, Zollner H. Chemistry and biochemistry of 4-hydroxynonenal, malonaldehyde and related aldehydes. Free Radic Biol Med 1991; 11: 81-128.

95) Eckl PM, Ortner A, Esterbauer H. Genotoxic properties of 4-hydroxyalkenals and analogous aldehydes. Mutat Res 1993; 290: 183-192.

96) Carini M, Aldini G, Facino RM. Mass spectrometry for detection of 4-hydroxynonenal (HNE) adducts with peptides and proteins. Mass Spectrom Rev 2004; 23: 281-305.

97) Parola M, Bellomo G, Robino G, Barrera G, Dianazani MU. 4-Hydroxynonenal as a biological signal: molecular basis and pathophisiological implications. Antioxid Redox Signal 1999; 1: 255-284.

98) Feng Z, Hu W, Tang MS. Trans-4-Hydroxy-2-nonenal inhibits nucleotide excision repair in human cells: a possible mechanism for lipid peroxidation-induced carcinogenesis. Proc Natl Acad Sci USA 2004; 101: 8598-8602.

99) Siems W, Grune T. Intracellular metabolism of 4-hydroxynonenal. Mol Aspects Med 2003; 24: 167-175.

100) Uchida K, Stadtman ER. Modification of histidine residues in proteins by reactions with 4-hydroxynonenal. Proc Natl Acad Sci USA 1992; 89: 4544-4548.

101) Butterfield DA. Amyloid _-peptide(1-42)-induced oxidative stress and neurotoxicity: implications for neurodegeneration in Alzheimer's disease brain. Free Radic Res 2002; 36: 1307-1313.

102) Uchida K. Role of reactive aldehyde in cardiovascular diseases. Free Radic Biol 2000; 28: 1685-1696.

103) Shao B, O'brien KD, McDonald TO, Fu X, Oram JF, Uchida K, Heinecke JW. Acrolein modifies apolipoprotein A-I in the human artery wall. Ann N Y Acad Sci 2005; 1043: 396-403.

104) Rahman I, van Schadewijk AA, Crowther AJ, Hiemstra PS, Stolk J, MacNee W, De Boer WI. 4-Hydroxy-2-nonenal, a specific lipid peroxidation product, is elevated in lungs of patients with chronic obstructive pulmonary disease. Am J Respir Crit Care Med 2002; 166: 490-495.

105) Barreiro E, de la Puente B, Minguella J, Corominas JM, Serrano S, Hussain SN, Gea J. Oxidative stress and respiratory muscle dysfunction in severe chronic obstructive pulmonary disease. Am J Respir Crit Care Med 2005; 171: 1116-1124.

106) Cracowski JL, Durand T, Bessard G. Isoprostanes as a biomarker of lipid peroxidation in humans: physiology, pharmacology and clinical implications. Trends Pharmacol Sci 2002; 23: 360-366.

107) Montuschi P, Barnes PJ, Roberts LJ. Isoprostanes: markers and mediators of oxidative stress. FASEB J 2004; 18: 1791-1800.

108) Basu S, Helmersson J. Factors regulating isoprostane formation in vivo. Antioxid Redox Signal 2005; 7: 221-235.

109) Janssen LJ. Isoprostanes:an overview and putative roles in pulmonary pathophysiology. Am J Physiol Lung Cell Mol Physiol 2001; 280: 1067-1082.

110) Montine TJ, Montine KS, McMahan W, Markesbery WR, Quinn JF, Morrow JD. F2-Isoprostanes in Alzheimer and other neurodegenerative diseases. Antioxid Redox Signal 2005; 7: 269-275.

111) Schwedhelm E, Boger RH. Application of gas chromatography-mass spectrometry for analysis of isoprostanes: their role in cardiovascular disease. Clin Chem Lab Med 2003; 4: 1552-1561.

112) Morrow JD. Quantification of isoprostanes as indices of oxidant stress and the risk of atherosclerosis in humans. Arterioscler Thromb Vasc Biol 2005; 25: 279-286.

113) Kadiiska MG, Gladen BC, Bard DD, Germolec D, Graham LB, Parker CE, Nyska A, Wachsman JT, Ames BN, Basu S, Brot N, Fitzgerald GA, Floyd RA, George M, Hinecke JW, Hatch GE, Hensley K, Lawson JA, Marnett LJ, Morrow JD, Murray DM, Plastaras J, Roberts LJ 2nd, Rokach J, Shigenaga MK, Sohal RS, Sun J, Tice RR, Van Thiel DH, Wellner D, Walter PB, Tomer KB, Mason RP, Barrett JC. Biomarkers of oxidative stress study II. Are oxidation products of lipids, proteins and DNA markers of CCL4 poisoning? Free Radic Biol Med 2005; 38: 698-710.

114) Wang Z, Ciabattoni G, Creminon C, Lawson J, Fitzgerald GA, Patrono C, Maclouf J. Immunological characterization of urinary 8-epiprostaglandin F2_ excretion in man. J Pharmacol Exp Ther 1995; 275: 94-100.

115) Pratico D, Lawson JA; Rokach J, Fitzgerald GA. The isoprostanes in biology and medicine. Trends Endocrinol Metab 2001; 12: 243-247.

116) Pratico D, Rokach J, Lawson J, Fitzgerald GA. F2-Isoprostanes as indices of lipid peroxidation in inflammatory diseases. Chem Phys Lipids 2004; 128: 165-171.

117) Richelle M, Turini ME, Guidoux R, Tavazzi I, Metairon S, Fay LB. Urinary isoprostane excretion is not confounded by the lipid content of the diet. FEBS Lett 1999; 459: 259-262.

118) Higdon JV, Liu J, Du SH, Morrow JD, Ames BN, Wander RC. Supplementation of postmenopausal women with fish oil rich in eicosapentaenoic acid and docosahexaenoic acid is not associated with greater *in vivo* lipid peroxidation compared with oils rich in oleate and linoleate as assessed by plasma malondialdehyde and F(2)-isoprostanes. Am J Clin Nutr 2000; 72: 714-722.

119) Montuschi P, Collins JV, Ciabattoni G, Lazzeri N, Corradi M, Kharitonov SA, Barnes PJ. Exhaled 8-isoprostane as an *in vivo* biomarker of lung oxidative stress in patients with COPD and healthy smokers. Am J Respir Crit Care Med 2000; 162: 1175-1177.

120) Montuschi P, Kharitonov SA, Ciabattoni G, Corradi M, van Rensen L, Geddes DM, Hodson ME, Barnes PJ. Exhaled 8-isoprostanes as new non-invasive biomarker of oxidative stress in cystic fibrosis. Thorax 2000; 55: 205-209.

121) Stojiljkovic MP, Lopes HF, Zhang D, Morrow JD, Goodfriend TL, Egan BM. Increasing plasma fatty acids elevates F2-isoprostanes in humans: implications for the cardiovascular risk factor cluster. J Hypertens 2002; 20: 1215-1221.

122) Schwedhelm E, Bartling A, Lenzen H, Tsikas D, Maas R, Brummer J, Gutzki FM, Berger J, Frolich JC, Boger RH. Urinary 8-iso-prostaglandin F2a as a risk marker in patients with coronary heart disease: a matched case-control study. Circulation 2004; 109: 843-848.

123) Noll T, de Groot H, Sies H. Distinct temporal relation among oxygen uptake, malondialdehyde formation, and low-level chemiluminescence during microsomal lipid peroxidation. Arch Biochem Biophys 1987; 252: 284-291.

124) Marshall PJ, Warso MA, Lands WEM. Selective microdetermination of lipid hydroperoxides. Anal Biochem 1985; 145: 192-199.

125) Frei B, Yamamoto Y, Niclas D, Ames BN. Evaluation of an isoluminol chemiluminescence assay for the detection of hydroperoxides in human blood plasma. Anal Biochem 1988; 175: 120-130.

126) Esterbauer H, Zollner H. Methods for determination of aldehydic lipid peroxidation products. Free Radic Biol Med 1989; 7: 197-203.

127) Hughes H, Smith CV, Tsokos-Kuhn JO, Mitchell JR. Quantitation of lipid peroxidation products by gas chromatography-mass spectrometry. Anal Biochem 1986; 152: 107-112.

128) Van Kuijk FJ, Thomas DW, Stephens RJ, Dratz EA. Occurrence of 4-hydroxyalkenals in rat tissues determined as pentafluorobenzyl oxime derivatives by gas chromatography-mass spectrometry. Biochem Byophys Res Commun 1986; 139: 144-149.

129) Mowri H, Chinen K, Ohkuma S, Takano T. Peroxidized lipids isolated by HPLC from atherosclerotic aorta. Biochem Int 1986; 12: 347-352.

130) Steinbrecher UP, Lougheed M, Kwan WC, Dirks M. Recognition of oxidized low density lipoprotein by the scavenger receptor of macrophages results from derivatization of apolipoprotein B by products of fatty acid peroxidation. J Biol Chem 1989; 264: 15216-15223.

131) Capitan P, Malmezat T, Breuille D, Obled C. Gas chromatographic-mass spectrometric analysis of stable isotopes of cysteine and glutathione in biological samples. J Chromatogr B 1999; 732: 127-135.

132) Steghens JP, Flourie F, Arab K, Collombel C. Fast liquid chromatography-mass spectrometry glutathione measurement in whole blood: micromolar GSSG is a sample preparation artifact. J Chromatogr B 2003; 798: 343-349.

133) Rossi R, Milzani A, Dalla-Donne I, Giustarini D, Lusini L, Colombo R, Di Simplicio P. Blood glutathione disulfide: *in vivo* factor or *in vitro* artifact? Clin Chem 2002; 48: 742-753.

134) Giustarini D, Dalle Donne I, Colombo R, Milzani A, Rossi R. Interference of plasmatic reduced glutathione and hemolysis on glutathione disulfide levels in human blood. Free Radic Res 2004; 38: 1101-1106.

135) Finkel T, Holbrook NJ. Oxidants, oxidative stress and the biology of ageing. Nature 2000; 408: 239-247.

136) Bharath S, Hsu M, Kaur D, Rajagopalan S, Andersen JK. Glutathione, iron and Parkinson's disease. Biochem Pharmacol 2002; 64: 1037-1048.

137) Warner DS, Sheng H, Batinic-Haberle I. Oxidants, antioxidants and the ischemic brain. J Exp Biol 2004; 207: 3221-3231.

138) Mills BJ, Weiss MM, Lang CA, Liu MC, Ziegler C. Blood glutathione and cysteine changes in cardiovascular disease. J Lab Clin Med 2000; 135: 396-402.

139) Navarro J, Obrador E, Carretero J, Petschen I, Avino J, Perez P, Estrema JM. Changes in glutathione status and the antioxidant system in blood and in cancer cells associate with tumor growth in vivo. Free Radic Biol Med 1999; 26: 410-418.

140) Pantke U, Volk T, Schmutzler M, Kox WJ, Sitte N, Grune T. Oxidized proteins as a marker of oxidative stress during coronary heart surgery. Free Radic Biol Med 1999; 27: 1080-1086.

141) Comhair SA, Erzurum SC. Antioxidant responses to oxidant-mediated lung diseases. Am J Physiol Lung Cell Mol Physiol 2002; 283: 246-255.

142) Lang CA, Mills BJ, Mastropaolo W, Liu MC. Blood glutathione decreases in chronic diseases. J Lab Clin Med 2000; 135: 402-405.

143) Lang CA, Mills BJ, Lang HL, Liu MC, Usui WM, Richie J Jr, Mastropaolo W, Murrell SA. High blood glutathione levels accompany excellent physical and mental health in women ages 60 to 103 years. J Lab Clin Med 2002; 140: 413-417.

144) Beckman JS, Koppenol WH. Nitric oxide, superoxide, and peroxynitrite: the good, the bad and ugly. Am J Physiol 1996; 271: C1424-1437.

145) van der Vliet A, Nguyen MN, Shigenaga MK, Eiserich JP, Marelich GP, Cross CE. Myeloperoxidase and protein oxidation in cystic fibrosis. Am J Physiol 2000; 279: 537-546.

146) Gaut JP, Yeh GC, Than HD, Byun J, Henderson JP, Richter GM, Brennan ML, Lusis AJ, Belaaouaj A, Hotchkiss RS, Heinecke JW. Neutrophils employ the myeloperoxidase system to generate antimicrobial brominating and chlorinating oxidants during sepsis.Proc Natl Acad Sci USA 2001; 98:11961-11966.

147) Brennan ML, Wu W, Fu X, Shen Z, Song W, Frost H, Vadseth C, Narine L, Lenkiewicz E, Borchers MT, Lusis AJ, Lee JJ, Lee NA, Abu-Soud HM, Ischiropoulos H, Hazen SL. A tale of two controversies: defining both the role of peroxidases in nitrotyrosine formation in vivo using eosinophil peroxidase and myeloperoxidase-deficient mice, and the nature of peroxidase-generated reactive nitrogen species. J Biol Chem 2002; 277: 17415-17427.

148) Duncan MW. A review of approaches to the analysis of 3-nitrotyrosine. Amino Acids 2003; 25: 351-361.

149) Tsikas D, Caidahl K. Recent methodological advances in the mass spectrometric analysis of free and protein-associated 3-nitrotyrosine in human plasma. J Chromatogr B 2005; 814: 1-9.

150) Schwedhelm E, Tsikas D, Gutzki FM, Frolich JC. Gas chromatographic-tandem mass spectrometric quantification of free 3-nitrotyrosine in human plasma at the basal state. Anal Biochem 1999; 276: 195-203.

151) Frost MT, Halliwell B, Moore KP. Analysis of free and protein-bound nitrotyrosine in human plasma by a gas chromatography/mass spectrometry method that avoids nitration artifacts. Biochem J 2000; 345: 453-458.

152) Tsikas D, Schwedhelm E, Stutzer FK, Gutzki FM, Rode I, Mehls C, Frolich JC. Accurate quantification of basal plasma levels of 3-nitrotyrosine and 3-nitrotyrosinoalbumin by gas chromatography-tandem mass spectrometry. J Chromatogr B 2003;784:77-90.

153) Wu W, Samoszuk MK, Comhair SA, Thomassen MJ, Farver CF, Dweik RA, Kavuru MS, Erzurum SC, Hazen SL. Eosinophils generate brominating oxidants in allergen-induced asthma. J Clin Invest 2000; 105: 1455-1463.

154) MacPherson JC, Comhair SAA, Erzurum SC, Klein DF, Lipscomb MF, Kavuru MS, Samoszuk MK, Hazen SL. Eosinophils are a major source of nitric oxide-derived oxidants in severe asthma: characterization of pathways available to eosinophils for generating reactive nitrogen species. J Immunol 2001; 166: 5763-5772.

155) Aldridge RE, Chan T, Van Dalen CJ, Senthilmohan R, Winn M, Venge P, Town GI, Kettle AJ. Eosinophil peroxidase produces hypobromous acid in the airways of stable asthmatics. Free Radic Biol Med 2002; 33: 847-856.

156) Kettle AJ, Chan T, Osberg I, Senthilmohan R, Chapman AL, Mocatta TJ, Wagener JS. Myeloperoxidase and protein oxidation in the airways of young children with cystic fibrosis. Am J Respir Crit Care Med 2004; 170: 1317-1323.

157) Bergt C, Pennathur S, Fu X, Byun J, O'Brien K, McDonald TO, Singh P, Anantharamaiah GM, Chait A, Brunzell J, Geary RL, Oram JF, Heinecke JW. The myeloperoxidase product hypochlorus acid oxidizes HDL in the human artery wall and impairs ABCA1-dependent cholesterol transport. Proc Natl Acad Sci USA 2004; 101: 13032-13037.

158) Zheng L, Nukuna B, Brennan ML, Sun M, Goormastic M, Settle M, Schmitt D, Fu X, Thomson L, Fox PL, Ischiropoulos H, Smith JD, Kinter M, Hazen SL. Apolipoprotein A-I is a selective target for myeloperoxidase-catalyzed oxidation and functional impairment in subjects with cardiovascular disease. J Clin Invest 2004; 114: 529-541.

159) Ischiropoulos H. Biological selectivity and functional aspects of protein tyrosine nitration. Biochem Biophys Res Commun 2003; 305: 776-783.

160) Shishehbor MH, Brennan ML, Aviles RJ, Fu X, Penn MS, Sprecher DL, Hazen SL. Statins promote potent systemic antioxidant effects through specific inflammatory pathways. Circulation 2003; 108: 426-431.

161) Gole MD, Souza JM, Choi I, Hertkorn C, Malcom S, Foust RF 3rd, Finkel B, Lanken PN, Ischiropoulos H. Plasma proteins modified by tyrosine nitration in acute respiratory distress syndrome. Am J Physiol Lung Cell Mol Physiol 2000; 278: 961-967.

162) Pignatelli B, Li CQ, Boffetta P, Chen Q, Ahrens W, Nyberg F, Mukeria A, Bruske-Hohlfeld I, Fortes C, Constantinescu V, Ischiropoulos H, Ohshima H. Nitrated and oxidized plasma proteins in smokers and lung cancer patients. Cancer Res 2001; 61: 778-784.

163) Masri FA, Comhair SAA, Koeck T, Xu W, Janocha A, Ghosh S, Dweik RA, Golish J, Kinter M, Stuehr DJ, Erzurum SC, Aulak KS. Abnormalities in nitric oxide and its derivatives in lung cancer. Am J Respir Crit Care Med 2005; 172: 597-605.

164) Ischiropoulos H, Beckman JS. Oxidative stress and nitration in neurodegeneration: cause, effect, or association? J Clin Invest 2003; 111: 163-169.

165) Zhan X, Desiderio DM. The human pituitary nitroproteome: detection of nitrotyrosyl-proteins with two-dimensional Western blotting, and amino acid sequence determination with mass spectrometry. Biochem Biophys Res Commun 2004; 325: 1180-1186.

166) Greenacre SA, Ischiropoulos H. Tyrosine nitration: localisation, quantification, consequences for protein function and signal transduction. Free Radic Res 2001; 34: 541-581.

167) Dean RT, Fu S, Stocker R, Davies MJ. Biochemistry and pathology of radical-mediated protein oxidation. Biochem J 1997; 324: 1-18.

168) Dalle-Donne I, Giustarini D, Colombo R, Rossi R, Milzani A. Protein carbonylation in human diseases. Trends Mol Med 2003; 9: 169-176.

169) Aldini G, Dalle-Donne I, Vistoli G, Maffei Facino R, Carini M. Covalent modification of actin by 4-hydroxy-trans-2-nonenal (HNE): LC-ESI-MS/MS evidence for Cys374 Michael adduction. J Mass Spectrom 2005; 40: 946-954.

170) Dalle-Donne I, Rossi R, Giustarini D, Milzani A, Colombo R. Protein carbonyl groups as a biomarkers of oxidative stress. Clin Chim Acta 2003; 329: 23-28.

171) Levine RL. Carbonyl modified proteins in cellular regulation, aging, and disease. Free Radic Biol Med 2002; 32: 790-796.

172) Butterfield DA, Castegna A. Proteomics for the identification of specifically oxidized proteins in brain: technology and application to the study of neurodegenerative disorders. Amino Acids 2003; 25: 419-425.

173) Renke J, Popadiuk S, Korzon M, Bugajczyk B, Wozniak M. Protein carbonyl groups' content as a useful clinical marker of antioxidant barrier impairment in plasma of children with juvenile chronic arthritis. Free Radic Biol Med 2000; 29: 101-104.

174) Oberg BP, McMenamin E, Lucas FL, McMonagle E, Morrow J, Ikizler TA, Himmelfarb J. Increased prevalence of oxidant stress and inflammation in patients with moderate to severe chronic kidney disease. Kidney Int 2004; 65: 1009-1016.

175) Winterbourn CC, Bonham MJ, Buss H, Abu-Zidan FM, Windsor JA. Elevated protein carbonyls as plasma markers of oxidative stress in acute pancreatitis. Pancreatology 2003; 3: 375-382.

176) Dizdaroglu M, Jaruga P, Birincioglu M, Rodriguez H. Free radical-induced damage to DNA: mechanisms and measurement. Free Radic Biol Med 2002; 32: 1102-1115.

177) Halliwell B. Effect of diet on cancer development: is oxidative DNA damage a biomarker? Free Radic Biol Med 2002; 32: 1102-1115.

178) Valko M, Izakovic M, Mazur M, Rhodes CJ, Telser J. Role of oxygen radicals in DNA damage and cancer incidence. Mol Cell Biochem 2004; 266: 37-56.

179) Cathcart R, Schwiers E, Saul RL, Ames BN. Thymine glycol and thymidine glycol in human and rat urine: a possible assay for oxidative DNA damage. Proc Natl Acad Sci USA 1984; 81: 5633-5637.

180) Collins AR, Cadet J, Moller L, Poulsen HE, Vina J. Are we sure we know how to measure 8-oxo–7,8-dihydroguanine in DNA from human cells? Arch Biochem Biophys 2004; 423: 57-65.

181) Papa A, Danese S, Sgambato A, Ardito R, Zannoni G, Rinelli A, Vecchio FM, Gentiloni-Silveri N, Cittadini A, Gasbarrini G, Gasbarrini A. Role of *Helicobacter pylori* CagA+ infection in determining oxidative DNA damage in gastric mucosa. Scand J Gastroenterol 2002; 37: 409-413.

182) Iida T, Furuta A, Kawashima M, Nishida J, Nakabeppu Y, Iwaki T. Accumulation of 8-oxo-2'-deoxyguanosine and increased expression of hMTH1 protein in brain tumors. Neuro-Oncol 2001; 3: 73-81.

183) Fairbarian DW, Olive PL, O'Neill KL. The comet assay: A comprehensive review. Mutat Res 1995; 339: 37-59.

184) Sutherland JC, Monteleone DC, Trunk JG, Bennett PV, Sutherland BM. Quantifying DNA damage by gel electrophoresis, electronic imaging and number-average lenght analysis. Electrophoresis 2001; 22: 843-854.

185) Zhang Y, Chen SY, Hsu T, Santella RM. Immunohistochemical detection of malondialdehyde-DNA adducts in human oral mucosa cells. Carcinogenesis 2002; 23: 207-211.

186) Sodum RS, Chung FL. Structural characterization of adducts formed in the reaction of 2,3-epoxy-4-hydroxynonanal 1 with deoxyguanosine. Chem Res Toxicol 1989; 2: 23-28.

187) Wayner DD, Burton GW, Ingold KU, Barclay LR, Locke SJ. The relative contributions of vitamin E, urate, ascorbate and proteins to the total peroxyl radical-trapping antioxidant activity of human blood plasma. Biochim Biophys Acta 1987; 924: 408-419.

188) Prior RL, Cao G. *In vivo* total antioxidant capacity: comparison of different analytical methods. Free Radic Biol Med 1999; 27: 1173-1181.

189) Berry EM, Kohen R. Is the biological antioxidants system integrated and regulated? Med Hypoth 1999; 53: 397-401.

190) Akerboom TP, Sies H. Assay of glutathione, glutathione disulfide, and glutathione mixed disulfides in biological samples. Methods Enzymol 1981; 77: 373-382.

191) Krebs HA. The redox state of nicotinamide adenine dinucleotide in the cytoplasm and mitochondria of rat liver. Adv Enzyme Regul 1967; 5: 409-434.

192) Motchnik PA, Frei B, Ames BN. Measurement of antioxidants in human blood plasma. Methods Enzymol 1994; 234: 269-279.

193) Rice-Evans CA, Miller NJ. Total antioxidant status in plasma and body fluids. Methods Enzymol 1994; 234: 279-293.

194) Lissi E, Salim-Hanna M, Pascual C, del Castillo MD. Evaluation of total antioxidant potential (TRAP) and total antioxidant reactivity from luminol-enhanced chemiluminescence measurements. Free Radic Biol Med 1995; 18: 153-158.

195) Niculescu L, Stancu C, Sima A, Toporan D, Simionescu M. The total peroxyl radical trapping potential in serum-an assay to define the stage of atherosclerosis. J Cell Mol Med 2001; 5: 285-294.

196) Benzie IFF, Strain JJ. Ferric reducing/antioxidant power assay: Direct measure of total antioxidant activity of biological fluids and modified version for simultaneous measurements of total antioxidant power and ascorbic acid concentration. Methods Enzymol 1999; 299: 15-27.

197) Benzie IF, Strain JJ. The ferric reducing ability of plasma (FRAP) as a measure of "antioxidant power": the FRAP assay. Anal Biochem 1996; 239: 70-76.

198) Cao G, Prior RL. Comparison of different analytical methods for assessing total antioxidant capacity of human serum. Clin Chem 1998; 44: 1309-1315.

199) Halliwell B, Murcia MA, Chirico S, Aruoma OI. Free radicals and antioxidants in food and *in vivo*: what they do and how they work. Crit Rev Food Sci Nutr 1995; 35: 7-20.

200) Rice-Evans CA. Measurement of total antioxidant activity as a marker of antioxidant status *in vivo*: procedures and limitations. Free Radic Res 2000; 33 Suppl: S59-66.

201) Horoz M, Bolukbas C, Bolukbas FF, Sabuncu T, Aslan M, Sarifakioglulari S, Gunaydin N, Erel O. Measurement of the total antioxidant response using a novel automated method in subjects with nonalcoholic steatohepatitis. BMC Gastroenterology 2005; 5: 35.

202) Harma M, Harma M, Erel O. Increased oxidative stress in patients with hydatidiform mole. Swiss Med Wkly 2003; 133: 563-566.

203) Erel O. A novel automated direct measurement method for total antioxidant capacity using a new generation, more stable ABTS radical cation. Clin Biochem 2004; 37: 277-285.

204) Whitehead TP, Thorpe GH, Maxwell SR. Enhanced chemiluminescent assay for anti-oxidant capacity in biological fluids. Anal Chim Acta 1992; 266: 265-277.

205) Sies H, Akerboom TP. Glutathione disulfide (GSSG) efflux from cells and tissues. Methods Enzymol 1984; 105: 445-451.

206) Sheehan TM, Gao M. Simplified fluorometric assay of total selenium in plasma and urine. Clin Chem 1990; 36 :2124-2126.

207) Kahan J. Automated fluorimetric assay of serum vitamin A. Int J Vitam Nutr Res 1973; 43: 127-141.

208) Omaye ST, Turnbull JD, Sauberlich HE. Selected methods for the determination of ascorbic acid in animal cells, tissues and fluids. Methods Enzymol 1979; 62: 3-11.

209) Desai ID. Vitamin E analysis methods for animal tissues. Methods Enzymol 1984; 105: 138-147.

210) Skoog DA, West DM, Holler FJ. An introduction to electrochemistry. In: Fundamentals of Analytical Chemistry, 7th edition. Saunders College Publishing, New York, pp 303-29, 1988.

211) Bard AJ, Faulkner LR. Electrochemical Methods: Fundamentals and Applications. John Wiley & Sons, Inc., New York, 1980.

212) O'Neill RD, Lowry JP, Mas M. Monitoring brain chemistry in vivo: voltammetric techniques, sensors, and behavioral applications. Crit Rev Neurobiol 1998; 12: 69-127.

213) Kohen R, Vellaichamay E, Hrbac L, Gati I, Tirosh O. Quantification of the overall reactive oxygen species scavenging capacity of biological fluids and tissues. Free Radic Biol Med 2000; 28: 871-879.

214) Noel M, Vasu KI. Cyclic voltammetry technique. In: Cyclic Voltammetry and the Frontiers of Electrochemistry, 1990. Aspect Publications, UK, pp 62-92.

215) Heineman WR, Kissinger PT. Laboratory Techniques in Electroanalytical Chemistry, 5th edition. Marcel Dekker, 1996, New York.

216) Kilmartin PA. Electrochemical detection of natural antioxidants: principles and protocols. Antioxid Redox Signal 2001; 3: 941-955.

217) Kohen R, Tirosh O, Gorodetzky R. The biological reductive capacity of tissues is decreased following exposure to oxidative stress: a cyclic voltammetry study of irradiated rats. Free Radic Res Commun 1992; 17: 239-248.

218) Dubois RN, Abramson SB, Crofford L, Gupta RA, Simon LS, Van De Putte LB, Lipsky PE. Cyclooxygenase in biology and disease. FASEB J 1998; 12: 1063-1073.

219) Ornoy A, Zaken V, Kohen R. Role of reactive oxygen species (ROS) in the diabetes-induced anomalies in rat embryos in vitro: reduction in antioxidant enzymes and low-molecular-weight antioxidant (LMWA) may be the causative factor for increased anomalies. Teratology 1999; 60: 376-386.

220) Blau S, Kohen R, Bass P, Rubinstein A. Relation between colonic inflammation severity and total low-molecular-weight antioxidant profile in experimental colitis. Dig Dis Sci 2000; 45: 1180-1187.

221) Shohami E, Gati I, Beit-Yannai E, Trembovler V, Kohen R. Closed head injury in the rat induces whole body oxidative stress:overall reducing antioxidant profile. J Neurotrauma 1999; 16: 365-376.

222) Kohen R, Gati I. Skin low molecular weight antioxidants and their role in aging and in oxidative stress. Toxicology 2000; 148: 149-157.

223) Zaken V, Kohen R, Ornoy A. Vitamins C and E improve rat embryonic antioxidant defense mechanism in diabetic culture medium. Teratology 2001; 64: 33-44.

224) Battino M, Ferrerio MS, Gallardo I, Newman HN, Bullon P. The antioxidant capacity of saliva. J Clin Periodontol 2002; 29: 189-194.

225) Raina AK, Perry G, Nunomura A, Sayre LM, Smith MA. Histochemical and immunocytochemical approaches to the study of oxidative stress. Clin Chem Lab Med 2000; 38: 93-97.

226) Barnes PJ, Karin M. Nuclear factor _B: a pivotal transcription factor in chronic inflammatory diseases. N Engl J Med 1997; 336:1066-1071.

227) Hur GM, Ryu YS, Yun HY, Jeon BH, Kim YM, Seok JH, Lee JH. Hepatic ischemia/reperfusion in rats induces iNOS gene transcription by activation of NF-kappaB. Biochem Biophys Res Commun 1999; 261: 917-922.

228) Salvemini D, Settle SL, Masferrer JL, Seibert K, Currie MG, Needleman P. Regulation of prostaglandin production by nitric oxide; an in vivo analysis. Br J Pharmacol 1995; 114: 1171-1178.

229) Ochiai M, Oguri T, Isobe T, Ishioka S, Yamakido M. Cyclooxygenase-2 (COX-2) mRNA expression levels in normal lung tissues and non-small cell lung cancers. Jpn J Cancer Res 1999; 90: 1338-1343.

230) Zimmermann KC, Sarbia M, Weber AA, Borchard F, Gabbert HE, Schror K. Cyclooxygenase-2 expression in human esophageal carcinoma. Cancer Res 1999; 59: 198-204.

231) Rioux N, Castonguay A. Prevention of NNK-induced lung tumorigenesis in A/J mice by acetylsalicyclic acid and NS-398. Cancer Res 1998; 58: 5354-5360.

232) Enomoto A, Itoh K, Nagayoshi E, Haruta J, Kimura T, O'Connor T, Harada T, Yamamoto M. High sensitivity of Nrf2 knockout mice to acetaminophen hepatotoxicity associated with decreased expression of ARE-regulated drug metabolizing enzymes and antioxidant genes. Toxicol Sci 2001; 59: 169-177.

233) Prestera T, Talalay P. Electrophile and antioxidant regulation of enzymes that detoxify carcinogens. Proc Natl Acad Sci USA 1995; 92: 8965-8969.

234) Henderson CJ, McLaren AW, Moffat GJ, Bacon EJ, Wolf CR. Pi-class glutathione S-transferase: regulation and function. Chem Biol Interact 1998; 111-112: 69-82.

235) Coles B, Ketterer B. The role of glutathione and glutathione transferases in chemical carcinogenesis. Crit Rev Biochem Mol Biol 1990; 25: 47-70.

236) Moncada S, Higgs A. The L-arginine-nitric oxide pathway. N Engl J Med 1993; 329: 2002-2012.

237) Orucevic A, Bechberger J, Green AM, Shapiro RA, Billiar TR, Lala PK. Nitric-oxide production by murine mammary adenocarcinoma cells promotes tumor-cell invasiveness. Int J Cancer 1999; 81: 889-896.

238) Baltaci S, Orhan D, Gogus C, Turkolmez K, Tulunay O, Gogus O. Inducible nitric oxide synthase expression in benign prostatic hyperplasia, low- and high-grade prostatic intraepithelial neoplasia and prostatic carcinoma. BJU Int 2001; 88: 100-103.

239) Gottschling BC, Maronpot RR, Hailey JR, Peddada S, Moomaw CR, Klaunig JE, Nyska A. The role of oxidative stress in indium phosphide-induced lung carcinogenesis in rats. Toxicol Sci 2001; 64: 28-40.

240) Immenschuh S, Ramadori G. Gene regulation of heme oxygenase-1 as a therapeutic target. Biochem Pharmacol 2000; 60: 1121-1128.

241) Liu Y, Zhu B, Luo L, Li P, Paty DW, Cynader MS. Heme oxygenase-1 plays an important protective role in experimental autoimmune encephalomyelitis. Neuroreport 2001; 12: 1841-1845.

242) Harrison R. Structure and function of xanthine oxidoreductase: when are we now? Free Radic Biol Med 2002; 33: 774-797.

243) Wu XW, Muzny DM, Lee CC, Caskey CT. Two independent mutational events in the loss of urate oxidase during hominoid evolution. J Mol Evol 1992; 34: 78-84.

244) Egan BM, Weder AB, Petrin J, Hoffman RG. Neurohumoral and metabolic effects of short-term dietary NaCl restriction in men. Relationship to salt-sensitivity status. Am J Hypertens 1991; 4(5 Pt 1): 416-421.

245) Del Rio A, Rodriguez-Villamil JL. Metabolic effects of strict salt restriction in essential hypertensive patients. J Intern Med 1993; 233: 409-414.

246) Peixoto MR, Monego ET, Jardim PC, Carvalho MM, Sousa AL, Oliveira JS, Balestra Neto O. Diet and medication in the treatment of hyperuricemia in hypersensitive patients. Arq Bras Cardiol 2001; 76: 463-472.

247) Granger DN, Parks DA. Role of oxygen radicals in the pathogenesis of intestinal ischemia. Physiologist 1983; 26: 159-164.

248) McCord JM. Oxygen-derived free radicals in postischemic tissue injury. N Engl J Med 1985; 312: 159-163.

249) Koj A. A re-evaluation of the tissue distribution and physiology of xanthine oxidoreductase. Histochem J 1994; 26: 159-163.

250) Fenton HJH. Oxidation of tartaric acid in presence of iron. J Chem Soc 1894; 65: 899.

251) Halliwell B, Gutteridge JMC. The chemistry of free radicals and related "reactive species". In: Halliwell B, Gutteridge JMC, editors. Free Radicals in Biology and Medicine. Oxford: Oxford University Press 1999; 43-45.

252) Harrison R. Human xanthine oxidoreductase: in search of a function. Biochem Soc Trans 1997; 25: 786-791.

253) Godber BL, Doel JJ, Sapkota GP, Blake DR, Stevens CR, Eisenthal R, Harrison R. Reduction of nitrite to nitric oxide catalyzed by xanthine oxidroreductase. J Biol Biochem 2000; 275: 7757-7763.

254) Truglio JJ, Theis K, Leimkuhler S, Rappa R, Rajagopalan KV, Kisker C. Crystal structures of the active and alloxanthine-inhibited forms of xanthine dehydrogenase from *Rhodobacter capsulatus*. Structure (Camb) 2002; 10: 115-125.

255) Ames BN, Cathcart R, Schwiers E, Hochstein P. Uric acid provides an antioxidant defense in humans against oxidant-and radical-caused aging and cancer: a hypothesis. Proc Natl Acad Sci USA 1981; 78: 6858-6862.

256) Becker BF. Towards the physiological function of uric acid. Free Radic Biol Med 1993; 14: 615-631.

257) Re R, Pellegrini N, Proteggente A, Pannala A, Yang M, Rice-Evans C. Antioxidant activity applying an improved ABTS radical cation decolorization assay. Free Radic Biol Med 1999; 26: 1231-1237.

258) Halliwell B, Gutteridge JMC. Antioxidant protection by low-molecular-mass agents: compounds synthesized in vivo. In: Halliwell B, Gutteridge JMC, editors. Free Radicals in Biology and Medicine. Oxford: Oxford University Press 1999: 195-197.

259) Davies KJ, Sevanian A, Muakkassah-Kelly SF, Hochstein P. Uric acid-iron ion complexes. A new aspect of the antioxidant functions of uric acid. Biochem J 1986;235:747-754.

260) Miura T, Muraoka S, Ogiso T. Inhibitory effect of urate on oxidative damage induced by adriamycin-Fe^{3+} in the presence of H_2O_2. Res Commun Chem Pathol Pharmacol 1993; 79: 75-85.

261) Squadrito GL, Cueto R, Splenser AE, Valavanidis A, Zhang H, Uppu RM, Pryor WA. Reaction of uric acid with peroxynitrite and implications for the mechanism of neuroprotection by uric acid. Arch Biochem Biophys 2000; 376: 333-337.

262) Gow AJ, Farkouh CR, Munson DA, Posencheg MA, Ischiropoulos H. Biological significance of nitric oxide-mediated protein modifications. Am J Physiol Lung Cell Mol Physiol 2004; 287: L262-268.

263) Skinner KA, White CR, Patel R, Tan S, Barnes S, Kirk M, Darley-Usmar V, Parks DA. Nitrosation of uric acid by peroxynitrite. Formation of a vasoactive nitric oxide donor. J Biol Chem 1998; 273: 24491-24497.

264) Watanabe S, Kang DH, Feng L, Nakagawa T, Kanellis J, Lan H, Mazzali M, Johnson RJ. Uric acid, hominoid evolution, and the pathogenesis of salt-sensitivity . Hypertension 2002; 40: 355-360.

Acknowledgements

The Authors thanks to Dr. Carla Torri from Callegari Spa-Catellani group, Parma, for a permission of reviewing their bibliography archives.