

**NEW MARKERS OF OXIDATIVE DAMAGE TO MACROMOLECULES**

## NOVI MARKERI OKSIDATIVNOG OŠTEĆENJA MAKROMOLEKULA

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**Summary:** The presence of free radicals in biological material has been discovered some 50 years ago. In physiological conditions, free radicals, in the first place the ones of oxygen and nitrogen, are continuously synthesized and involved in the regulation of a series of physiological processes. The excess of free radicals is efficiently eliminated from the body in order to prevent their toxic effects. Toxic effects of free radicals may be classified into three groups: a) change of intracellular redox potential, b) oxidative modification of lipids, proteins and DNA, and c) gene activation. Lipid peroxidation involving cell membranes, lipoproteins and other molecules leads to the production of primary high-reactive intermediaries (alkyl radicals, conjugated dienes, peroxy- and alkoxyl radicals and lipid hydroperoxide), whose further breakdown generates the secondary products of lipid peroxidation: short-chain evaporable hydrocarbons, aldehydes and final products of lipid peroxidation: isoprostanes, MDA, 4-hydroxy-2,3-transnonenal and 4,5-dihydroxydecenal which are important mediators of atherosclerosis, coronary disease, acute myocardial infarction, rheumatoid arthritis, systemic sclerosis and lupus erythematoses. Oxidative modification of proteins is manifested by changes in their primary, secondary and tertiary structures. Proteins have a specific biological function, and therefore their modification results in unique functional consequences. The nature of protein modification may provide valid information on the type of oxidants causing the damage. Chlorotyrosyl is a specific marker of oxidative damage to tyrosine caused by HOCl action, which most commonly reflects the involvement of neutrophils and monocytes in oxidative stress, while nitrotyrosyl indicates the presence of higher peroxy-nitrite synthesis. Methionin and cysteine are the amino acids most sensitive to oxidative stress, carbonyl groups are markers of severe damage caused by free radicals, and di-tyrosyl is the most significant and sensitive marker of oxidative modification made by  $\gamma$  rays. »Carbo-

**Kratik sadržaj:** Prisustvo slobodnih radikala u biološkim materijalima je otkriveno pre nešto više od 50 godina. U fiziološkim uslovima, slobodni radikali, pre svega kiseonika i azota, stalno se sintetišu i pri tom učestvuju u regulaciji niza fizioloških procesa. Višak slobodnih radikala se efikasno uklanja iz organizma u cilju sprečavanja njihovih toksičnih efekata. Toksični efekti delovanja slobodnih radikala se mogu podeliti u tri grupe: a) pomeranje intraćelijskog redoks stanja, b) oksidativna modifikacija lipida, proteina i DNK i c) aktivacija gena. Lipidna peroksidacija koja zahvata ćelijske membrane, lipoproteine i druge molekule dovodi do stvaranja primarnih visokoreaktivnih intermedijera (alkil radikala, konjugovanih diena, peroksi i alkoksi radikala i lipidnih hidroperoksida), koji daljom razgradnjom daju sekundarne produkte lipidne peroksidacije: kratkolančane isparljive ugljovodonike, aldehide i krajnje proizvode lipidne peroksidacije: izoprostane, MDA (malondialdehid), 4-hidroksi-2,3-transnonenal i 4,5-dihidroksidecenal, koji su značajni medijatori ateroskleroze, koronarne bolesti, akutnog infarkta miokarda, reumatoidnog artritisa, sistemske skleroze, lupusa eritematodesa. Oksidativna modifikacija proteina manifestuje se promenama njihove primarne, sekundarne i tercijarne strukture. Proteini imaju specifičnu biološku funkciju, stoga njihova izmena ima jedinstvene funkcionalne posledice. Priroda proteinske modifikacije može nam dati validan podatak o vrsti oksidansa koji je doveo do oštećenja. Hlorotirozil je specifičan marker oksidativnog oštećenja tirozina dejstvom hipohlorita (HOCl), koji najčešće reflektuje učešće neutrofila i monocita u oksidativnom stresu, dok nitrotirozil ukazuje na prisustvo povećane sinteze peroksinitrita. Metionin i cistein su najosetljivije aminokiseline na dejstvo oksidativnog stresa, karbonilne grupe su markeri teškog oštećenja slobodnim radikalima, dok je di-tirozil najznačajniji i najosetljiviji marker oksidativne modifikacije dejstvom  $\gamma$ -zraka. »Karbonilni stres« je značajan vid sekundarne oksidacije proteina u kojem redukujući šećeri neenzimski reaguju sa amino grupama proteina i lipida dovodeći do stvaranja kovalentnih jedinjenja poznatih kao uznapredovani proizvodi glikozilacije ili AGE-proizvodi (advanced glycosylated end products). Hidroksilni radikal oštećuje i molekul DNK dovodeći do gubitka baze i stvaranja abazičnih mesta (AP site-ova), cepanja lanca DNK i modifikacije šećera. Krajnji proizvodi lipidne peroksidacije (MDA) mogu se kovalentno vezivati za DNK, stvara-

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nyl stress« is an important form of the secondary oxidation of proteins, where reducing sugars non-enzymatically react with amino groups of proteins and lipids and give rise to the production of covalent compounds known as advanced glycosylated end products (AGE-products). A hydroxyl radical damages the DNA, leading to a loss of base and the formation of abasic sites (AP sites), break of DNA chain and sugar modification. Final lipid peroxidation products (MDA) may covalently bind to DNA, producing the »DNA radicals« which are responsible for mutations. Measurement of an adequate oxidative stress biomarker may not only point to an early onset of disease, its progression and assessment of therapy effectiveness, but can also help in the clarification of the pathophysiological mechanisms of tissue damage caused by oxidative stress, prediction of disease prognosis and choice of appropriate treatment in the early stages of disease.

**Keywords:** oxidative modification of lipids, proteins, DNA, biomarkers, free radicals, lipid peroxidation, carbonyl stress

## Introduction

The presence of free radicals in biological materials was discovered some 50 years ago (1). Soon after that discovery, Harman (2) reported his hypothesis that free radicals were the products of enzymatic reactions *in vivo*, describing them as a »Pandora' box«, responsible for the majority of cell damages, mutagenesis, carcinogenesis and degenerative processes inducing the aging (3).

The research of free radicals began its second era when McCord and Fridovich (4), in 1969, discovered the enzyme superoxide dismutase (SOD), and documented the significance of free radicals for biological systems. Many studies then initiated were aimed at investigating the oxidative damage to DNA, proteins, lipids and other cell components caused by free radicals (5).

The third era of research on free radicals began with the first description of their biological effects. Mittal and Murard (6), in 1977, confirmed that superoxide anion ( $O_2^{\cdot -}$ ), via its hydroxyl radical derivatives, stimulated the activation of guanylate cyclase and synthesis of the secondary messenger of cGMP. In 1987, Ignaro and Kadowitz (7) and Moncada (8) discovered an independent role of nitrogen monoxide (NO) as a regulatory molecule in the control of the relaxation of smooth muscle cells and inhibition of platelet adhesion. Roth and Dröge (9) proved that superoxide anion or a low concentration of hydrogen peroxide in activated T-lymphocytes increased the synthesis of interleukin-2 (IL-2), an important immunological protein of T-lymphocytes. Keyse and Tyrrel (10) found that hydrogen peroxide induced the expression of hem oxygenase gene (HO-1 gene). Storz and associates (11), in 1990, showed that hydrogen peroxide induced the expression of different genes in bacteria, while Schreck and Baeuerle (12),

jući »DNK radikale« koji su odgovorni za nastanak mutacija. Merenje adekvatnog biomarkera oksidativnog stresa može nam ukazati ne samo na ranu pojavu bolesti, njenu progresiju i procenu efikasnosti terapije, već i pomoći u rasvetljavanju patofizioloških mehanizama oštećenja tkiva dejstvom oksidativnog stresa, u predikciji prognoze bolesti i izboru adekvatnog lečenja u ranim stadijumima bolesti.

**Ključne reči:** oksidativna modifikacija, lipidi, proteini, DNK, biomarkeri, slobodni radikali, lipidna peroksidacija, karbonilni stres

in 1991, described the activation of transcriptional nuclear factor  $\kappa$ B (NF- $\kappa$ B) in mammalian cells by  $H_2O_2$  action.

Signal transduction is a process of transmission of signals from the cell surface into the cell interior. Free radicals and their metabolites are included in a complex network of different signal pathways and most probably operate as intracellular and intercellular mediators, transforming the initial signal into a biochemical response. Free radicals and redox stress also participate actively in cell signalization as secondary messengers in the activation of transcription factors and induction of gene expression.

The term »redox-signalization« is used in the description of a regulatory process where a signal originates during redox-reaction. The principle mechanism of redox homeostasis is based on the induction of redox sensitive signaling cascade which leads to a higher expression of antioxidative enzymes or to increased cysteine transport system, which results in an elevated intracellular glutathione concentration.

Cells and tissues are stable if the rate of production of free radicals and capacity of »cleansing« are in continuous balance. Redox signals demonstrate that such balance is impaired either by an increased production of free radicals or lower activity of one or more antioxidant systems. If the initial increase of free radicals were relatively small, the antioxidant response would be sufficient to neutralize it and restore the balance. In some circumstances, production of free radicals may be more intensive and persistent, therefore, the antioxidant response will not be adequate to neutralize them and restore the system to the initial level of redox-homeostasis. In such cases, the system may still be recovered and restore its balance, but the newly developed »quasi-stable« state is now followed by a high production of free radicals and varying gene expression. Pathological conditions

occur exclusively in extreme situations, with continuously high concentrations of free radicals. These conditions do not necessarily mean an immediate loss of homeostasis, but rather a »chronic shift of the homeostasis level«. Pathological symptoms may result from the noxious effect of free radicals, as well as altered gene expression due to their effect (13–15).

Under physiological conditions, free radicals, in the first place oxygen and nitrogen, are constantly synthesized and simultaneously involved in the regulation of a series of physiological processes. An excess of free radicals is efficiently eliminated from body in order to prevent their toxic effects. The toxic effects of free radicals are associated with the development of over 100 different diseases such as: cardiovascular diseases, myocardial infarction, atherosclerosis, diabetes, neurodegenerative diseases, rheumatoid arthritis, cystic fibrosis, etc. (16). The toxic effects of free radicals may be divided into three groups:

1. shift of intracellular redox condition
2. oxidative modification of lipids, proteins and DNA
3. gene activation.

### Lipid peroxidation

Lipid peroxidation is an oxidative damage involving the cell membranes, lipoproteins and other molecules containing lipids under conditions of oxidative stress. Cell membrane lipids (phospholipids, glycolipids and cholesterol) are the most common substrates of oxidative attack. Once initiated, the reaction of peroxidation is continued autocatalytically, and has a progradient course, finally resulting in structural-functional changes of substrates (17).

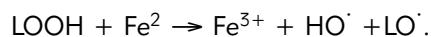
Lipid peroxidation in controlled conditions modifies the cell membrane permeability, affects the intensity of the metabolism of membrane lipids and proteins, and provides control of the synthesis of biologically active eicosanoids, control of cell proliferation and initiation of cell death (18, 19).

Polyunsaturated fatty acids in phospholipids and glycolipids are the basic substrate of oxidative damage to lipids caused by free radicals. The process of lipid peroxidation proceeds through the phases of *initiation*, *propagation* and *termination*.

*Initiation* of the lipid peroxidation process implies an elimination of a hydrogen atom from a methyl group ( $-\text{CH}_2-$ ), which is in an  $\alpha$ -position in relation to the  $-\text{C}=\text{C}-$  bond in the chain of fatty acids (LH), in which process a lipid radical ( $\text{L}^\cdot$ ) is produced. With the aim of molecule stabilization, double bonds are arranged intramolecularly in lipid radicals and conjugated dienes are formed. Addition of a molecular oxygen to conjugated dienes produces a peroxy radical ( $\text{LOO}^\cdot$ ). A peroxy radical may further initiate the

removal of hydrogen from adjacent lipid molecules, at which point the reaction of lipid peroxidation enters the second phase of *propagation* and autooxidation, resulting in the formation of lipid hydroperoxide (LOOH) and new lipid (alkyl) radicals (20).

Lipid hydroperoxide is the next potential source of high-reactive  $\text{OH}^\cdot$ . In the presence of  $\text{Fe}^{2+}$  in the classical Fenton's reaction, breakdown of LOOH yields  $\text{OH}^\cdot$  and alkoxy (oxyl) radical ( $\text{LO}^\cdot$ ).



In the presence of  $\text{Fe}^{3+}$  LOOH are degraded to peroxy radical ( $\text{LOO}^\cdot$ ).



Alkoxy and peroxy radicals are responsible for further initiation and propagation of lipid peroxidation.

Lipid hydroperoxides may be produced by one-electron reduction of oxyl radical ( $\text{LO}^\cdot$ ) in a  $\text{Fe}^{2+}$ -mediated reaction (21).

There are three options for the further breakdown of alkoxy (oxyl) radical:

1. by elimination of hydrogen from adjacent molecules of unsaturated fatty acids, an oxyl radical ( $\text{LO}^\cdot$ ) is transformed into the hydroperoxyl radical (LOOH);
2. in the process of  $\beta$ -oxidation, they are transformed into aldehydes and new alkyl radicals;
3. oxygenation and rearrangement of double bonds result in epoxy peroxide radicals ( $\text{OLOO}^\cdot$ ).

Malondialdehyde (MDA) is the final product of lipid peroxidation, which may react further with protein amino groups, forming the intramolecular cross-over bonds, or may bind two different proteins through intramolecular bonds thus changing their structural-functional characteristics (22).

Lipid peroxidation reduces the fluidity of biological membranes which leads to their higher permeability for mono- and bivalent ions and inactivation of membrane enzymes (23). Fragmentation of fatty acid chains to intermediaries such as aldehydes and short-chain evaporable hydrocarbons (i.e., pentane) gives rise to the loss of membrane integrity, while the rupture of lysosomal membranes leads to a release of hydrolytic enzymes, which further damages the cell (24).

### End products of lipid peroxidation

Lipid peroxidation products may be divided into primary high-reactive intermediaries (alkyl radicals, conjugated dienes, peroxy and alkoxy /oxyl/ radicals and lipid hydroperoxide) and, upon further degradation, secondary products, i.e. short-chain evaporable hydrocarbons, aldehydes and end products of lipid peroxidation: isoprostanes, MDA, 4-hydroxy-2,3, trans nonenal and 4,5-dihydroxydecenal.

**Malondialdehyde** (MDA) is physiological ketoaldehyde, secondary product of peroxidative breakdown of unsaturated fatty acids, particularly arachidonic acid (25). MDA in a higher concentration reacts with free amino groups of proteins (especially with lysine residue). It is the path of the generation of MDA-modified proteins, which have altered biological characteristics. Such modified protein structures have immunogenic features. It has been validated by the isolation of autoantibodies against MDA-modified lysine residue in human and animal serum (26). Some studies have shown that an increased titer of these autoantibodies directly correlates with the extent of damage and may predict the progression of atherosclerosis, coronary disease and acute myocardial infarction. Reaction of MDA and proteins is especially important for atherosclerosis, which is the major cause of coronary heart disease and brain insult. Bonding of MDA to LDL particle initiates several proinflammatory and proatherogenic processes, leading to the generation of foam cells (27). MDA concentration is elevated in diabetes mellitus, plasma and atherosclerotic plaque as well as the concentration of new compounds developed by bonding of MDA to the lysine residue of apolipoproteins B-100 and 4,5-dihydroxy nonenal (HNE) (28, 29). Higher MDA concentrations were found in pre-eclamptic women (30), in plasma samples and expired air of asthmatics (31), in patients with Parkinson's disease, amyotrophic lateral sclerosis and Alzheimer's disease (32).

**4-hydroxynonenal** (HNE) is a major and most toxic aldehyde, which is produced by the oxidation of  $\omega$ -6 polyunsaturated fatty acids (arachidonic, linolic and linolenic acids) (33), and considered the second toxic messenger of free oxygen radicals (34,35). HNE reacts with proteins, peptides, phospholipids and nucleic acids, and, therefore, it has multiple cytotoxic, mutagenic, genotoxic and signal effects including the inhibition of proteins and DNA synthesis, enzyme inactivation, stimulation of phospholipase C and chemotaxis of neutrophils, modulation of platelet aggregation, as well as modulation of various gene expression (36). HNE is considered a significant mediator of apoptosis induced by oxidative stress, cell proliferation and signaling pathways (37). Physiologically, HNE is continuously synthesized in minimal quantities, but its synthesis is considerably enhanced in the pathological conditions of increased lipid peroxidation. In physiological conditions, HNE concentration ranges from 0.1 to 3.0  $\mu\text{mol/L}$ . During intensive oxidative stress, such as rheumatoid arthritis, systemic sclerosis, lupus erythematoses, chronic lymphedema, chronic renal failure, the concentration of HNE rises 3–10 times above physiological concentrations (38, 39). HNE forms stable compounds with residues of histidine, lysine and cysteine (covalent bonds), known as advanced lipoxidation end products (ALE), and initiates the generation of protein carbonyl groups (40–42).

**Acrolein** is a  $\alpha,\beta$ -unsaturated aldehyde which emerges as a secondary product of lipid peroxidation, but also in the reaction of oxidation of amino acids catalyzed by myeloperoxidases. It is present in a variety of human surrounding resources, particularly in cigarette smoke. Acrolein reacts with lysine residues of apolipoprotein A-I, the key protein of HDL particle, that has a critical role in mobilizing the cholesterol from macrophages localized on the blood vessel walls. Accordingly, the products of oxidative modification of proteins by acroleins are localized in human atherosclerotic lesions and have a significant role in atherogenesis (43). Past studies showed that exposure of vascular smooth muscle cells to different acrolein doses led to significant damage of cell morphology whose extent was relative to acrolein concentration and length of exposure (44). Higher acrolein concentrations (5–10  $\mu\text{g/mL}$ ) cause activation of MAP and tyrosine kinase, that has an important role in vascular pathogenesis and acrolein-induced cell damage. Acrolein inhibits the synthesis of interleukin IL-2, IL-10, granulocytic-macrophage colony stimulating growth factor, interferon  $\gamma$ , TNF- $\alpha$  and NF- $\kappa$ B1. The latter blocks bonding of DNA to NF- $\kappa$ B1, that has a direct implication to the modulation of gene expression and modification of gene transcription (45, 46).

**Hexanoyl-lysine** (HEL) is a recently discovered product of lipid peroxidation which is formed via an oxidative modification of the lysine residue of a protein by hexanoyl action. Hexanoyl lysine is generated by metabolizing of  $\omega$ -6 fatty acids (linolic and arachidonic) and it is a marker of the initial phase of lipid peroxidation. Numerous studies have verified the presence of hexanoyl lysine in oxidatively modified LDL particles, atherosclerotic lesions, and in human urine and serum. It has been proved that HEL may arise in muscles during intensive exercise. Antioxidants (e.g., flavonoids) may inhibit its synthesis.

**Isoprostanes** are prostaglandin  $F_2$ -like substances ( $F_2$ -IsoPs) which are produced *in vivo* by non-enzymatic peroxidation of arachidonic acid via the action of free radicals without cyclooxygenase action (47, 48). Isoprostane synthesis, during auto-oxidation of polyunsaturated fatty acids, was first described in mid 1970s of the 20th century, but their *in vivo* synthesis was not explained until 1990 (49, 50).  $F_2$ -isoprostanes are a group of 64 isomers, structurally similar to prostaglandin  $F_{2\alpha}$  ( $\text{PGF}_{2\alpha}$ ). Peroxidation of arachidonic acid first results in arachidonyl-radicals, which by endo-cyclization, yield 4 bis-cyclic endoperoxide intermediaries, similar to prostaglandin  $H_2$  ( $\text{PGH}_2$ ). Upon reduction of these intermediaries, 4 series of isoprostanes (series 5, 12, 8 and 15), i.e. 4 F-rings of regioisomers are formed; each of them consists of 8 racemic diastereoisomers. According to the nomenclature system approved by the Committee of Eicosanoids Nomenclature, the names of these series come from the number of C-atoms in the chain

the OH-group is bound to, and where the C-atom of carboxyl group is designated as C-1 (51). F<sub>2</sub>-isoprostanes always develop esterified, *in situ*, in the composition of phospholipids. Upon the action of phospholipase A<sub>2</sub>, they are released from phospholipid composition and pass into all body fluids (52). Rearrangement of first developed endoperoxides results in isoprostanes with E or D ring or compounds with thromboxane ring (53). Reactive cyclopentanes A<sub>2</sub> and J<sub>2</sub> isoprostanes (54) as well as acyclic keto-aldehydes, termed isoketals, are the result of E<sub>2</sub> and D<sub>2</sub>-isoprostanes dehydration, the latter being highly reactive and unstable compounds (55). The main metabolite of isoprostane in humans, which is excreted via urine, is 2, 3-dinor-5,6-dihydro-15-F<sub>2t</sub>-isoprostane (56). Oxidation of docohexaenol acid (C<sub>22</sub>:6ω3), most prevalent polyunsaturated fatty acid in the central nervous system, produces isoprostane-like compounds called neuroprostanes that may be a unique marker of oxidative CNS damage (57).

#### Determination of lipid peroxidation end products

The spectrophotometry method based on the measurement of MDA concentration in reaction with thiobarbituric acid was previously used very often for the assessment of the intensity of the lipid peroxidation process in »*in vivo*« and »*in vitro*« conditions (17, 20). The knowledge that MDA may bind to protein molecules and modify them, where newly developed complexes assume immunogenic features, has led to the development of tests which are based on immunochemical principles (28). MDA binding to DNA chain, particularly to guanine, adenine and cytosine bases, causes the formation of oxidatively modified DNA chains that may be determined by gas chromatography with mass detection (58, 59).

Determination of protein conjugates developed from a reaction with 4-hydroxynonenal and histidine and cysteine, respectively, is completed by means of liquid chromatography with electro-spray ionization together with mass detection or HPLC technique (60, 61), and recently ELISA has been used (62).

Acrolein bound to carnosine, homocarnosine or histidine, and especially to DNA chain, may be determined by liquid chromatography with mass detection (63) or liquid chromatography with electrospray ionization together with mass detection (64). Binding of acrolein to lysine residue of protein conditioned the development of ELISA assays using the monoclonal antibodies which specifically react with Acr-lys product, in the form of a competitive reaction with formaldehyde-hydro piperidine-lysine (FDP-Lys). Similar to acrolein-lysine, hexanoyl-lysine may be determined by an ELISA assay using the monoclonal antibodies which enable determination of this compound in serum, urine and even in tissues.

F<sub>2</sub>-isoprostanes are presently considered very reliable parameters of oxidative stress and lipid peroxidation *in vivo*. Other products of the isoprostane pathway, such as D<sub>2</sub>- and E<sub>2</sub>-, are less stable, and consequently less used for determination. F<sub>2</sub>-isoprostanes may be determined in esterified form in all tissues, and in free form in all biological fluids, as a marker of the »physiological« level of oxidative stress (65). In order to examine the endogenous production of F<sub>2</sub>-isoprostanes in humans, it is necessary to determine free non-metabolized F<sub>2</sub>-isoprostane in biological fluids such as plasma and urine, esterified F<sub>2</sub>-isoprostane in tissue and plasma lipoproteins, as well as to measure the major metabolite 2,3-dinor-5,6-dihydro-15-F<sub>2t</sub>-isoprostane in urine. The referent analytical procedure for the determination of isoprostanes in tissues and biological fluids is gas chromatography with mass detection (GC/MS), which is long-lasting and expensive. Alternative methods that have been developed over time are based on immunochemical and radioimmunochemical principles (66, 67).

Isoprostane determination has several advantages over the determination of other markers of oxidative stress. F<sub>2</sub>-isoprostanes are chemically stable, specific products of lipid peroxidation, produced *in vivo*, their concentration is detectable in all tissues and biological fluids, therefore enabling the determination of a reference interval; their values significantly increase in oxidative stress, and their concentration is not dependable upon the volume of lipid intake (68, 69). Isoprostanes are commonly determined in urine, because the method is completely non-invasive, they are not subject to auto-oxidation and are very stable in urine (70). No significant daily variations of concentration or day-to-day variations have been reported in healthy people (17). F<sub>2</sub>-isoprostanes may be determined in the expired air condensate, what makes them very suitable for the determination of inflammatory conditions as well as the effect of oxidative stress on the development of pulmonary diseases in both children and adults with severe pulmonary function impairment (72, 73). Determination of esterified F<sub>2</sub>-isoprostanes in plasma lipoproteins may be a significant tool in the investigation of LDL particle oxidation *in vivo*, as the central event of atherosclerosis (74).

Quantitative determination of F<sub>2</sub>-isoprostanes in tissues and different biological fluids (serum, plasma, urine, cerebrospinal and synovial fluids, bronchoalveolar lavage, etc.) is a new approach to the quantification of oxidative stress, which is the biological foundation for the application of adequate therapy in specific pathological conditions.

#### Oxidative modification of proteins

Proteins are highly sensitive to free-radicals action. The reactions of protein structure modifications by the action of free radicals proceed in »*in vivo*«

conditions and are responsible for the physiological processes of aging, degradation and protein restoration, as well as the regulation of aerobic and anaerobic metabolism. Oxidative modification of proteins has been verified in the following diseases and conditions: aging, ischemic-reperfusion lesions, coronary and cerebral occlusion, pulmonary emphysema, cataract, rheumatoid arthritis, chronic alcoholism, neurological conditions followed by demyelination, diabetes mellitus, liver and kidney disorders, hemochromatosis, etc. (75–80). Unlike lipid peroxidation, modification of proteins is fast and linear (in time and concentration), and therefore it is a more sensitive parameter of oxidative modification of biomolecules than lipid peroxidation (81–83).

Oxidative modification of proteins is manifested by changes of their primary, secondary and tertiary structure. Modification of primary protein structure results from the modification or deficit of some amino acids, aggregation and fragmentation of proteins, while modification of secondary and tertiary structure causes the changes of solubility and charge (84, 85).

Stadtman's classification established several types of modification of proteins and enzymes, that are illustrated in *Table I* (*Table I*).

Oxidative modification of amino acids often leads to the development of some other forms of amino acids, what may affect the functional activity of the protein itself. Nevertheless, not every oxidative modification of amino acids is disastrous for its function. Oxidations regularly occur in the cell, which may lead to the production of amino acid derivatives that may be involved in further metabolic pathways in the body (*Table II*). Amino acids most susceptible to oxidative attack are cysteine and methionine: they both have the sulfur atom (86, 87). All kinds of oxidizing agents may induce modification of cysteine and methionine that is reflected in the formation of

**Table I** Types of oxidative modification of proteins (Stadtman's classification).

1. loss of catalytic function
2. modification of amino acids
3. formation of carbonyl groups
4. increase of acidity
5. methionine conversion into methionine sulfide
6. reduced thermic stability
7. changed viscosity
8. fluorescence change
9. fragmentation
10. formation of interprotein bonds – aggregation
11. formation of S-S bridges
12. higher proteolytic sensitivity

**Table II** Oxidative modification of some amino acid residua.

Amino acid residue	Oxidatively modified amino acid residue
Arginyl	Glutamyl semialdehyde
Prolyl	Glutamyl semialdehyde Pyroglutamyl/Glutamyl Cis/Trans-4-hydroxi prolyl
Lysyl	$\alpha$ -Amino adipyl-semialdehyde
Histidyl	Asparaginyl/Aspartyl
Cysteinyl	-S-S- Protein bonds
	Mixed disulfide protein
	-S-S-R (thiyl-radicals)
Methionyl	Methionyl-sulphoxide
Tyrosyl	Tyrosyl-Tyrosyl interaction
Leucyl	Hydroxy-leucyl
Tryptophan	N-formyl kynurenine
Threonyl	2-amino-3-ketobutyric acid

disulfide bonds, mixed disulfides and thiyl radicals. Other amino acids may also be oxidized but they require more strict oxidative conditions (88).

### Direct oxidation of amino acids

Several systems have been evidenced in the cell which are involved in the oxidative modification of proteins. Mostly, these are the oxidative systems of mixed functions that may contain some metal (usually reduced iron or copper ions), and they were named accordingly: metal catalyzed oxidation (MCO). Within these processes, the reduced metal form ( $\text{Fe}^{2+}$ ,  $\text{Cu}^+$ ) reduces  $\text{H}_2\text{O}_2$  to hydroxyl radical and pheryl radical (Fenton's reaction) which thereupon acts on amino acids. Aromatic amino acids are especially sensitive to such oxidation, and in the process oxohistidine or aspartate results from histidine, while carbonyl derivatives (aldehydes or ketones) develop from amino acids, such as lysine, arginine, proline and threonine, which are termed due to their carboxyl groups (88).

A significant oxidant is hypochlorite ( $\text{HOCl}$ ), which is the product of peroxidase reaction. Hypochlorite reacts with tyrosine, tryptophane, lysine and methionine (in protein composition), resulting in chlorotyrosyl, chloramine, aldehyde and methionine sulfoxide, while a reaction with free tyrosine produces p-hydroxy-phenyl-acetaldehyde (pHA), which is covalently bound to lysine residua, thus modifying them (90).

Peroxynitrite, resulting from a reaction of nitric oxide and superoxide anion, most frequently nitrates the tyrosine residua, as well as cysteine, tryptophane, methionine and phenylalanine residua, and it is a very poor inductor of carbonyl groups (91–94).

$\gamma$ -rays induce the direct formation of hydroxyl radicals which cause generalized damages to proteins, particularly protein aggregation, degradation and metal-catalyzed oxidation. Accordingly, for example, hydrophobe amino acids: valine, leucin, tyrosine are oxidized in the hydroxy and hydroperoxy derivatives after  $\gamma$ -ray and O<sub>2</sub> exposition. The most significant marker of oxidative modification by  $\gamma$ -rays is di-tyrosyl which is at the same time the best, i.e. most sensitive marker of such oxidation (78).

#### *Secondary mechanism of protein oxidative modification*

Intracellular proteins may also be oxidized by secondary mechanisms as the result of interaction of free radicals and other cellular constituents such as lipids, hydrocarbons and nucleic acids. Lipid peroxidation and oxidative modification are two independent processes. Their interaction is reflected in the fact that the reactive aldehydes, lipid peroxidation products: MDA, glyoxal and 4-HNE may react with amino groups of proteins forming the intermediaries (36, 95). The most common ligand of these lipid radicals is the  $\epsilon$ -amino group of lysine, producing the lipoxidation products, such as N-carboxymethyl lysine (CML), which may also be a glucose derivative. Similar reaction was confirmed on histidine. Such oxidative modification of proteins may occur in plasma lipoprotein metabolism, especially of apoprotein B of the LDL particle, which is extremely significant for the development of atherosclerosis (43).

«**Carbonyl stress**» is an important form of the secondary oxidation of proteins. Reduced sugars may react non-enzymatically with amino groups of proteins and lipids, through a series of complex biochemical reactions that include oxidative and non-oxidative rearrangement, based on Maillard's reaction, which finally result in the formation of covalent compounds known as advanced glycosylated end products (AGE-products) (96, 97).

The basic biochemical reaction leading to AGE synthesis proceeds between reduced sugars and proteins by the formation of unstable »Schiff base«. This reaction is fast and reversible. Isomerization of reaction product may be continued via a reaction that is now slower, converting to a ketoamine derivative termed an »Amadori product«. One typical Amadori product is glycosylated hemoglobin (HbA<sub>1c</sub>), which is used as a classic so-called long-term marker of glycemia in diabetes (i.e., metabolic control marker). This early product may be further involved in complex oxidative reactions of degradation and condensation, where rearrangement occurs within the molecule resulting in AGE products that are now stable and irreversible. These processes may take weeks and months to complete (98). The main substrate of this reaction is glucose, although other sugars may be

**Table III** Biologically significant AGE

- |  |
|--|
| 1) Monolysyl-products (without cross-link reactions) |
| a) N <sup>ε</sup> -carboxy methyl lysine (CML)       |
| b) N <sup>ε</sup> -carboxy ethyl lysine (CEL)        |
| c) Pyralin   |
| 2) Bis-lysyl products (cross-linkage)                |
| a) Glyoxal-lysine dimer (GOLD)                       |
| b) Methyl-glyoxal lysine dimer (MOLD)                |
| c) 3-Deoxy-glukozon lysine dimer (DOLD)              |
| 3) Hydroimidazolones                                 |
| a) Glyoxal-hydroimidazolone (G-H)                    |
| b) Methylglyoxal-hydroimidazole (MG-H)               |
| c) 3-deoksiglukozon lysine dimer (3DG-H)             |
| 4) Other cross-linking bonds                         |
| a) Pentosidine                                       |

involved: fructose, threose, glucose-6 phosphate and glyceraldehyde-3 phosphate. The most important AGE products are presented in Table III (Table III).

Synthesis of AGE products begins slowly and goes through several different phases, leading to the damage of protein structure and change of superficial molecule topology, which largely alter the biochemical characteristics of the molecule. The use of specific antibodies against a specific AGE epitope allowed for the detection of AGE in human tissue under physiological conditions, where their concentration tends to rise with aging, showing the association of AGE with degeneration of long-lived proteins and biological aging (99). Theoretically, any protein may become a target of glyco-oxidative modification »*in vivo*«. Long-lived proteins including the matrix and structural proteins in the ocular lens and extracellular matrix (ECM) are particularly susceptible to AGE modification. Increased AGE accumulation in tissues may occur due to higher production, lower degradation or longer survival (reduced clearance). Elevated tissue or plasma AGE concentration has been detected in many diseases, such as: DM, chronic renal failure, atherosclerosis, arterial hypertension and Alzheimer's disease. Moreover, AGE products have been detected in primary rheumatoid arthritis and in the course of osteoporosis (100, 101). Binding of AGE to specific receptors (RAGE) which recognize AGE-modified proteins leads to the activation of intracellular signaling cascades and activation of the entire spectrum of proinflammatory and profibrotic cellular responses (102). AGE product binding to specific receptors is the major pathogenic factor of complications in diabetes. They are also involved in the pathophysiology of other non-diabetic renal conditions. AGE products are considered to play an important role in glomerular and tubulointerstitial damages, due to the amplification of local immune and inflammatory response of the kidney. Because of impaired renal functioning, AGE products are the major »culprits« for an abrupt rise in concentration of these products in plasma, which will actually be a vicious circle of disease, deepening the condition even more and aggravating the survival (103–108).

In diabetes, hyperglycemia triggers the activation of alternative intracellular metabolic pathways (e.g., polyol pathway), wherein the synthesis of these AGE products occurs, and especially: N<sup>ε</sup>-carboxy methyl lysine (CML), N<sup>ε</sup>-carboxy ethyl lysine (CEL), and pentosidine which are the major representatives of this AGE class. In addition, pentosidine is a »cross-linking« molecule that covalently binds distant lysine and arginine residues in proteins, through a C5 ring, thus binding different proteins, together or by forming an intramolecular covalent bond. Alternatively, these products may be generated during fat metabolism, i.e. via oxidation of polyunsaturated fatty acids and arachidonic acid in ion-catalyzed reactions. Products resulting from this type of reaction are called »advanced lipoperoxidation end« (ALE) products. Compounds such as malondialdehyde (MDA)-lysine, hydroxy-nonenal-lysine or acrolein produced by oxidation of hydroxy-amino acids, L-serine or L-threonine, belong to this class of terminally modified proteins. The term AGE is otherwise used for both groups of products. Gluco-oxidative and lipo-oxidative pathways, independent from each other, end in the same final product: CML, while pentosidine develops exclusively from a carbohydrate precursor. Conversely, imidazolone and pyraline may be the result of oxidative stress processes. Non-oxidative compounds may also produce AGE from methylglyoxal during non-oxidative anaerobic glycolysis or on the basis of 3-deoxyglucosone that is released from an Amadori rearrangement (109–110).

Diabetes mellitus and uremia are conditions inducing a significant rise of AGE concentrations in tissues and plasma (111). Pentosidine plasma concentration may be even 10 times higher in patients with end-stage renal disease in comparison with healthy controls (112). In diabetics, an increased glucose level may simply account for a propensity towards the generation of AGE products, by shifting the balance of the biochemical reaction to glycation, i.e. formation of Schiff bases, and irreversible AGE products in accord with the concentration gradient of glucose.

#### *Determination of oxidatively modified amino acids*

Methodological approaches to the determination of oxidatively modified amino acids are illustrated in Table IV (Table IV).

Tests for the determination of dityrosine, chlorotyrosine and dihydro-phenylalanine are very sensitive and specific; however, the products of tyrosine oxidative modification are not so present in biological samples as carbonyl proteins may be. For instance, 3-chlorotyrosine level in atherosclerotic plaque reaches the mean value of 424 μmol/mol of tyrosine, that is equivalent to one modified residue per 2500 tyrosine residues. Dityrosine level may even be lower, which is the

**Table IV** Methods of determination of oxidatively modified proteins.

Modification	Detection methods
Disulfides	SDS-gel electrophoresis
Thiyl-radicals	electron spin resonance/ spectroscopy
Glutathionylation	HPLC/with mass detection
Methionine sulfoxide	cleavage with CNBr/determination of amino acid
Carbonyl groups	bonding to DNPH/Western blot/ELISA/HPLC/A <sub>370</sub>
2-oxo-histidin	reduction by NaBH <sub>3</sub> /determination of amino acid
Dityrosine	fluorescence: proteolysis or hydrolysis/HPLC
Chlorotyrosine	hydrolysis/nitrosonaphthol/HPLC HBr hydrolysis-GH/MD
Nitrotyrosine	ELISA; hydrolysis/HPLC; HPLC/electrochemical detection
Tryptophanyl	fluorescence; alkaline hydrolysis; proteolysis/MS
Hydroperoxides	KI/I <sub>3</sub> <sup>-</sup> /spectroscopy; NaBH <sub>4</sub> /hydrolysis/OPA/HPLC
Products of lipid peroxidation	NaBH <sub>4</sub> /hydrolysis/OPA/HPLC GH/MS; DNPH; ELISA
Products of glyco-oxidation	derivation/GH/MS
Cross-link aggregates	SDS-gel electrophoresis; HPLC

result of a low content of tyrosine in proteins in comparison to other amino acids such as lysine, arginine, proline and threonine. Nevertheless, the products of tyrosine oxidative modification are elevated in oxidative stress, and are a significant parameter for its determination (113, 114).

Methionine sulfoxide is also a good and sensitive marker of protein oxidative modification. So far, an ELISA assay for the determination of this marker has not been developed in spite of some attempts, because a specific methionine antibody has not been isolated to this day.

Carbonyl products are the most commonly used markers of protein oxidation in biological samples. Carbonyl derivatives of proline, arginine, lysine and threonine are very stable, which facilitates the process of determination and storage. They are present in almost all proteins, in concentrations of approximately 1 nmol/mg of protein, which is equivalent to a concentration of 0.05 mol of carbonyl/mol of protein mass of 50 kDa. In conditions of elevated oxidative stress, their concentration rises 2–8 times in relation to basic values (115). Carbonyl groups may be indu-



ced by various types of free radicals both »*in vivo*« and »*in vitro*«, including the metal-catalyzed oxidation, ozone, HOCl, singlet oxygen and ionizing radiation (113, 116). High-sensitive tests for their detection are available. Many methods include derivation of carbonyl groups with dinitrophenyl hydrazine (DNPH), followed by synthesis of stable dinitrophenyl hydrazone that may be detected by different methods: UV absorption at 370 nm, protein fractionating by HPLC technique, immunochemical ELISA technique, immunohistochemically, one- or two-dimensional SDS-gel electrophoresis with immunoblotting (Western blotting). The last two methods are extremely sensitive and specific (comparing with others), but they are merely semiquantitative.

#### *Advantages and disadvantages of the determination of oxidatively modified proteins*

One of the greatest current challenges is the determination of oxidative stress »*in vivo*«. Given that proteins are ubiquitous, i.e. present in all cells and tissues, their determination may serve as a good marker of oxidative stress, considering they are highly susceptible to oxidative stress. Products of protein oxidative modification are determined in different pathological conditions. In some cases, the source of free radicals is known, and sometimes it is not.

In relation to products of lipid peroxidation and DNA oxidative damage, proteins show some advantages:

1. proteins have specific biological functions, therefore, their modification has exceptional functional sequelae (i.e., loss of coagulation function due to fibrinogen oxidative damage or reduced synthesis of ATP due to G3PDH oxidation).
2. Products of protein oxidative damage are relatively stable and sensitive which makes them suitable for determination.
3. The nature of protein modification may provide valid data on the type of oxidant causing the damage:
  - a) e.g. *chlorotyrosyl* is a specific marker of oxidative damage to tyrosine by HOCl action, reflecting most commonly the involvement of neutrophils and monocytes in oxidative stress (117, 118);
  - b) *nitrotyrosyl* residua indicate the presence of increased synthesis of peroxynitrite, i.e. nitric oxide and superoxide (119, 120);
  - c) *carbonyl groups* may be induced by all ROS types, so they may not suggest the type of oxidant, but since they are too difficult to be induced (i.e. they are produced only in extreme conditions of oxidative stress), their presence points to severe oxidative stress-related damage (121);

d) although *methionine* and *cysteine* are most sensitive to oxidative modification, they are not always the markers of altered protein function, because in some cases methionine acts as an internal »scavenger«, that is, amino acid protecting the critical acyl residua against oxidative damage (122).

4. High specificity of protein oxidative damage lies in the occasional use of these macromolecules as markers of oxidative damage. There is no »universal marker« of oxidative damage, since there is a large number of causative agents of oxidation as well as various parameters of oxidative damage. Therefore, it would be pointless to determine chlorotyrosyl, and so far neutrophils and monocytes have not been included in the studies of oxidative stress. Singlet oxygen has no effect on carbonyl group induction, but it may oxidize methionine, histidine, tyrosine and tryptophane, so these markers are recommended for the respective process (113).

#### **Oxidative modification of DNA**

Free radicals may also bring about the oxidative damages of DNA that are manifested by the development of cancerogenic phenotype if specific proto-oncogenes are involved in a series of degenerative processes, but also in normal processes of aging and apoptosis (123). Although DNA is the most worthy target of action of reactive oxygen species, not all types of radicals are equally toxic and their mode of action is not unique.

The most common types of oxidative modification of the DNA structure are presented in *Table V* (*Table V*).

The largest number of DNA damages occur through hydroxyl ion action, while superoxide ion and

**Table V** Most common types of DNA oxidative modification.

Break of DNA chain (unilateral or bilateral)
Interaction of bases within one DNA chain spiral
Interaction of bases between two different DNA chain spirales
Base change
Base loss
Base modification
Production of pyrimidine dimer
Generation of 6-4 photoproducts
Formation of massive obstacles within DNA chain
Interaction with proteins
Interaction with lipid peroxides (MDA)
Interaction with glycooxidation products
Oxidative modification of deoxyribose

hydrogen peroxide are not generally noxious unless  $\text{Fe}^{2+}$  or  $\text{Cu}^+$  are present, when it comes to a Fenton's reaction where these free radicals are converted to hydroxyl ion; thereupon, this ion renders multiple modifications of DNA molecules (124, 125). These modifications are most frequently manifested by base loss and formation of abasic sites (AP site), cleavage of DNA chain and sugar modification (126).

AP sites often emerge spontaneously, through depurination. It is estimated that, at least, 10,000 processes of depurination per cell occur within 24 hours under physiological conditions (127).

The double bond between C4-C5 pyrimidine is sensitive to oxidative action of ROS (reactive oxygen species), resulting in a spectrum of pyrimidine derivatives: thymine-glycol, uracil-glycol, urea residue, 5-hydroxy-deoxy-uracil, 5-hydroxy-deoxy-cytosine, while the reaction of free radicals and purines produces the following: 8-hydroxy-2-deoxy guanosine, 8-hydroxy-2-deoxy adenine, form-amino pyrimidine and other less known derivatives (128, 129). Overall changes occurring in a cell are estimated at 200,000 per cell daily. Biological consequences of these modifications are generally known; thymine-glycol blocks DNA replication, which is disastrous for the cell, while 8-oxo guanosine, if not restored by reparatory mechanisms, leads to mutagenesis (130, 131).

Sugar molecule damage as a result of a Fenton's reaction causes the break of DNA structure and release of base and MDA.  $\text{OH}^-$  is responsible for such modification. This mechanism gives rise to a »collapse« of DNA structure and again the action of  $\text{Fe}^{2+}$  and  $\text{Cu}^+$  ions is initially responsible, causing the break of 5' or 3' phospho-diester bond (132, 133).

As early as in the beginning of 1970s, it was established that the product of peroxidation of fatty acids, MDA, covalently binds to the DNA chain, leading to a break of structure of the DNA chain (134). Subsequently, it was found that it was the reaction according to the type of Schiff base, accomplished by amino-groups of DNA bases and MDA carboxyl group. In this way, it comes to the formation of labile sites on the DNA molecule – so called *DNA radicals* (135). It is believed that the effects of ozone, X-rays and other exogenous generators of radicals on the DNA molecule are indirect, following generation of MDA which interacts with the DNA molecule, leading to mutations (136).

Ionizing radiation often causes a break of the DNA chain and modification of bases, as well as chromosomal aberrations. UV radiation (254 nm) causes the production of pyrimidine dimers, i.e. formation of a covalent bond between two pyrimidine bases and so-called 6-4 photo-products. Therefore, deletion occurs within a chromosome – formation of dicentric chromosomes and changes in homologue chromosomes. If it is the question of the insufficiency of reparatory system

DNA, most commonly the chromosomes are sensitive to UV beams and x-rays (137).

Free radicals may activate the system of poly-ADP-ribosylation, owing to an over-consumption of reduced NADPH at the expense of NADP increase which is a significant stimulus of poly-ribosylation. The rearrangement of genetic material in DNA sequences is especially important, where regulatory genes and proto-oncogenes are coded, because it induces the impairment of cell proliferation, which is one of the signals of cancerogenic phenotype (138).

The cell is protected against toxic DNA damage through a specific localization in the nucleus where free iron as well as superoxide anions can hardly penetrate. Significant protection of DNA structure is provided by histones as well as free radical scavengers which are universal mechanisms of antioxidative defense, either belonging to enzymatic or nonenzymatic antioxidants. Complex mechanisms of DNA repair imply the presence of adaptation systems of tolerance as well as specific reparatory systems.

Even in the regular process of DNA synthesis, more precisely in polymerization, formation of a wrong base is possible, although mistakes are rare ( $10^9$ – $10^{10}$  bases). Reparation enzymes are present in conditions of regular functioning and synthesis of DNA. It is the inducible enzymatic system known as »SOS response« or »SOS regulon« which includes a large number of important enzymes, the most significant of which are: DNA endonucleases, AP endonucleases, pyrimidine-hydrate-DNA glycosylase,  $\beta$ -lyase,  $\delta$ -lyase, deoxy-phospho-diesterase, reparatory 3' diesterase, DNA polymerases and DNA ligases.

In the human genome, reparation may be completed within only 24 hours if it is exposed to UV rays, where essential genes priority and are repaired in 8 hours.

In a large number of diseases such as Alzheimer's disease, Huntington's chorea, Parkinson's disease, multiple sclerosis, atherosclerosis, etc., reparatory power of DNA is poor, and therefore these patients are hypersensitive to UV rays, radiation or some chemical noxae. Reparatory system insufficiency in the genome is the most important risk factor for a cancerogenic phenotype, especially in conditions of cell exposure to UV rays, gamma radiation or hydrogen peroxide (139, 140).

#### *Determination of oxidatively modified products of DNA*

The most commonly used marker of oxidatively modified DNA molecule is 8-hydroxy-2'-deoxyguanosine (8-OHdG). Biological material most often includes serum or urine, as well as plasma, saliva and tissues. The initial and reference method for this biomarker was HPLC technique. Today, this biomarker

has been increasingly determined quantitatively by an immunochemical method using monoclonal antibodies (141), or the immunohistochemical method of fluorescent flow cytometry for the detection of oxidatively modified DNA molecules in the composition of cells and tissues (142).

*A-purine or a-pyrimidine* loci in the DNA molecule are very often sites of damage occurring during base excision and during the repair of oxidatively deaminated and alkylated bases. It is estimated that  $20 \times 10^5$  of these lesions are formed in a cell daily. They are also determined by an immunochemical method termed »ARP assay« (Aldehyde Reactive Probe). Addition of a reagent, containing N-amino-oxymethyl-carbonyl-hydrazine-D-biotin, leads to a specific reaction with the aldehyde group of the damaged base in which the ring is open and the aldehyde group is free. Aldehyde group binds to biotin from ARP reagent, which is then quantified by ELISA testing, using the avidin-biotin complex conjugated with peroxidase or alkaline phosphatase as an indicatory enzyme (143).

#### *The choice of oxidative stress biomarker and method validation*

A biomarker of oxidative stress is defined as a biological molecule whose chemical structure is subject to changes, through the action of free radicals, and which may be used reliably for the determination of oxidative stress status in humans and animals. The usefulness of a perfect biomarker of oxidative stress will be shown in its capacity to indicate early onset of the disease or its progression and evaluate the effectiveness of therapy. The measurement of an oxidative stress biomarker may also be of assistance in the elucidation of pathophysiological mechanisms of the oxidative stress-related damage of tissues, in prediction of disease prognosis and selection of adequate treatment in the early stage of disease. A perfect biomarker should be measurable in biological material samples that are easily accessible, such as blood and urine. It would have to possess certain characteristics so as to be a valid biomarker of oxidative stress. It must be:

- a) a stable product of oxidative stress, which is not susceptible to artificial induction, oxidation or loss during handling with samples, transport or storage;
- b) the key product of oxidative stress, having direct implications for the development and/or progression of disease;
- c) detectable in target tissue where it causes oxidative modification and damage;
- d) present in sufficient, measurable concentrations;
- e) specific for some types of free radicals and its concentrations should not be affected by dietary factors;
- f) biological material for testing should be easily obtainable by noninvasive methods and procedures;
- g) determinable by methods adequately specific, sensitive and reproducible;
- h) relatively easily measurable in the sample of a studied population;
- i) its concentration should not vary considerably in the same person, when measured under same conditions but at different times (intraindividual variations); and,
- j) have relatively small (acceptable) analytic variation in relation to interindividual (biological) variation.

Validation of an oxidative stress biomarker proceeds through several steps. The first step is *analytical validation*, implying the development of procedures, analysis of reference material and quality control. The next step is *clinical validation*, meaning that changes of biomarker concentrations have to correlate with the course of disease, so it can be used with certainty for the prediction of disease progress (144). Determination of reference values and basal values in healthy tissues, cells or body fluids has to be carried out most carefully. The use of a biomarker »panel« may, to a large extent, increase positive predictive test values and reduce the frequency of false-positive and false-negative results. The last step is validation in »*epidemiological study*«, that is often very difficult to accomplish. Due to the complex development of human diseases associated with oxidative stress, there is an extremely low probability that a single biomarker may fully replace the significance of the clinical outcome. Therefore, development of biomarkers is presently considered the baseline of the better understanding of disease pathogenesis, as well as the process of synthesizing and production of future drugs. The possibility of measurement of biomarkers with the intention of accurate determination of the degree of oxidative damage is of huge importance in clinical trials studying the effectiveness of antioxidant therapy for the prevention and reduction of risk of various complications.

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