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Reviews

Biomarkers of Oxidative Damage in Human Disease

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Oxidative/nitrosative stress, a pervasive condition of increased amounts of reactive oxygen/nitrogen species, is now recognized to be a prominent feature of many acute and chronic diseases and even of the normal aging process. However, definitive evidence for this association has often been lacking because of recognized shortcomings with biomarkers and/or methods available to assess oxidative stress status in humans. Emphasis is now being placed on biomarkers of oxidative stress, which are objectively measured and evaluated as indicators of normal biological processes, pathogenic processes, or pharmacologic responses to therapeutic intervention. To be a predictor of disease, a biomarker must be validated. Validation criteria include intrinsic qualities such as specificity, sensitivity, degree of inter- and intraindividual variability, and knowledge of the confounding and modifying factors. In addition, characteristics of the sampling and analytical procedures are of relevance, including constraints and noninvasiveness of sampling, stability of potential biomarkers, and the simplicity, sensitivity, specificity, and speed of the analytical method. Here we discuss some of the more commonly used biomarkers of oxidative/nitrosative damage and include selected examples of human studies.

Increased oxidative/nitrosative stress generally describes a condition in which cellular antioxidant defenses are inadequate to completely inactivate the reactive oxygen species (ROS)[3] and reactive nitrogen species (RNS) generated because of excessive production of ROS/RNS, loss of antioxidant defenses, or both. A major consequence of oxidative/nitrosative stress is damage to nucleic acid bases, lipids, and proteins, which can severely compromise cell health and viability or induce a variety of cellular responses through generation of secondary reactive species, ultimately leading to cell death by necrosis or apoptosis. Oxidative damage of any of these biomolecules, if unchecked, can theoretically contribute to disease development. Indeed, an increasing amount of evidence suggests that oxidative/nitrosative stress is linked to either the primary or secondary pathophysiologic mechanisms of multiple acute and chronic human diseases *(1–6)* (Table 1). However, definitive evidence for this association has often been lacking because of recognized shortcomings with methods available to assess oxidative stress status in vivo in humans *(7)*.

Substantial evidence suggests that ROS/RNS participate in the normal aging process as well as in age-related diseases such as atherosclerosis and ophthalmologic and neurodegenerative diseases *(8, 9)*. Furthermore, observations in vitro and in cultured cell systems indicate that oxidative stress contributes to cancer risk by numerous mechanisms that are independent of genotoxicity *(10)*. Recent evidence has further supported the association between the cellular response to oxidants and the mechanisms that regulate longevity. Recent studies suggest that 3 gene products linked to aging—Forkhead transcrip-

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[3] Nonstandard abbreviations: ROS, reactive oxygen species; RNS, reactive nitrogen species; MDA, malondialdehyde; HNE, 4-hydroxy-2-nonenal; PD, Parkinson disease; TBARS, thiobarbituric acid-reactive substance(s); ALS, amyotrophic lateral sclerosis; AD, Alzheimer disease; ALE, advanced lipoxidation end product; LC, liquid chromatography; NICI, negative-ion chemical ionization; apoA-I, apolipoprotein A-I; COPD, chronic obstructive pulmonary disease; F_2-IsoP, F_2-isoprostane; 8-iso-$PGF_{2\alpha}$, 8-iso-prostaglandin $F_{2\alpha}$; COX, cyclooxygenase; GC, gas chromatography; MS, mass spectrometry; MS/MS, tandem mass spectrometry; LC, liquid chromatography; BAL, bronchoalveolar lavage; ARDS, adult (acute) respiratory distress syndrome; CNS, central nervous system; GSH, reduced glutathione; GSSG, glutathione disulfide; NO_2-Tyr, 3-nitrotyrosine; Cl-Tyr, 3-chlorotyrosine; EC-NCI, electron capture-negative chemical ionization; di-Tyr, *o,o*′-dityrosine; APCI, atmospheric pressure chemical ionization; 8OHdG, 8-hydroxy-20-deoxyguanosine; HNA, 4-hydroxynonenoic acid; DHN, 1,4-dihydroxynonene; NHPA, 3-nitro-4-hydroxyphenylacetic acid; and PHPA, *para*-hydroxyphenylacetic acid.

Table 1. Human diseases that were found to be associated with increased oxidative stress on the basis of (potential) biomarkers of oxidative damage.

Biomarker	Reference(s)
MDA	
AD	*(11, 43)*
ALS	*(43)*
Asthma	*(42)*
Atherosclerosis	*(3, 40)*
Cutaneous leishmaniasis	*(176)*
Diabetes mellitus	*(39)*
Preeclampsia	*(41)*
HNE	
AD	*(5, 50, 51, 53)*
Atherosclerosis	*(3)*
Cardiovascular disease	*(57)*
COPD	*(59, 60)*
Mild cognitive impairment	*(53)*
PD	*(56)*
Acrolein	
Atherosclerosis	*(58)*
Cardiovascular disease	*(57)*
Mild cognitive impairment	*(53)*
F_2-IsoPs	
ARDS	*(23, 24, 64)*
Acute and chronic alcoholic liver disease	*(24)*
Acute chest syndrome of sickle cell disease	*(23)*
AD	*(23, 24, 64, 68, 83)*
Asthma	*(24, 42)*
Atherosclerosis	*(23, 24, 64)*
Cardiopulmonary bypass	*(64)*
Cardiovascular disease	*(80)*
Chronic kidney disease	*(147)*
COPD	*(5, 23, 24, 79)*
Coronary artery disease	*(24)*
Creutzfeldt–Jakob disease	*(23, 24)*
Crohn disease	*(23, 24)*
Cystic fibrosis	*(23, 24, 64, 78)*
Diabetes (types 1 and 2)	*(23, 24, 64)*
Down syndrome	*(23, 24)*
Heart failure	*(24, 64)*
Hepatic cirrhosis	*(23)*
Huntington disease	*(23, 24)*
Hypercholesterolemia	*(23, 24, 64)*
Hyperhomocysteinemia	*(23, 24)*
Ischemia/Reperfusion injury	*(24)*
Interstitial lung disease	*(23)*
Multiple sclerosis	*(24)*
Myocardial infarction	*(23)*
Obesity	*(24)*
Osteoarthritis	*(64)*
Osteoporosis	*(24)*
Pancreatitis	*(23)*
Primary biliary cirrhosis	*(24)*
Psoriatic arthritis	*(64)*
Pulmonary hypertension	*(23, 64)*
Reactive arthritis	*(23, 64)*
Rheumatoid arthritis	*(23, 64)*
Sickle cell disease	*(23, 64)*
Spinal cord injury	*(23, 64)*
Systemic lupus erythematosus	*(23)*
Systemic sclerosis (scleroderma)	*(23, 24, 64)*
Unstable angina	*(23)*
Zellweger syndrome	*(82)*
Decrease in GSH concentration and/or GSH:GSSG ratio	
ARDS	*(101)*
Alcoholic liver disease	*(90)*
AD	*(90)*
ALS	*(90)*
Asbestosis	*(101)*
Asthma	*(101)*
Ataxia telangiectasia	*(90, 93)*
Cancer	*(99)*
Cardiovascular disease	*(90, 98)*
Cataract genesis	*(90)*
Diabetes mellitus (both types)	*(5, 90, 93)*
HIV-positive patients	*(90, 93, 101)*
Idiopathic pulmonary fibrosis	*(101)*
Ischemic brain	*(97)*
PD	*(96)*
Preeclampsia	*(93)*
Respiratory distress syndrome	*(90)*
Retinopathy of prematurity	*(93)*
Rheumatoid arthritis	*(90)*
Werner syndrome	*(90)*
S-Glutathionylated proteins	
Cataract genesis	*(105, 177)*
Diabetes (types 1 and 2)	*(104)*
Friedreich ataxia	*(90)*
HIV infections	*(178)*
Hyperlipidemia	*(104)*
Renal cell carcinoma	*(104)*
Spherocytosis	*(5)*
Uremia associated with hemodialysis or peritoneal dialysis	*(104)*
NO_2-Tyr	
ARDS	*(5)*
AD	*(132)*
ALS	*(5, 132)*
Asthma	*(5)*
Atherosclerosis	*(5)*
Cardiovascular disease	*(125)*
COPD	*(5)*
Coronary artery disease	*(123–125)*
Crohn disease	*(5)*
Cystic fibrosis	*(107, 118)*
Diabetes (types 1 and 2)	*(179, 180)*
Hypercholesterolemia	*(5)*
Lung cancer	*(129–131)*
Lung injury	*(31)*
Multiple sclerosis	*(5)*
Myocardial inflammation	*(5)*

Table 1. Continued

Biomarker	Reference(s)
Osteoarthritis	*(5)*
Preeclampsia	*(5)*
Rheumatoid arthritis	*(5)*
Severe bronchopulmonary dysplasia in neonates	*(181)*
Synucleinopathies	*(5, 132)*
Tauopathies	*(5)*
Cl-Tyr	
ARDS	*(5)*
Asthma	*(5)*
Atherosclerosis	*(40, 126)*
Cardiovascular disease	*(125)*
Chronic renal failure	*(5)*
Coronary artery disease	*(123, 125)*
Cystic fibrosis	*(5)*
Rheumatoid arthritis	*(5)*
Di-Tyr	
ARDS	*(5)*
Atherosclerosis	*(14, 28, 40, 182)*
Cystic fibrosis	*(5)*
End-stage renal disease	*(5)*
Carbonylated proteins	
Aceruloplasminemia	*(5)*
ARDS	*(4, 11)*
Acute autoimmune myocarditis	*(183)*
Acute pancreatitis	*(148)*
AD	*(145)*
ALS	*(5)*
Asthma	*(5)*
Bronchopulmonary dysplasia	*(144)*
Cataractogenesis	*(143)*
Chronic fatigue syndrome	*(184)*
Chronic hepatitis C	*(143)*
Chronic kidney disease	*(147)*
COPD	*(60)*
Chronic renal failure	*(4)*
Crohn disease	*(143)*
Cystic fibrosis	*(118)*
Diabetes (types 1 and 2)	*(4)*
Helicobacter pylori infection and inflammation	*(143)*
Idiopathic pulmonary fibrosis	*(185)*
Juvenile chronic arthritis	*(144, 146)*
Lung cancer	*(5)*
Meningitis	*(186)*
PD	*(143)*
Preeclampsia	*(144)*
Progeria	*(11)*
Psoriasis	*(143)*
Rheumatoid arthritis	*(143)*
Sarcoidosis	*(185)*
Sepsis	*(4)*
Systemic amyloidosis	*(143)*
Uremia	*(143)*
Werner syndrome	*(11)*

tion factors, the adaptor protein p66Shc, and the histone deacetylase Sir2—are all involved in either regulating the concentrations of intracellular ROS in mammalian cells or increasing oxidative stress resistance *(9)*. It is now well established that biological aging correlates with the accumulation of oxidized biomolecules in most tissues *(11–13)*. In the study of age-related increases in concentrations of oxidized biomolecules, disparities have been observed between intracellular and extracellular proteins. The concentrations of oxidative markers were found to increase more with age in extracellular proteins than in intracellular proteins *(12)*. This disparity might be explained by a difference in turnover between extracellular (hours to days) and intracellular proteins (minutes to hours). The difference in homeostatic control between extra- and intracellular proteins might also play a role.

The localization and effects of oxidative stress, as well as information regarding the nature of the ROS/RNS, may be gleaned from the analysis of discrete biomarkers of oxidative/nitrosative stress/damage isolated from tissues and biological fluids. Biomarkers are defined as characteristics that can be objectively measured and evaluated as indicators of normal biological processes, pathogenic processes, or pharmacologic responses to a therapeutic intervention. 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 direct detection methods are not yet applicable for clinical examination because of the instability of many reactive species and the need for expensive equipment. Furthermore, ROS/RNS are generally too reactive and/or have a half-life too short (even much shorter than seconds) to allow direct measurement in cells/tissues or body fluids. Because molecular products formed from the reaction of ROS/RNS with biomolecules are generally 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 *(5, 7, 14–20)*. 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 severity, so that they can be used as diagnostic tools.

Oxidative stress-induced peroxidation of membrane lipids can be very damaging because it leads to alterations in the biological properties of the membrane, such as the degree of fluidity, and can lead to inactivation of membrane-bound receptors or enzymes, which in turn may impair normal cellular function and increase tissue permeability. Moreover, lipid peroxidation may contrib-

ute to and amplify cellular damage resulting from generation of oxidized products, some of which are chemically reactive and covalently modify critical macromolecules. Products of lipid peroxidation have therefore commonly been used as biomarkers of oxidative stress/damage. Lipid peroxidation generates a variety of relatively stable decomposition end products, mainly α,β-unsaturated reactive aldehydes, such as malondialdehyde (MDA), 4-hydroxy-2-nonenal (HNE), and 2-propenal (acrolein) *(21, 22)*, and isoprostanes *(23, 24)*, which can then be measured in plasma and urine as an indirect index of oxidative stress. Compared with free radicals, the aldehydes are relatively stable and can diffuse within or even escape from the cell and attack targets far from the site of the original event. They therefore are not only end products and remnants of lipid peroxidation processes but also may act as "second cytotoxic messengers" for the primary reactions *(25)*. Some of these aldehydes have been shown to exhibit facile reactivity with various biomolecules, including proteins, DNA, and phospholipids, generating stable products at the end of a series of reactions that are thought to contribute to the pathogenesis of many diseases. Modification of amino acids by α,β-unsaturated aldehydes occurs mainly on the nucleophilic residues Cys and, to a lesser extent, His and Lys. Lipid hydroperoxides and aldehydes can also be absorbed from the diet and then excreted in urine. It follows that measurements of hydroxy fatty acids in plasma total lipids as well as plasma or urinary MDA and HNE can be confounded by diet and should not be used as an index of whole-body lipid peroxidation unless diet is strictly controlled *(26, 27)*.

Proteins are major targets for ROS/RNS because of their high overall abundance in biological systems and because they are primarily responsible for most functional processes within cells. It has been estimated that proteins can scavenge the majority (50%–75%) of reactive species (ROS/RNS) generated *(14)*. Exposure of proteins to ROS/RNS may alter every level of protein structure from primary to quaternary (if multimeric proteins), causing major physical changes in protein structure. Oxidative damage to proteins is induced either directly by ROS/RNS or indirectly by reaction of secondary byproducts of oxidative stress and can occur via different mechanisms, leading to peptide backbone cleavage, cross-linking, and/or modification of the side chain of virtually every amino acid *(11, 14, 28, 29)*. Most protein damage is irreparable, and oxidative changes of protein structure can have a wide range of downstream functional consequences, such as inhibition of enzymatic and binding activities, increased susceptibility to aggregation and proteolysis, increased or decreased uptake by cells, and altered immunogenicity *(28, 30–34)*. Individual proteins may display different susceptibilities to oxidative attack, linked to the variable proportions and distributions of sulfhydryl groups, Fe-S clusters, reduced heme moieties, copper prosthetic groups, sequence motifs, and residues exposed on the molecular surface. Oxidative damage to proteins may be important in vivo not only in its own right (affecting, for example, the functions of receptors, enzymes, and transport proteins), but also because it can contribute to secondary damage to other biomolecules; for example, inactivation of DNA repair enzymes and loss of fidelity of damaged DNA polymerases in replicating DNA *(1)*. The major fate of oxidized proteins is catabolism by proteasomal and lysosomal pathways, but some functionally inactive proteins appear to be poorly degraded, form protein aggregates, and accumulate in separate compartments within cells or the extracellular environment *(28, 30)*. The accumulation of such damaged material increases during the normal aging process, may contribute to a range of human pathologies, and is able to act as an inhibitor of the proteasome *(35–37)*. Because of this decreased capacity for removal of oxidized proteins, the accumulation of misfolded and damaged proteins is accelerated. The vicious circle of decreased proteolysis and accumulation of increasing amounts of oxidatively damaged proteins continues until the protein aggregates cause metabolic dysfunctions or the initiation of apoptotic or necrotic events.

When investigating oxidative/nitrosative damage in relation to disease conditions, what are the most appropriate biomarkers? What are the best ways to measure them? At present, the biomarkers of oxidative/nitrosative stress/damage and the methods used to measure them to determine an individual's oxidative status in relation to disease conditions frequently vary among studies, making comparisons of study findings difficult. In addition, the validity of many biomarkers remains to be established. Assays that have been developed have several shortcomings related to (*a*) the limited specificity of the assay itself for the product of oxidative/nitrosative damage being measured; (*b*) the fact that the analyte being measured is not a specific product of a specific ROS/RNS; (*c*) the lack of sufficient sensitivity to detect concentrations of the product being measured in healthy individuals, thus not allowing the definition of a reference interval; (*d*) concentrations of the product being measured being influenced by external factors such as the lipid content of the diet; or (*e*) the assay being too invasive for in vivo investigations in humans.

In the following sections, we briefly discuss some of the more commonly used biomarkers of oxidative/nitrosative stress and includes selected examples of human studies.

MDA, HNE, and Acrolein

MDA (Fig. 1) is 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

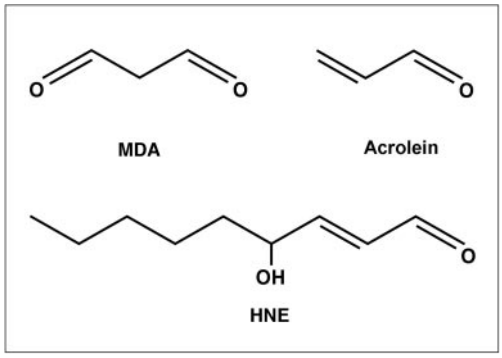

Fig. 1. Chemical structures of MDA, acrolein, and 4-HNE.
For simplicity, stereochemistries are not indicated.

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 *(3)*. Some studies have reported that the titer of these autoantibodies is associated with the burden of, and may predict progression of, atherosclerosis and myocardial infarction. Higher titers of autoantibodies have also been associated with coronary artery disease *(3)*.

The clinical relevance of the reaction between MDA and proteins is highlighted in atherosclerosis, which is a major cause of coronary heart disease and strokes. MDA-LDL, in addition to oxidized LDL, mediates several proinflammatory and proatherogenic processes, all of which ultimately lead to foam cell generation *(38)*. Plasma MDA concentrations are increased in diabetes mellitus, and MDA can be found in the atherosclerotic plaques promoted by diabetes *(39)*. Adducts of apolipoprotein B-100 Lys residues with MDA and HNE have been characterized extensively in human atherosclerotic lesions *(3, 40)*. Increased MDA concentrations have been found in samples from women with preeclampsia *(41)*, in plasma and breath condensates from asthmatics *(42)*, and in the brains of patients suffering from Parkinson disease (PD), whereas increased thiobarbituric acid-reactive substances (TBARS)—the most prevalent substrate of which is MDA—have been observed in the plasma of patients with amyotrophic lateral sclerosis (ALS) as well as in the brains of persons afflicted with Alzheimer disease (AD) *(43)*.

Acrolein (Fig. 1) is present in various environmental sources, the largest source being cigarette smoke. HNE (Fig. 1) is a major and toxic aldehyde generated by free radical attack on ω-6 polyunsaturated fatty acids (arachidonic, linoleic, and linolenic acids) *(21)* and is considered a second toxic messenger of oxygen free radicals *(44, 45)*. HNE undergoes many reactions with proteins, peptides, phospholipids, and nucleic acids; it therefore has a high biological activity and exhibits numerous cytotoxic, mutagenic, genotoxic, and signal effects *(21, 44, 45)*, 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 various genes *(21, 22)*. In addition, HNE may be an important mediator of oxidative stress–induced apoptosis *(22)*, cellular proliferation, and signaling pathways *(46)*. HNE is permanently formed at basal concentrations under physiologic conditions, but its production is greatly enhanced in pathologic conditions related to increased lipid peroxidation. Under physiologic conditions, the cellular concentration of HNE ranges from 0.1 to 3 μmol/L *(21, 44)*. Under conditions of oxidative stress, HNE concentrations are significantly increased in plasma, various organs, and cell types *(21, 44, 47)*. During heavy oxidative 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 *(48)*. HNE and acrolein are highly reactive toward proteins (in particular, 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) *(49–51)*. This process introduces carbonyl groups into proteins.

Numerous studies have demonstrated increased lipid peroxidation in the brains of persons with AD relative to age-matched controls, whereas lipid peroxidation is not a significant feature of normal aging *(21, 43, 50–52)*. Increased concentrations of HNE and acrolein have been measured in the brains of individuals with mild cognitive impairment (which is considered to be a transition between normal aging and AD) and early AD compared with age-matched controls *(53)*. In particular, binding of the glial glutamate transporter GLT-1 (EAAT2) by HNE is increased in brains of persons with AD *(50, 51)*. Neurofilament proteins are major targets of HNE modification *(54)*. Notably, it has recently been shown that the phosphorylation-dependent adduction/carbonylation of tau protein by HNE promotes and contributes to the generation of major conformational changes associated with neurofibrillary tangles *(55)*. The concentrations of both free and protein-bound HNE are also increased in the brain tissue of PD patients, in the cerebrospinal fluid of ALS patients, and in human atherosclerotic lesions *(3, 43, 50, 51, 56)*. Furthermore, concentrations of acrolein– and HNE–protein adducts are increased in cardiovascular disease *(57)*. Acrolein reacts with Lys residues of apolipoprotein A-I (apoA-I), the major protein of HDL, which plays a critical role in mobilizing cholesterol from artery wall macrophages. Acrolein adducts colocalize with apoA-I in human atherosclerotic lesions. Moreover, the ability 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 *(58)*. 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 associated with lung function *(59)*. 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 correlated with disease severity) and between HNE–protein adducts and respiratory muscle strength (i.e., HNE–protein adduct formation correlated with respiratory muscle function) *(60)*.

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 compounds generated 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. Isoprostanes containing alternative ring structures, such as E_2/D_2-IsoPs, A_2/J_2-IsoPs, and thromboxane-like compounds (isothromboxanes), can also be formed by this mechanism. F_2-IsoPs, however, have been the most studied class of isoprostanes and, because of their stability, afford the most accurate measure of oxidative stress. 8-iso-Prostaglandin $F_{2\alpha}$ (8-iso-$PGF_{2\alpha}$; Fig. 2), also known as $iPF_{2\alpha}$-III and 15-F_{2t}-IsoP, is one abundant endogenous $F_{2\alpha}$-IsoP excreted into the urine of humans and, at present, is the most thoroughly investigated $F_{2\alpha}$-IsoP. Actually, to date, contradictory results have been reported on the mechanisms leading to F_2-IsoP formation in vitro and in vivo, in humans and in animals, suggesting both enzymatic and nonenzymatic formation. Notably, 8-iso-$PGF_{2\alpha}$ has also been shown to be formed enzymatically by the action of cyclooxygenase (COX). This has been shown, for example, in isolated rat kidney glomeruli *(61)* and with isolated COX enzymes *(62)*. Evidence for enzyme-catalyzed formation of 8-iso-$PGF_{2\alpha}$ is usually based on the inhibition of 8-iso-$PGF_{2\alpha}$ formation by COX inhibitors such as acetylsalicylic acid, indomethacin, and naproxen *(62)*. Nevertheless, from the knowledge available at present, it can be assumed that the origin of urinary and circulating 8-iso-$PGF_{2\alpha}$ and other $F_{2\alpha}$-IsoPs in humans is free-radical–catalyzed peroxidation of arachidonic acid esterified to lipids, but not COX-dependent peroxidation of arachidonate *(62)*. Several of these compounds possess potent biological activity as pulmonary and renal vasoconstrictors and modulators of platelet activation. In general, they have short half-lives. 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 *(7, 23, 24, 63–68)*. F_2-IsoPs have also been used to assess in vivo oxidative response to various drugs, antioxidants, or dietary interventions for their free-radical–scavenging properties *(7, 23, 24, 63–68)*. Different methods for F_2-IsoP quantification are available *(23, 68, 69)*. In addition to being markers of oxidative stress and antagonists of the action of prostaglandins, they may also exert unique biological effects *(63, 66)*. Notable examples include the disease rhabdomyolysis, in which myoglobin is released into the plasma, oxidizes arachidonic acid in the presence of lipid peroxides, and leads to vasoconstriction in the kidney *(70)*.

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, although plasma and urine are the sample types that are commonly analyzed, being the most convenient to obtain and the least invasive *(64, 71)*. In particular, F_2-IsoPs, including 8-iso-$PGF_{2\alpha}$, are present in human plasma in 2 forms: esterified to lipids and as free acids, with the esterified form being the most abundant, whereas only hydrolyzed isoprostanes are excreted into the urine [for an example, see Ref. *(62)*].

At present, measurement of F_2-IsoPs is regarded as one of the most reliable approaches for the assessment of oxidative stress status or free-radical–mediated lipid peroxidation in vivo *(7, 24, 64, 66, 71)*. Available data indicate that quantification of F_2-IsoPs in either plasma or urine gives a highly precise and accurate index of oxidative stress *(18, 71, 72)*. Whereas the biological validity of F_2-IsoPs as biomarkers of oxidative stress is well established, it is technically quite complicated to measure F_2-IsoPs and their metabolites in body fluids, and some limitations with respect to their measurement must be taken into account *(69)*. F_2-IsoPs are chemically stable in vivo and ex vivo, but once they generated and released into the circulation, they are rapidly metabolized (although not as rapidly or as extensively as prostaglandins) and eliminated. Their rapid disappearance from plasma

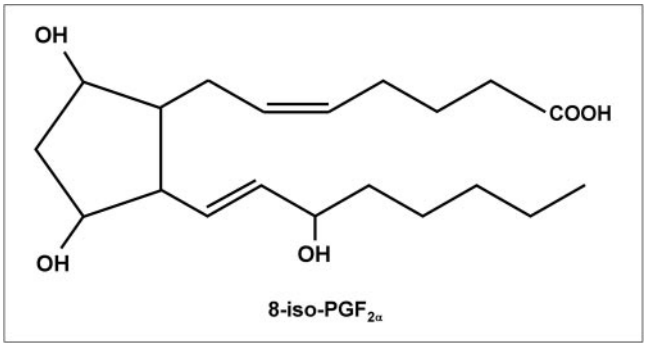

Fig. 2. Chemical structure of 8-iso-$PGF_{2\alpha}$.
For simplicity, stereochemistry is not indicated.

may hamper practical application. Current methodologies [gas chromatography–mass spectrometry (GC-MS), GC–tandem MS (GS-MS/MS), liquid chromatography (LC)-MS, LC-MS/MS, enzyme immunoassays, and RIAs] are able to detect the steady-state concentrations of F_2-IsoPs in many tissues and body fluids, even in the basal state, which allows researchers to assess any fluctuation in their concentrations after any degree of oxidant stress or lipid peroxidation in vivo. Several internal standards (^{18}O- or ^{2}H-labeled analogs of specific isoprostane isomers) are available from commercial sources to quantify the isoprostanes by MS methods.

Alternative approaches 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 *(71)*. A potential drawback of these methods is that limited information is currently available regarding their precision and accuracy. In addition, few data exist comparing F_2-IsoP concentrations measured by immunoassay with MS results. Furthermore, the sensitivity and/or specificity of these assays may vary substantially among manufacturers. However, although MS methods 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. In addition to commercial immunoassays, some investigators have generated polyclonal antibodies and have developed assays for F_2-IsoP *(73)*. It appears that there is good correlation between these methods and MS.

Different analytical approaches are available for the analysis of isoprostanes, the most sensitive, highly specific, and reliable method being GC with negative-ion chemical ionization (NICI) MS *(69, 71)*. For quantification of lipid peroxidation, measurements of F_2-IsoPs have a clear advantage over currently available methods such as assays for MDA, TBARS, lipid hydroperoxides, or conjugated dienes, which are hampered by various methodologic limitations *(64)*.

F_2-IsoPs are very well suited as biomarkers of oxidative stress for the following reasons: (*a*) The in vivo formation of isoprostanes increases as a function of oxidative stress *(71, 74, 75)*. (*b*) They can be measured accurately down to picomolar concentrations with analytical techniques such as GC-MS, GC-MS/MS, LC-MS, LC-MS/MS, or RIA. The first 4 techniques can easily differentiate among the different 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. (*c*) They are stable in isolated samples of body fluids, including urine and exhaled breath condensates, providing an exceedingly noninvasive route for their measurement. (*d*) Their measured values do not exhibit diurnal variations and are not affected by lipid content in the diet *(76, 77)*; however, they do vary markedly in clinical and experimental conditions characterized by oxidative stress and closely parallel disease severity. Some diurnal variation in urinary F_2-IsoP excretion does occur within individual humans, although this variation is not present when F_2-IsoPs are evaluated on a group level. Furthermore, although 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 F_2-IsoP excretion *(63, 65, 71)*. (*e*) They are specific products of peroxidation. (*f*) They are present in detectable amounts in all healthy tissues and biological fluids, thus allowing definition of a reference interval.

Because of the free-radical–catalyzed conversion of arachidonic acid to isoprostanes, precautions must be taken to avoid artifactual formation during sample storage and processing *(69)*. 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 hydroxytoluene 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 compounds 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 *(69)*.

Dozens of diseases and experimental conditions with diverse etiologies have been shown to be associated with marked increases in urinary, plasma, and tissue concentrations of F_2-IsoPs *(24, 64, 67)*. 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. In fact, the isoprostanes may mediate many of the features of the disease states for which they are used as indicators *(67)*. However, we should consider that, in human plasma, the concentration of free 8-iso-$PGF_{2\alpha}$, the biologically active component, amounts to <10 ng/L *(62)*; at this concentration, it cannot develop considerable biological activity. 8-iso-$PGF_{2\alpha}$ is generated in substantial amounts in otherwise "normal" individuals exposed to cigarette smoke, allergens, 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 method for direct measurement of oxidative stress in the lungs) in several 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 *(24, 42, 64, 78, 79)*. Systemic and synovial fluid concentrations of 8-iso-$PGF_{2\alpha}$ are higher in patients with rheumatoid arthritis *(23, 64)*, psoriatic arthritis *(64)*, reactive arthritis *(23, 64)*, and osteoarthritis *(64)* 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 *(80)*. Similarly, several cardiovascular conditions feature marked increases in F_2-IsoP concentrations, including during and after cardiopulmonary bypass *(64)*, renal, cerebral, and myocardial ischemia-reperfusion injury *(24)*, unstable angina *(24)*, heart failure *(24, 64)*, coronary heart disease *(24)*, acute ischemic stroke *(24)*, hypercholesterolemia *(23, 24, 64)*, and atherosclerosis *(23, 24, 64)*. Recently, 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, in addition to known risk factors of this pathology, i.e., diabetes mellitus, hypercholesterolemia, hypertension, obesity, and smoking *(81)*. Increased concentrations of 8-iso-$PGF_{2\alpha}$ have also been found in plasma or urine samples from patients with type 2 diabetes *(64)*. To date, the highest reported concentrations of urinary 8-iso-$PGF_{2\alpha}$ have been measured, by a validated GC-NICI-MS/MS method, in children with congenital Zellweger syndrome, which is attributable to impaired peroxisomal β-oxidation of various compounds, including prostaglandins and leukotrienes *(82)*.

Concentrations of F_2- and F_4-isoprostanes [which arise from oxidation of docosahexaenoic acid, an abundant unsaturated fatty acid in the central nervous system (CNS) and are often called neuroprostanes], are increased in the cerebrospinal fluid from AD patients *(2, 43, 50–52, 83, 84)* and may even be increased before the development of AD *(74, 85)*, consistent with the view that peroxidation may be an important step in progressive neuronal injury leading to clinically manifested disease *(2, 50, 51, 74, 85)*. Some authors have claimed that urinary and plasma concentrations of isoprostanes are also increased in AD patients to an extent correlated with the degree of cognitive impairment *(84, 85)*, although this was not confirmed in other studies *(86, 87)*. The reason for this discrepancy is possibly because the samples were not taken at the same stage of the disease or because various medications that the patients were taking might have affected the outcome. Because F_2-IsoPs are generated by every cell, peripheral production unrelated to CNS disease could easily confound interpretation of blood or urine concentrations in AD patients. Consequently, comparison of plasma/urine F_2-IsoP concentrations with cerebrospinal fluid concentrations raises serious concerns about the relevance of peripheral F_2-IsoPs to CNS disease. The majority of studies involving AD have shown that isoprostanes are localized in the tissues or in the cerebrospinal fluid *(24, 64)*. Finally, 8-iso-$PGF_{2\alpha}$ has been found to be a useful marker of oxidative damage and lipid peroxidation in disease states as diverse as multiple sclerosis, systemic lupus erythematosus, several hepatic pathologies (e.g., acute and chronic alcoholic liver disease), and inflammatory diseases *(24, 67, 75)*.

8-Iso-$PGF_{2\alpha}$ has also been used as a reliable biochemical marker of oxidative stress to assess the in vivo reduction of oxidative stress after pharmacologic treatment at therapeutically relevant doses. For example, nebivolol (a vasodilating and highly selective β_1-adrenergic receptor antagonist) and olmesartan medoxomil (an angiotensin II subtype-1 receptor antagonist) have been shown to decrease systemic oxidative stress in healthy persons and patients with type 2 diabetes, respectively, as evidenced by decreases in the urinary or plasma concentrations of 8-iso-$PGF_{2\alpha}$ *(88, 89)*.

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; Fig. 3) in blood has been considered essential as an index of whole-body GSH status and

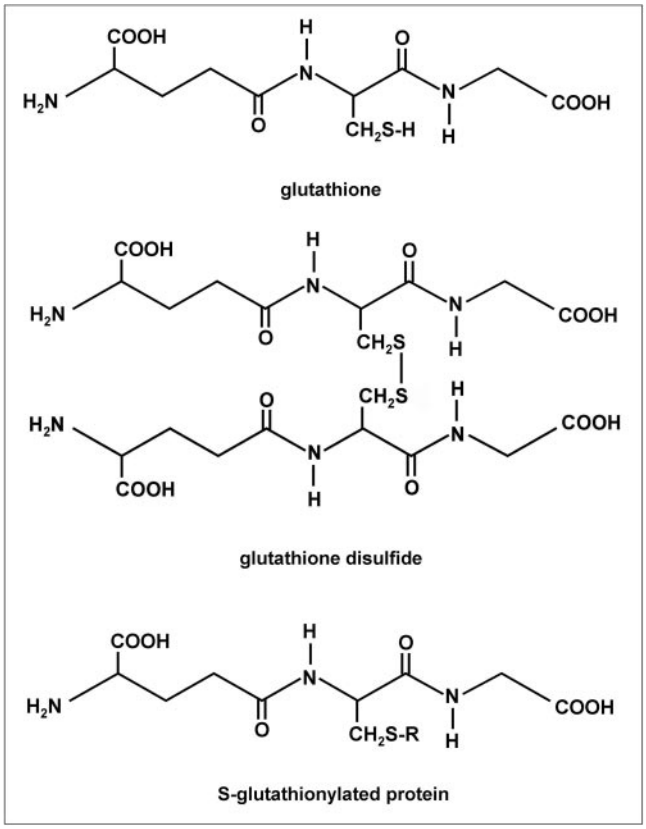

Fig. 3. Chemical structures of glutathione, GSSG and S-glutathionylated proteins.

For simplicity, stereochemistries are not indicated.

a useful indicator of oxidative stress status in humans. Several methods 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 *(90–92)*. Furthermore, a wide variety of methods have been introduced for the determination of GSH and GSSG in human blood, the measurement of which, particularly that of GSSG, could be overestimated if samples are not properly processed *(93–95)*. In particular, scarce attention is usually paid to sample manipulation and prevention of artifactual GSH oxidation. We 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 *(93–95)*. 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 [reviewed in Refs. *(93–95)*]. As a consequence, 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 has been well established that a decrease in GSH concentration may be associated with aging *(8)* and the pathogenesis of many diseases, including rheumatoid arthritis, ALS, AIDS, AD, alcoholic liver disease, cataract genesis, respiratory distress syndrome, cardiovascular disease, and Werner syndrome *(90)*. Furthermore, there is a drastic depletion in cytoplasmic concentrations of GSH within the substantia nigra of PD patients *(96)*. 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 *(97)*, cardiovascular diseases *(98)*, and cancer *(99)*, and decreased concentrations of GSH are consistently observed in both types of diabetes mellitus *(90)*. 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 *(90, 100)*. 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 rapidly 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 *(101)*. Total GSH was markedly decreased in older patients with chronic diseases, including cancer and genitourinary, gastrointestinal, cardiovascular, and musculoskeletal diseases *(90, 98, 99, 102)*, the deficit being attributable to lower GSH concentrations and not to higher GSSG. These findings 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 health *(103)*. Furthermore, a high blood GSH concentration was correlated with long life-span in mice, rats, and healthy elderly humans *(102, 103)*.

Under conditions of moderate oxidative stress, oxidation of Cys residues can lead to the reversible formation of mixed disulfides between protein thiol groups and low–molecular-mass thiols (S-thiolation), particularly with GSH (S-glutathionylation), the most abundant (0.5–10 mmol/L) low–molecular-mass thiol in mammalian cells. Protein S-glutathionylation can directly alter or regulate protein function (redox regulation) and may also have a role in protection from irreversible (terminal) oxidation. S-Glutathionylated proteins accumulate under oxidative/nitrosative stress conditions, but they can be readily reduced to free thiol groups when normal cellular redox status is recovered by glutaredoxins (thioltransferases) or reducing agents. As highlighted above, a characteristic hallmark of many pathophysiologic conditions is a decrease in the GSH:GSSG ratio. When GSSG accumulates in cells, it can undergo disulfide exchange reactions with protein thiols, leading to their S-glutathionylation.

S-Glutathionylated proteins (Fig. 3) have been investigated as possible biomarkers of oxidative/nitrosative stress in some human diseases *(104, 105)*, such as renal cell carcinoma and diabetes *(5, 104)*. Significant increases in glutathionylated hemoglobin and glutathionylated actin have been found in the blood and fibroblasts, respectively, of patients with Friedreich ataxia *(106)*. Glutathionylated hemoglobin is also increased in patients with type 1 and type 2 diabetes, hyperlipidemia, and uremia associated with hemodialysis or peritoneal dialysis *(104)*.

Tyrosine Oxidation, Nitration, and Halogenation

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 (Fig. 4) has been performed in various diseases, and several

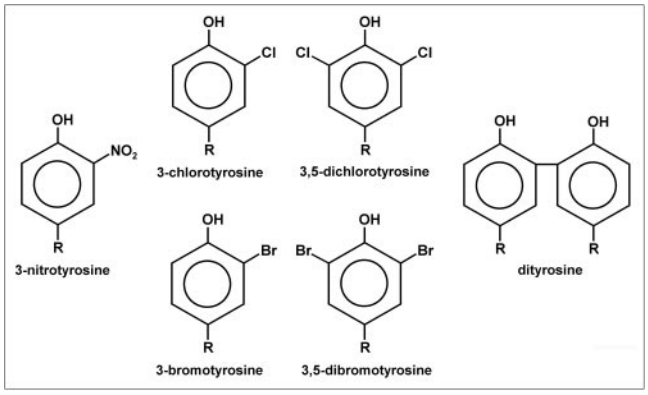

Fig. 4. Chemical structures of NO_2-Tyr, halogenated tyrosines, and di-Tyr.

For simplicity, stereochemistries are not indicated.

diverse methods have been developed for such measurements *(107–113)*. The quantitative determination of NO_2-Tyr is hindered by severe methodologic problems, which have been critically and exhaustively discussed in 2 recent reviews *(111, 112)*. The majority of the data available on NO_2-Tyr in tissues and fluids have been derived from antibody-based methods, which however, are usually not rigorously validated. In particular, in most of these works, the reliability of the method is difficult to assess, and its specificity is often not addressed *(111)*. Therefore, such immunologic methods 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 *(110, 114–117)*. However, reported concentrations of NO_2-Tyr in normal human plasma vary 30-fold among different studies [reviewed in Refs. *(111, 112)*]. One possible reason is that NO_2-Tyr can be generated readily ex vivo during sample preparation and analysis as a result of acidification or contribution of proteolytic enzymes by self-digestion.

Cl-Tyr and 3-bromotyrosine can also be formed artifactually during sample preparation and analysis. In this regard, MS analyses permit the use of a stable, isotopically labeled internal standard that, apart from its heavy isotope, is structurally identical to the target analyte and therefore behaves identically during extraction, processing, and chromatographic analyses. Including such an internal standard corrects for analyte loss during processing and increases the precision of quantitative measurements because it permits evaluation of artifactual production of the analytes that form ex vivo during sample preparation and analysis.

LC-MS/MS and EC-NCI GC-MS were specifically compared as techniques for quantifying Cl-Tyr, 3-bromotyrosine, and NO_2-Tyr in human plasma *(110)*. Artifact generation was shown to be negligible with either technique. EC-NCI GC-MS was found to be ~100-fold more sensitive than LC-MS/MS for analyzing authentic Cl-Tyr, 3-bromotyrosine, and NO_2-Tyr and allowed accurate, highly sensitive quantification of all 3 biomarkers in a single, rapid analysis. However, evidence of interference in the GC-MS analysis despite use of an HPLC step for sample purification have suggested that the reported GC-MS methods do not provide accurate values for free NO_2-Tyr *(111, 112)*. The overall discussion on the quantitative determination of both free and protein-bound NO_2-Tyr leads to the conclusions that, at present, only MS/MS-based methods, both GC-MS/MS and LC-MS/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 *(111–113, 117)*.

Another methodologic problem, in addition to artifactual NO_2-Tyr formation, is considerable interference by coeluting substances, which can be eliminated only by use of MS/MS. On the basis of the data provided by GC-MS/MS and LC-MS/MS, NO_2-Tyr concentrations of 0.5–2.9 nmol/L and protein-associated NO_2-Tyr concentrations of 0.6 pmol/mg of plasma protein or $0.4–1.6 \times 1{:}10^6$, i.e., the molar ratio of NO_2-Tyr to Tyr in plasma proteins, seem to be justified for use as reference intervals in healthy humans *(112)*. Highly specific, interference-free, and accurate quantitative determination of albumin-bound NO_2-Tyr in plasma of healthy humans by GC-MS/MS gave a mean concentration of 24 nmol/L (range, 15–40 nmol/L) and a mean molar ratio of NO_2-Tyr to Tyr of $1.5 \times 1{:}10^6$ (range, $0.5–3.5 \times 1{:}10^6$) *(117)*.

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 *(3, 5, 107, 118–122)*. The major products are Cl-Tyr and 3-bromotyrosine, but dihalogenated compounds (3,5-dichlorotyrosine and 3,5-dibromotyrosine; Fig. 4) are formed with high excesses of HOCl and HOBr. Dramatic selective enrichment in protein-bound NO_2-Tyr and Cl-Tyr content within apoA-I, the major protein constituent within HDL, recovered from human plasma and atherosclerotic lesions has been demonstrated by proteomic and MS techniques. 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 artery disease *(122–125)*. These observations suggest that increased concentrations of Cl-Tyr and NO_2-Tyr in circulating HDL might represent specific markers for clinically significant atherosclerosis. Remarkably, HDL from human aortic atherosclerotic intima had an 8-fold higher concentration of Cl-Tyr than did plasma HDL. Moreover, the concentration of Cl-Tyr was 13-fold higher in HDL isolated from the plasma of persons with established coronary artery disease than in HDL from the plasma of healthy persons *(123)*. Increased concentrations of Cl-Tyr have also been detected in LDL isolated from human atherosclerotic lesions *(40, 126)*. The mean concentration of protein-bound NO_2-Tyr in HDL isolated from human aortic atherosclerotic intima was 6-fold higher than that in circulating HDL. Moreover, plasma HDL from patients with established coronary artery disease contained twice as much protein-bound NO_2-Tyr as did HDL from plasma of healthy persons *(124)*. It is noteworthy that the concentration of NO_2-Tyr in lesion HDL was very similar to that reported previously for lesion LDL, which contains much higher concentrations of NO_2-Tyr than does circulating LDL *(40)*, indicating that both lipoproteins are nitrated to a similar extent in the human artery wall.

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 *(31)*. The clinical relevance of protein Tyr nitration has been emphasized recently 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) *(127, 128)*.

Patients with lung cancer have significantly higher serum concentrations of nitrated proteins, supporting the presence of oxidative and nitrosative stress *(129, 130)*. Specific locations and targets of Tyr nitration in lung cancer have recently been detailed *(131)*. 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 environment inside the tumor that may contribute to the disease process. This was noted in squamous cell carcinoma as well as in the well-differentiated adenocarcinoma. Using proteomic and genomic approaches, authors have identified the protein targets *(131)*. Most of the nitrated proteins fall into 4 categories: oxidant defense (such as manganese superoxide dismutase and carbonic anhydrase), energy production (many glycolytic 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 AD, in the plaques of brains from persons with multiple sclerosis, and in degenerating upper and lower motor neurons in ALS patients *(5, 132)*. Nitrated α-synuclein selectively accumulates in Lewy bodies and protein inclusions in a wide range of pathologies (AD, PD, synucleinopathies, and tauopathies) *(5)*. Nitrated proteins have been evidenced in diverse inflammatory diseases, including ARDS, severe asthma, inflammatory bowel disease, chronic renal failure, rheumatoid arthritis, type 1 and type 2 diabetes, and cystic fibrosis *(5)*. 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 these nitrated proteins have been identified. Two-dimensional Western blotting and LC-MS/MS analyses have recently been used to detect and characterize 4 nitrated proteins, including actin, in the healthy human pituitary, which participate in neurotransmission, cellular immunity, and cellular structure and motility *(133)*. These data are consistent with the emerging perspective that low-level Tyr nitration may be a physiologic regulator of a signaling pathway *(133–135)*. Although protein Tyr nitration is a low-yield process in vivo and, under inflammatory conditions, 1 to 5 NO_2-Tyr residues per 10 000 Tyr residues (100–500 μmol/mol) are detected, relatively limited numbers of proteins are preferential targets of nitration, and within these proteins, only one or a few specific Tyr residues can be nitrated *(31, 134)*.

A biomarker worth further development for human use might be *o,o′*-dityrosine (di-Tyr), which is apparently not metabolized and is also detectable in human urine, in which it is markedly increased in patients with sepsis *(136)*. Its concentration in urine might therefore serve as a noninvasive marker of protein oxidation. Increased concentrations of di-Tyr have been reported in atherosclerosis, in which its accumulation positively correlates with disease severity, and in AD, cystic fibrosis, end-stage renal disease, and acute inflammation with or without sepsis *(5, 136)*. Urinary concentrations of di-Tyr are also markedly increased (3- to 7-fold) in children with kwashiorkor (a severe form of protein-energy malnutrition), with or without infection *(137)*. Di-Tyr has also been proposed as a marker of whole-body oxidative stress status such as in atherosclerosis, acute inflammation, and systemic bacterial infections. In this regard, measured di-Tyr concentrations were 100-fold higher in LDL isolated from atherosclerotic lesions than in healthy individuals, and persons suffering from systemic bacterial infections had twice the concentration of di-Tyr in urine than did healthy individuals *(40)*. Moreover, di-Tyr has the advantage of being metabolically stable because, once the 3′–3′ carbon–carbon bond is formed, it is released only after enzymatic hydrolysis of the oxidatively modified protein *(138)*.

Several analytical methods have been developed to quantify di-Tyr in vivo and in vitro. These include HPLC with either ultraviolet or fluorescence detection, GC-MS analysis after derivatization, and LC-MS/MS analysis with atmospheric pressure chemical ionization (APCI) or electrospray ionization *(139, 140)*. Unfortunately, HPLC with ultraviolet or fluorescence detection does not have sufficient specificity for the analysis of urine or tissue extracts because several other protein modifications (e.g., the conversion of tryptophan to *N*-formylkynurenine and the conjugation of HNE with Lys residues) also yield products that exhibit fluorescence characteristics similar to those of di-Tyr. Furthermore, derivatization-based methods may introduce artifactual oxidation product(s) because they require extreme pH values and high temperatures during derivatization. LC-MS–based methods without derivatization may offer a solution to these problems. Recently, an isotope-dilution reversed-phase LC-APCI-MS/MS method and a triple-quadrupole LC-APCI-MS/MS method have been developed for the quantitative determination of di-Tyr in human urine *(140, 141)*. Triple-quadrupole LC-APCI-MS/MS, in particular, is a simple, rapid, sensitive, and reliable method that has a detection limit that is 2.5-fold lower than that of isotope-dilution reversed-phase LC-APCI-MS/MS. Furthermore, it offers a great advantage over other described methods, as it does not require any pretreatment other than centrif-

ugation of the urine sample and addition of the labeled internal standard before injection *(140)*. Actually, reliable quantification of di-Tyr, as well as of NO_2-Tyr, in urine and plasma is attainable only with the MS/MS technology *(113, 140)*

Carbonylated Proteins

Protein carbonyls may be generated by the oxidation of several amino acid side chains (e.g., in Lys, Arg, Pro, and Thr; Fig. 5); by the formation of Michael adducts between Lys, His, and Cys residues and α,β-unsaturated aldehydes, forming ALEs; and by glycation/glycoxidation of Lys amino groups, forming advanced glycation end products *(4, 11, 28, 29, 33, 142)*. The formation of carbonyl compounds is the most general and widely used marker of severe protein oxidation both in vitro and in vivo, with several assays developed for the quantification of these species *(15, 16, 143)*. The chemical stability of protein carbonyls makes them suitable targets for laboratory measurement and is also useful for their storage: their stability during storage for 10 years at −80 °C has been demonstrated *(33)*. 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 a variety of organisms *(4, 5, 11, 28, 118, 144)*.

Specific carbonylated proteins have been detected in both the brain tissue and plasma of AD patients *(145)*. The observation of carbonylated proteins in plasma—a body fluid easily obtainable without invasive procedures and, more importantly and unlike brain samples, before the death of the patient—suggests that these oxidized species may be useful as diagnostic biomarkers for (possibly early) AD.

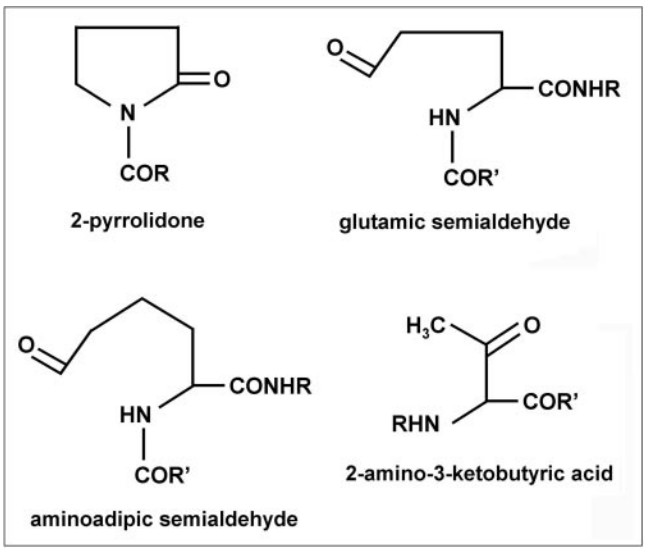

Fig. 5. Chemical structures of protein carbonyls arising from direct oxidation of amino acid side chains.

Shown are 2-pyrrolidone (resulting from direct oxidation of Pro residues), glutamic semialdehyde (resulting from direct oxidation of Arg and Pro residues), α-aminoadipic semialdehyde (resulting from direct oxidation of Lys residues), and 2-amino-3-ketobutyric acid (resulting from direct oxidation of Thr residues). For simplicity, stereochemistries are not indicated.

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 *(146)*.

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 persons; furthermore, such biomarkers of oxidative/nitrosative stress are significantly higher in patients with diabetes and hypercholesterolemia *(147)*.

Winterbourn et al. *(148)* determined that protein carbonyl concentrations were increased in both plasma and BAL 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 injury *(148)*.

Oxidative DNA Damage

Cellular DNA damage can be caused by ROS generated under different conditions, and several techniques have been developed to measure the oxidatively modified nucleobases in DNA *(7, 149, 150)*. Oxidative DNA damage seems to relate to an increased risk of cancer development later in life *(150, 151)*. DNA subjected to attack by hydroxyl radical generates a wide range of base and sugar modification products *(149)*. Such products can be measured by HPLC, GC-MS, LC-MS, and antibody-based techniques *(7, 18, 149, 152)*. Usually 8-hydroxy-20-deoxyguanosine (8OHdG) is measured as an index of oxidative DNA damage *(7, 18, 152)*. The advantages of and artifacts produced during measurement of 8OHdG in cellular DNA have been reviewed recently *(150, 152)*. Antibody-based methods have also been developed for 8OHdG and are useful for visualization of damage, but they seem likely to be only semiquantitative *(7)*.

DNA can also be damaged by RNS, undergoing mainly nitration and deamination of purines. Methods for the measurement of DNA base nitration and deamination products have been developed but may need more refinement and validation before they can be routinely applied to human materials *(7)*.

None of the analytical methods mentioned above identifies where the oxidative DNA damage is located. An-

other 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?) *(150)* that changes here are reflected in other tissues.

Measurement of 8OHdG in urine has been used to assess "whole-body" oxidative DNA damage. This can be achieved by HPLC and MS techniques *(7)*. However, 8OHdG 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 8OHdG is a partial measure of damage to guanine residues in DNA and its nucleotide precursor pool, and 8OHdG concentrations may not truly reflect rates of oxidative damage to DNA *(149)*. Nevertheless, 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 8OHdG 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 *(72)*.

Metabolism of Oxidized Biomolecules

An important question regarding the use of any oxidation product to monitor oxidative stress in vivo is its fate in the body. We therefore examined currently known aspects related to the in vivo metabolism of selected oxidized biomolecules.

The fate of HNE has been investigated both in various isolated cells/organs and cultured cells and in the whole animal following the disposition and biotransformation of exogenous radiolabeled HNE. However, the metabolic pattern of HNE produced within cell membranes or lipoproteins during lipoperoxidation is inevitably very different from that produced by parenteral administration.

HNE is removed mainly by its intracellular metabolism. Mammalian cells possess highly active pathways of HNE metabolism, the HNE degradation rate depending strongly on the cellular concentration and on the initial HNE concentration. The metabolic fate of HNE has been investigated in various mammalian cells and organs, such as hepatocytes, intestinal enterocytes, renal tubular cells, aortic and brain endothelial cells, synovial fibroblasts, neutrophils, thymocytes, the heart, and tumor cells *(48)*. In all cell types investigated, 90%–95% of 100 μmol/L HNE was degraded within 3 min of incubation. At 1 μmol/L HNE, the physiologic serum concentration of ~0.1–0.2 μmol/L was already restored after 10–30 s. Current knowledge indicates that the main primary metabolites of HNE are the HNE–GSH adduct, the corresponding carboxylic acid 4-hydroxynonenoic acid (HNA), and the corresponding alcohol 1,4-dihydroxynonene (DHN). The main enzymes involved in the metabolism are therefore glutathione *S*-transferases (Michael addition of thiols), aldehyde dehydrogenases (NAD^+-dependent oxidation of the carbonyl group), and alcohol dehydrogenases (NADH-dependent reduction of the carbonyl group) *(48)*. In most cell types, including hepatocytes, formation of HNE–GSH and HNA is much higher than that of DHN. Additionally, various other enzymes and nonenzymatic reactions should be taken into account for HNE metabolism. Glutathione *S*-transferase activity is more than 600 times faster than the rate of the nonenzymatic Michael addition reaction. Accelerated HNE formation or the addition of HNE to cells leads to a rapid decrease in intracellular GSH concentration *(48)*. The total rate of HNE degradation and the pattern of HNE metabolites vary depending on the pathophysiologically relevant conditions *(48)*. Despite the active and rapid metabolism of HNE, protein modification by HNE does occur. The measured percentages of HNE binding to proteins in different mammalian cell types and subcellular fractions after the addition of 100 μmol/L HNE were between 1% and 8.5% of total *(48)*. As to the fate of protein–HNE adducts, there are two general possibilities: one is the degradation of HNE-modified proteins by the 20S proteasome; the other is the cross-linking of proteins and their subsequent accumulation, if they are not or almost not degradable, finally leading to cytotoxicity.

The main urinary metabolites are represented by 2 groups of compounds. One group comes from the formation of mercapturic acid from (*a*) DHN-GSH, which originates from the conjugation of HNE with GSH by glutathione *S*-transferases and the subsequent reduction of the aldehyde by a member of the aldo-keto reductase superfamily; (*b*) the lactone of HNA-GSH (HNA-lactone–GSH), which originates from the conjugation of HNE followed by the oxidation of the aldehyde by aldehyde dehydrogenase; and (*c*) HNA-GSH, which originates from the hydrolysis of the corresponding lactone. The other group consists of metabolites issuing from the ω-hydroxylation of HNA or HNA-lactone by cytochromes P450 4A, followed eventually, in the case of ω-oxidized HNA-lactone, by conjugation with GSH and subsequent mercapturic acid formation *(153)*. The metabolism of HNE–protein adducts, involving probably the proteasome pathway, might take too long for the metabolites to be excreted in urine within 24 or 48 h, particularly because HNE has been shown to reduce some of the proteolytic activities of the proteasome *(154)*.

The electrophilic nature of HNE and the high concentrations of endogenous available compounds (thiols and His and Lys residues) can lead to several competitive reactions among nucleophiles; thus, the accessibility of the nucleophile at a given site of HNE generation is the more relevant factor in which metabolic pathway is followed. HNE is an endogenous electrophile permanently formed from lipoperoxidation of lipoproteins and cell membranes; it therefore can be expected that HNE is generated everywhere in the organism, but to a different extent. This and the above factors considered together imply that the metabolism of endogenous HNE likely

shows quantitative differences dependent on the site of generation and, especially, on the ratio of free GSH to reactive proteins *(153)*.

MDA is excreted mainly in the form of adducts with lysine and its N-acetylated derivative, both in humans and rats, indicating that its predominant reaction in vivo is with the ϵ-amino groups of the Lys residues of proteins. Smaller amounts of adducts with serine and ethanolamine and the nucleic acid bases guanine and deoxyguanosine reflect reactions with these compounds *(26)*.

Alterations in isoprostane biosynthesis, secretion, and excretion in physiologic and pathophysiologic states are attributable to several endogenous and exogenous regulatory mechanisms that control the availability of precursors required for isoprostane synthesis, such as dietary and tissue arachidonic acid content, oxygen concentration, and the generation of various free radical species *(65)*. After formation from esterified arachidonate, isoprostanes are quickly hydrolyzed via various phospholipases and further metabolized by β-oxidation. A substantial portion of isoprostanes appear to undergo β-oxidation in tissues before their release into the plasma. Intact isoprostanes, together with their β-oxidized metabolites, are efficiently excreted into the urine. Although urinary isoprostanes can come from lipid peroxidation in the kidney, evidence suggests that they originate mostly from free isoprostanes filtered from the circulation *(155, 156)*. It is generally assumed that excessive isoprostane production, rather than inefficient excretion or metabolism, accounts for increased concentrations of these compounds in pathophysiologic settings in vivo, although this has not been studied carefully *(65, 71)*. There are several factors, at least theoretically, that might regulate endogenous free isoprostane concentrations in tissues and biological fluids, including the initial oxidation step of arachidonate by free radicals, the deesterification process that leads to the formation of free isoprostanes, and the subsequent metabolism and excretion of both the parent compounds and degraded metabolites into the urine. After formation in tissues, esterified isoprostanes are enzymatically hydrolyzed in situ to form bioactive isoprostanes in their free acid form. This enzymatic cleavage step is also, at least theoretically, an important rate-limiting step for the formation of free isoprostanes in the circulation. Weight loss has been reported to lead to a decrease in F_2-IsoP concentrations. It has also been suggested that the lipolysis process during fasting might release 8-iso-$PGF_{2\alpha}$ from the adipose tissue, accounting for increased circulating concentrations of the compound seen during a 24-h fast *(65)*.

One of the major disadvantages of measuring NO_2-Tyr in tissues or biological fluids is that nitrated proteins are broken down at variable rates, and NO_2-Tyr is metabolized and excreted *(157)*. NO_2-Tyr is metabolized to 3-nitro-4-hydroxyphenylacetic acid (NHPA), which is excreted in the urine as the major urinary metabolite. This has led to the notion that measurement of urinary NHPA could be used as a marker of systemic NO_2-Tyr formation in vivo. However, NHPA is not derived exclusively from metabolism of NO_2-Tyr; it can also be formed via nitration of circulating *para*-hydroxyphenylacetic acid (PHPA), a metabolite of Tyr that is present at high concentrations in plasma *(158)*. In reality, because the plasma concentration of PHPA is markedly higher than that of free NO_2-Tyr (~400-fold), the nitration of endogenous PHPA to form NHPA becomes very significant and accounts for the majority of NHPA excreted in urine. After intravenous injection of NO_2-Tyr in rats, 0.5 (0.16)% [mean (SD)] of the injected dose was excreted as urinary free NO_2-Tyr, and 4.3 (0.2)% as urinary NHPA *(158)*.

A GC-MS/MS method has very recently been developed and validated for specific, interference-free, and accurate quantitative determination of basal concentrations of NO_2-Tyr in human urine *(113)*. This method represents an expansion of the GC-MS/MS method originally reported for circulating free and protein-bound NO_2-Tyr in the basal state *(114, 117)*. The NO_2-Tyr concentrations measured in urine of healthy humans by this new GC-MS/MS method are <33 nmol/L and are the lowest reported for urinary NO_2-Tyr to date. It is noteworthy that GC-MS/MS has also shown that ~30% of NO_2-Tyr is excreted in the urine without being metabolized *(113)*. Furthermore, it has been observed that both the plasma protein-bound and free NO_2-Tyr concentrations are not significantly affected by nitrate supplementation *(159)*.

Differently, di-Tyr released from proteins is relatively resistant to metabolism into other compounds, is rapidly cleared from the blood, and is excreted by the kidney into urine in near-quantitative yield rather than being recycled into proteins *(136, 160, 161)*.

The halogenated Tyr residues are ideal biomarkers for hypohalous acids because they retain chlorine and bromine and are stable under the acidic conditions required to hydrolyze proteins *(119, 162)*. Nothing is currently known about the metabolism of halogenated Tyr residues, but they are potential substrates for dehalogenases or glutathione *S*-transferases.

Choice of the Most Appropriate Biomarker(s) and Best Detection Method(s)

Whether ROS/RNS activity has a causal or propagating role in human diseases associated with oxidative stress remains generally unresolved *(3, 132)*. Nevertheless, the finding of increased oxidative stress in pathologic conditions has suggested the use of biomarkers of oxidative/nitrosative stress for the development of new diagnostic, therapeutic, and preventive strategies for impeding or delaying the development of complications such as atherosclerosis and coronary artery disease.

Biomarkers may yield information on three progressive levels of disease outcome: (*a*) as measurable endpoints of damage to biomolecules such as lipids and proteins; (*b*) as

functional markers of, for example, cognitive function; and (*c*) as endpoints related to specific disease. Although the clinical symptoms of a disease are endpoints in themselves, they are not suitable, in many cases, for early detection and, therefore, prevention of diseases associated with oxidative stress. A series of biomarkers would be preferred, each validated in sequence. To this end, the association between a biomarker and a disease should be defined.

The most intuitive goals for a biomarker are to help diagnose symptomatic and presymptomatic disease and to provide surrogate endpoints to demonstrate clinical efficacy of new treatments. The usefulness of the ideal biomarker of oxidative damage lies in its ability to provide early indication of disease and/or its progression (Fig. 6). A valid biomarker of oxidative/nitrosative stress should be (*a*) a stable product, not susceptible to artifactual induction, oxidation, or loss during sample handling, processing, analysis, and storage; (*b*) a major product of oxidative/nitrosative damage that may be implicated directly in the onset and/or progression of disease; (*c*) accessible in a target tissue or a valid surrogate that quantitatively reflects the oxidative modification of the target tissue; (*d*) present at concentrations high enough for the biomarker to be a significant product; (*e*) specific for the reactive species in question and free of confounding factors from dietary intake; (*f*) noninvasive; (*g*) measurable by an assay that is specific, sensitive, and reproducible; (*h*) easy to detect and measurable across populations; (*i*) present in concentrations that do not vary widely in the same persons under the same conditions at different times; (*j*) measurable with relatively small within-assay intrasample variation compared with between-person variations.

Validation of biomarkers requires multiple different steps (Fig. 7). One step is analytical validation, which includes development of procedures, analysis of reference materials, and quality control. Another is validation of the fact that changes in biomarker concentration do reflect the later development of disease. No currently used biomarker has yet fulfilled this key requirement of the ideal biomarker: that it is predictive of the later development of disease. No biomarker meets all of the "technical" requirements, but some are better than others. Case–control studies on stored samples should be used to test the efficiency of biomarkers. Care must be taken to define and establish reference values or baseline profiles in healthy tissues, cells, or body fluids. The use of a panel of biomarkers would enhance the positive predictive value of a test and minimize the proportion of false-positive and false-negative results.

From the purposes described above it can be inferred that the development and validation of suitable biomarkers of oxidative/nitrosative stress are important but time-consuming tasks. In particular, the last step in the validation process of a biomarker, i.e., its validation in an epidemiologic study (Fig. 7), is often difficult to achieve. Because of the complexity of human diseases associated with oxidative/nitrosative stress, it is highly unlikely that a single biomarker in diseases that completely substitutes for a meaningful clinical outcome will ever be found; for this reason (i.e., the complexity of these diseases), we believe the development of biomarkers is essential for better understanding of disease pathogenesis and for future drug development.

The availability of biomarkers that can provide an accurate assessment of the degree of oxidative damage will become important in clinical trials aimed at investigating the effectiveness of antioxidant therapy for preventing or reducing the risks of complications (Fig. 6). Although establishing the occurrence of oxidative stress

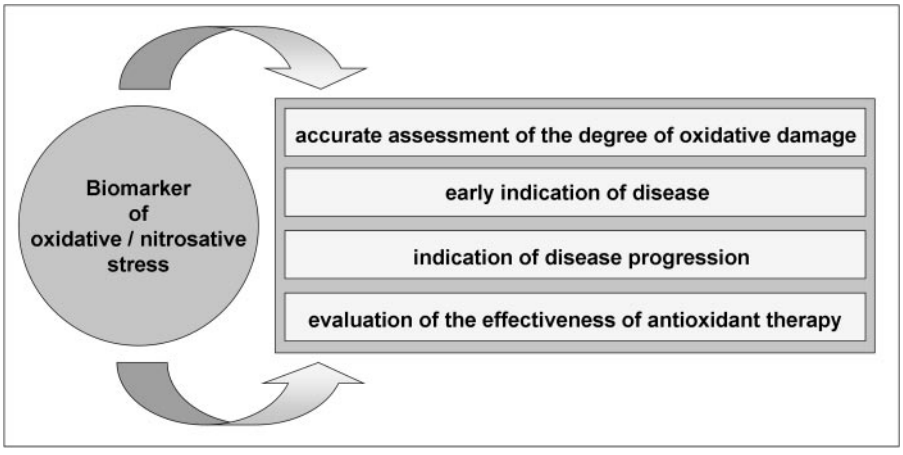

Fig. 6. Potential uses of biomarkers of oxidative/nitrosative stress.

A biomarker is defined as a characteristic that is objectively measured and evaluated as an indicator of normal biological processes, pathogenic processes, or pharmacologic responses to a therapeutic intervention. A biomarker of oxidative/nitrosative stress is classically defined as a biological molecule whose chemical structure has been modified by ROS/RNS and which can be used to reliably assess oxidative/nitrosative stress status in animal models and in humans. The most intuitive use for a biomarker is to help diagnose disease. The usefulness of the ideal biomarker of oxidative damage lies in its ability to provide early indication of disease and/or its progression, as well as to assess therapy effectiveness. Measurement of biomarkers of oxidative/nitrosative stress may also help in elucidating the pathophysiologic mechanisms mediating oxidative injury and may allow the prediction of disease outcome and the choice of the most adequate treatment in very early stages of the disease. Ideally, biomarkers of oxidative stress for human studies would be measurable in specimens that can be collected relatively easily, such as blood or urine. To serve these purposes, however, an ideal biomarker of oxidative damage should fulfill several conditions (see text and Fig. 7).

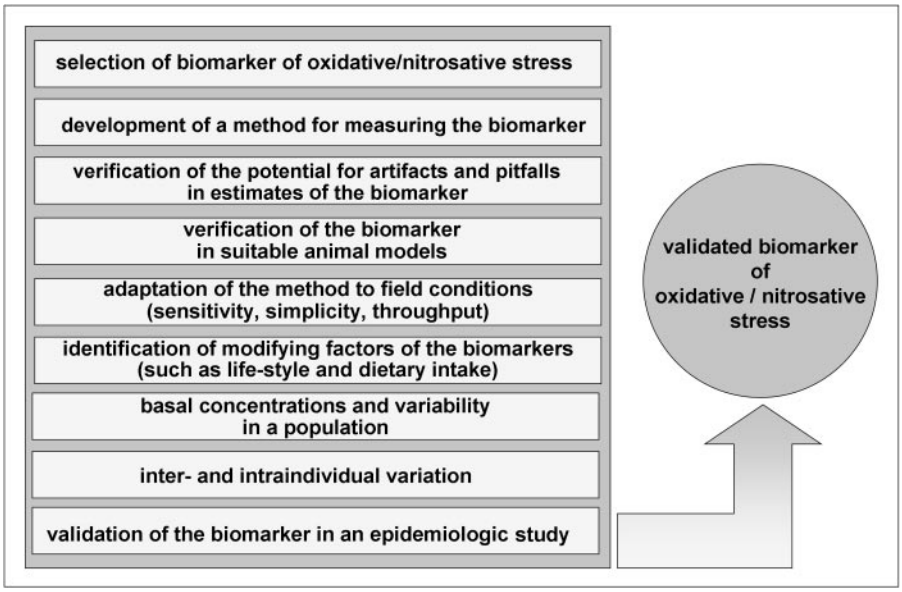

Fig. 7. Key criteria of the validation process of a suitable biomarker of oxidative/nitrosative stress.

in a human disease is an important first step, this alone does not determine whether oxidative stress plays a fundamental role in the pathogenesis of that disease. This can be determined only by demonstrating that amelioration of the oxidative stress via treatment with antioxidants mitigates manifestations of the disease. To accomplish that requires in-depth understanding of the clinical pharmacology of antioxidant agents, which currently is lacking. Although evidence from in vitro models (e.g., models of neurodegeneration) and from some animal models has demonstrated the protective effects of antioxidants, the clinical evidence that antioxidants act as protective drugs is still relatively scarce and/or controversial *(163–165)*. A key issue is that the effectiveness of a given biological antioxidant may depend on the ROS/RNS involved. Carotenoids, for example, are highly efficient antioxidants when the oxidizing species is singlet oxygen, but their effectiveness for other ROS/RNS is more questionable. Unquestionably, the relative importance of antioxidants as protective agents depends on which ROS/RNS is generated, how it is generated, where it is generated, what target of damage is measured, and the severity of the damage *(1)*. This is particularly relevant and extremely important in the setting of disease research, in that the key reactive species are rarely known (mainly because the possible sources for overproduction of reactive species are widespread), although there are a few limited situations in which the etiology is known (e.g., erythropoietic protoporphyria is known to be a singlet oxygen–mediated condition). Indeed, because methods currently available for the direct measurement of ROS/RNS are of limited applicability to humans *(7)*, most clinical studies have focused on the measurement of oxidative damage. This is to some extent logical because it is the damage caused by reactive species that is important rather than the total amount of such species generated. Relevant to this, some protein biomarkers of oxidative damage can be useful indicators of the type of ROS/RNS involved. For example, increased concentrations of Cl-Tyr in the bronchoalveolar fluid from children with cystic fibrosis led to the conclusion that HOCl is produced early in cystic fibrosis and that it is a candidate for precipitating the fatal decrease in lung function associated with this disease *(118)*.

A key methodologic issue concerns the potential for artifacts in estimates of biomarkers of oxidative/nitrosative stress. That is, oxidation of samples can occur during typical sample handling, processing, and analysis such that measured concentrations are not at all reflective of the concentrations encountered in vivo. This issue has plagued investigations of many different biomarkers of oxidation; for example, artifactual formation of NO_2-Tyr (see below).

The most widely used technique to evaluate lipid peroxidation is the TBARS assay, which includes MDA. TBA reacts with MDA to produce a stable chromogen that can be quantified by either spectrophotometry or HPLC. Although this technique is easy to use (mainly the spectrophotometric assay), the use of the TBARS test to assess oxidative stress status in human fluids is problematic for several reasons: (*a*) aldehydes other than MDA may react with TBA to produce a compound that absorbs in the same range as MDA; (*b*) decomposition of lipid peroxides during the test itself may mask the actual MDA content in the fluid before testing; (*c*) the presence or absence of metal ions or other undefined radicals affects the rate of this decomposition, making reliability a problem; and (*d*) most TBA-reactive material, including MDA, in human body fluids is not a specific product of lipid peroxidation and may produce false-positive results *(1, 7)*. The lack of specificity has rendered the simple spectrophotometric method rather obsolete. Instead, most researchers today

use an HPLC modification of the TBARS method. This approach uses HPLC to separate the MDA–TBA adduct from interfering chromogens, thus improving the sensitivity, specificity, and reproducibility *(1, 7)*. TBARS can be measured in tissues but are generally measured in plasma in the setting of epidemiologic research. Therefore, although the TBARS assay is accepted as an index of oxidative stress, this method quantifies MDA-like material and does not specifically measure MDA or lipid peroxidation.

The authors of many studies have measured whole-blood or erythrocyte GSH and GSSG, comparing concentrations obtained in healthy individuals with those measured in persons affected by various diseases, reporting a correlation between the disease and significantly different concentrations of GSSG, GSH, and/or GSH/GSSG ratios. However, the results of some of the studies we reviewed suggest that, because large differences in GSH and, particularly, in GSSG have been found in different investigations (also for control values in healthy people), accurate revision of the methods used is needed *(93)*. The fact that GSSG values in human blood can span up to 2 orders of magnitude, even in controls, based on the method used justifies new efforts to identify and eliminate all possible pitfalls. Furthermore, it is essential to revise the methods before attempting to interpret the role of an altered GSH or GSSG concentration in blood. Recent studies have examined the most frequent pitfalls in sample collection, manipulation, and GSH/GSSG titration in human blood or erythrocytes *(93, 95)*.

Recent reports have documented that NO_2-Tyr can be generated ex vivo during sample processing, in particular under acidic conditions *(110, 112, 114–116, 166, 167)*, illustrating the limitations of some quantitative methods that do not use isotopically labeled internal standards (e.g., immunochemical, spectroscopic, and electrochemical detection), which can distinguish in vivo from ex vivo generation. These findings highlight the importance of developing robust analytical methods and validating them in vivo. Indeed, some advances in the MS quantification of Tyr modifications have been described *(110, 112, 113, 115, 116, 168)*, in particular, in the MS/MS technology, which currently is the only technology that can guarantee reliable, sensitive, and interference-free quantification of free and protein-bound NO_2-Tyr in both plasma *(112)* and urine *(113)*. This technology seems to be applicable even to exhaled breath condensate; a reliable and sensitive LC-MS/MS method was developed for the quantitative detection of free NO_2-Tyr and revealed considerably lower values [NO_2-Tyr concentration ranged between 3.9 ng/L (the limit of quantification) and 184 ng/L] than could be measured with immunoassays *(169)*.

Numerous markers of oxidative stress and antioxidant status have been evaluated, but there has been little systematic effort to validate sensitive and specific biomarkers for oxidative damage in animal models. The recently completed BOSS study, which was organized and sponsored by the National Institute of Environmental Health Sciences (NIEHS), is the first comprehensive comparative study, to our knowledge, that has examined several proposed markers of oxidative stress in the same model system to determine which of the biomarkers used for noninvasive measurement of oxidative stress are most specific, sensitive, and selective *(72, 170)*. To this end, this multilaboratory validation study, performed by leading investigators in the field, used a well-documented animal model of oxidative stress, CCl_4, administered to rats. CCl_4 is a well-known hepatotoxin that induces free radical injury in the liver and other tissues when administered to rats. In this comprehensive study, the time- and dose-dependent effects of acute CCl_4 poisoning on the oxidation products of lipids, proteins, and DNA were measured in blood, plasma, and urine. The criterion used to identify a good marker for the measurement of oxidative stress was a significant effect seen at both tested doses at more than one time point. The authors of the trial concluded that plasma concentrations of MDA and 8-iso-$PGF_{2\alpha}$ (both measured by GC-NICI-MS) and urinary concentrations of 8-iso-$PGF_{2\alpha}$ (measured by immunoassay or LC-MS/MS) are promising candidates for general biomarkers of oxidative stress. Differently, many other putative markers of oxidative stress, including oxidation products of plasma proteins and leukocyte DNA, are not reliable biomarkers for free radical damage induced by CCl_4 *(72, 170)*.

CCl_4-mediated oxidative damage is just one example of the more general phenomenon of oxidative stress. Other models also need to be studied. It still needs to be determined whether the identified biomarkers of oxidative damage in the present rodent model of CCl_4 poisoning would be applicable to other oxidative insults. Therefore, other models of oxidative stress must be developed and studied for comparison.

Prospects and Challenges

There is growing interest in identifying biomarkers for diseases in which oxidative stress is involved. Various invasive and semi-invasive means of assessing oxidative biomarkers are available; these include measurements in cerebrospinal fluid, synovial fluid, BAL fluid, urine, and tissue biopsies. These other body fluids share some of the protein characteristics of plasma, with specific local additions that reveal interesting clinical information. Unfortunately, these samples are more difficult to obtain or preserve in a useful state than is plasma. For example, the procedures for collecting cerebrospinal fluid and synovial fluid are invasive, involving pain and some risk, whereas urine is more difficult to process to a useful sample quickly in a clinical setting (processing requires centrifugation to remove cells that can undergo lysis if left in suspension, prevention of microbial growth, and concentration). Recent studies have therefore focused on noninvasive techniques to evaluate oxidative stress, e.g., in inflammatory lung diseases such as asthma, COPD, and

cystic fibrosis. The assessment of biomarkers of oxidative stress in exhaled breath condensate is one such approach and is emerging as a promising area of future research in inflammatory lung diseases *(171, 172)*.

The collection of exhaled breath condensate has several advantages over the more traditional methods of sampling the pulmonary airspaces, such as BAL fluid. It is a simple, noninvasive approach, less expensive in terms of both equipment and personnel costs, may be repeated frequently, and can be applied to children, including neonates, and patients with severe disease in whom more invasive procedures are not possible *(171, 172)*. It is currently used as a research and diagnostic tool in the free radical field, yielding information on redox disturbance and the degree and type of inflammation in the lung. However, the standardization of measured concentrations of potential oxidative stress biomarkers (e.g., NO_2-Tyr) in exhaled breath condensate is currently an unresolved problem *(169)*, unlike in urine and plasma (see above). With further technical developments, such an approach might ultimately have a role in the clinic, in helping to diagnose specific lung diseases.

The application of any new technical procedure in investigative studies or to the clinic requires its validation in terms of sensitivity, specificity, reproducibility, and correlation of the measurements with disease status. The validity of exhaled breath condensate as a tool for the assessment of airway oxidative stress is still questionable because of limitations in the reproducibility of analyzed oxidative biomarkers, with respect to both intra- and interindividual variability *(172)*.

Quantification of chemical markers of protein oxidation in human urine, plasma, and other biological fluids also may identify pathways that promote oxidative stress in vivo and might indicate the extent of protein damage if these markers are liberated by proteolysis and excreted into such fluids *(111, 112)*. Di-Tyr and NO_2-Tyr are promising markers for these purposes because they are excreted in the urine in near quantitative yields and are not generated ex vivo during analysis by isotope-dilution GC-MS *(40)*. Nevertheless, as highlighted above, whereas GC-MS is susceptible to interference by coeluting compounds, both GC-MS/MS and LC-MS/MS are not and, therefore, should be regarded as the methods of choice for the accurate quantification of NO_2-Tyr in urine as well as in plasma *(111, 112)*. These observations raise the possibility that assaying oxidized amino acids in urine and plasma might be a useful approach to monitoring oxidative stress for human clinical and epidemiologic studies.

Development of new biomarkers based on disease-induced protein modifications is limited by the lack of easy access to tissues from patient populations; therefore, the majority of discovery work will need to be carried out in animal models. The advantage provided by animal models is the ability to control and define disease stages; correlating these model diseases to actual patient populations has been difficult, however. The process of biomarker discovery in animal models, through clinical validation to translation into a clinical test or panel of tests, has become known as "clinical proteomics" *(173)*. As the technology develops to allow higher throughput screening, these candidate markers can be more easily tested against large groups.

The successful development of effective antioxidant therapies remains a key goal, the attainment of which is required to elucidate the role played by accumulation of (toxic) oxidized molecules in the clinical picture of diseases associated with oxidative stress. The use of biomarkers provides a logical scientific basis for major intervention trials of antioxidants; such trials could, in turn, eventually validate or disprove the biomarker concept. Any intervention trial that does take place should be accompanied by measurements of one or more relevant biomarkers at intervals during the study. If the endpoint of the trial is disease incidence or mortality, such studies could help to validate or disprove the biomarker concept. However, with regard to this matter, we must highlight the disappointing clinical evidence showing the failure of antioxidant therapy for oxidative stress-associated pathologies such as cardiovascular disease, coronary artery disease, AD, and COPD *(163–165, 174, 175)*. At best, use of antioxidants is controversial and the ideal antioxidant strategy is still unknown.

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References

1. Halliwell B, Gutteridge JMC. Free radicals in biology and medicine. New York: Oxford University Press, 1999:936pp.
2. Halliwell B. Role of free radicals in the neurodegenerative diseases: therapeutic implications for antioxidant treatment [Review]. Drugs Aging 2001;18:685–716.
3. Stocker R, Keaney JF Jr. Role of oxidative modifications in atherosclerosis [Review]. Physiol Rev 2004;84:1381–478.
4. Dalle-Donne I, Giustarini D, Colombo R, Rossi R, Milzani A. Protein carbonylation in human diseases [Review]. Trends Mol Med 2003;9:169–76.
5. Dalle-Donne I, Scaloni A, Giustarini D, Cavarra E, Tell G, Lungarella G, et al. Proteins as biomarkers of oxidative stress in diseases: the contribution of redox proteomics [Review]. Mass Spectrom Rev 2005;24:55–99.
6. Klaunig JE, Kamendulis LM. The role of oxidative stress in carcinogenesis [Review]. Annu Rev Pharmacol Toxicol 2004;44: 239–67.
7. Halliwell B, Whiteman M. Measuring reactive species and oxidative damage in vivo and in cell culture: how should you do it and what do the results mean? [Review]. Br J Pharmacol 2004;142: 231–55.
8. Finkel T, Holbrook NJ. Oxidants, oxidative stress and the biology of ageing [Review]. Nature 2000;408:239–47.

9. Finkel T. Oxidant signals and oxidative stress [Review]. Curr Opin Cell Biol 2003;15:247–54.

10. Dedon PC, Tannenbaum SR. Reactive nitrogen species in the chemical biology of inflammation [Review]. Arch Biochem Biophys 2004;423:12–22.

11. Stadtman ER, Berlett BS. Reactive oxygen–mediated protein oxidation in aging and disease [Review]. Chem Res Toxicol 1997;10:485–94.

12. Linton S, Davies MJ, Dean RT. Protein oxidation and ageing [Review]. Exp Gerontol 2001;36:1503–18.

13. Schoneich C. Mass spectrometry in aging research [Review]. Mass Spectrom Rev 2005;24:701–18.

14. Davies MJ, Fu S, Wang H, Dean RT. Stable markers of oxidant damage to proteins and their application in study of human disease [Review]. Free Radic Biol Med 1999;27:1151–61.

15. Winterbourn CC, Buss H. Protein carbonyl measurement by enzyme-linked immunosorbent assay. Methods Enzymol 1999; 300:106–11.

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

17. Pryor WA. Bio-assays for oxidative stress status (BOSS). Amsterdam: Elsevier Science BV, 2001:286pp.

18. Griffiths HR, Moller L, Bartosz G, Bast A, Bertoni-Freddari C, Collins A, et al. Biomarkers [Review]. Mol Aspects Med 2002; 23:101–208.

19. Giustarini D, Dalle-Donne I, Colombo R, Milzani A, Rossi R. Adaptation of the Griess reaction for detection of nitrite in human plasma. Free Radic Res 2004;38:1235–40.

20. Tarpey MM, Wink DA, Grisham MB. Methods for detection of reactive metabolites of oxygen and nitrogen: in vitro and in vivo considerations [Review]. Am J Physiol Regul Integr Comp Physiol 2004;286:R431–44.

21. Uchida K. 4-Hydroxy-2-nonenal: a product and mediator of oxidative stress [Review]. Prog Lipid Res 2003;42:318–43.

22. Carini M, Aldini G, Facino RM. Mass spectrometry for detection of 4-hydroxy-*trans*-2-nonenal (HNE) adducts with peptides and proteins [Review]. Mass Spectrom Rev 2004;23:281–305.

23. Cracowski JL, Durand T, Bessard G. Isoprostanes as a biomarker of lipid peroxidation in humans: physiology, pharmacology and clinical implications [Review]. Trends Pharmacol Sci 2002;23: 360–6.

24. Montuschi P, Barnes PJ, Roberts LJ. Isoprostanes: markers and mediators of oxidative stress [Review]. FASEB J 2004;18:1791–800.

25. Uchida K. Histidine and lysine as targets of oxidative modification [Review]. Amino Acids 2003;25:249–57.

26. Draper HH, Csallany AS, Hadley M. Urinary aldehydes as indicators of lipid peroxidation in vivo. Free Radic Biol Med 2000;29: 1071–7.

27. Wilson R, Lyall K, Smyth L, Fernie CE, Riemersma RA. Dietary hydroxy fatty acids are absorbed in humans: implications for the measurement of 'oxidative stress' in vivo. Free Radic Biol Med 2002;32:162–8.

28. Dean RT, Fu S, Stocker R, Davies MJ. Biochemistry and pathology of radical-mediated protein oxidation [Review]. Biochem J 1997;324:1–18.

29. Davies MJ. The oxidative environment and protein damage [Review]. Biochim Biophys Acta 2005;1703:93–109.

30. Grune T, Merker K, Sandig G, Davies KJA. Selective degradation of oxidatively modified protein substrates by the proteasome. Biochem Biophys Res Commun 2003;305:709–18.

31. Ischiropoulos H. Biological selectivity and functional aspects of protein tyrosine nitration [Review]. Biochem Biophys Res Commun 2003;305:776–83.

32. Requena JR, Levine RL, Stadtman ER. Recent advances in the analysis of oxidized proteins [Review]. Amino Acids 2003;25: 221–6.

33. Stadtman ER, Levine RL. Free radical-mediated oxidation of free amino acids and amino acid residues in proteins [Review]. Amino Acids 2003;25:207–18.

34. Stadtman ER, Moskovitz J, Levine RL. Oxidation of methionine residues of proteins: biological consequences [Review]. Antiox Redox Signal 2003;5:577–82.

35. Keck S, Nitsch R, Grune T, Ullrich O. Proteasome inhibition by paired helical filament-τ in brains of patients with Alzheimer's disease. J Neurochem 2003;85:115–22.

36. Sitte N, Huber M, Grune T, Ladhoff A, Doecke WD, Zglinicki T, et al. Proteasome inhibition by lipofuscin/ceroid during postmitotic ageing of fibroblasts. FASEB J 2000;14:1490–8.

37. Grune T, Jung T, Merker K, Davies KJA. Decreased proteolysis caused by protein aggregates, inclusion bodies, plaques, lipofuscin, ceroid, and 'aggresomes' during oxidative stress, aging, and disease [Review]. Int J Biochem Cell Biol 2004;36:2519–30.

38. Berliner J, Heinecke JW. The role of oxidized lipoproteins in atherogenesis [Review]. Free Radic Biol Med 1996;20:707–27.

39. Slatter DA, Bolton CH, Bailey AJ. The importance of lipid-derived malondialdehyde in diabetes mellitus [Review]. Diabetologia 2000;43:550–7.

40. Heinecke JW. Oxidized amino acids: culprits in human atherosclerosis and indicators of oxidative stress [Review]. Free Radic Biol Med 2002;32:1090–101.

41. Yoneyama Y, Sawa R, Suzuki S, Doi D, Yoneyama K, Otsubo Y, et al. Relationship between plasma malondialdehyde levels and adenosine deaminase activities in preeclampsia. Clin Chim Acta 2002;322:169–73.

42. Wood LG, Gibson PG, Garg ML. Biomarkers of lipid peroxidation, airway inflammation and asthma [Review]. Eur Respir J 2003; 21:177–86.

43. Barnham KJ, Masters CL, Bush AI. Neurodegenerative diseases and oxidative stress [Review]. Nat Rev Drug Discov 2004;3:205–14.

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

45. Eckl PM, Ortner A, Esterbauer H. Genotoxic properties of 4-hydroxyalkenals and analogous aldehydes. Mutat Res 1993;290: 183–92.

46. Parola M, Bellomo G, Robino G, Barrera G, Dianzani MU. 4-Hydroxynonenal as a biological signal: molecular basis and pathophysiological implications [Review]. Antioxid Redox Signal 1999;1:255–84.

47. 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 U S A 2004;101:8598–602.

48. Siems W, Grune T. Intracellular metabolism of 4-hydroxynonenal [Review]. Mol Aspects Med 2003;24:167–75.

49. Uchida K, Stadtman ER. Modification of histidine residues in proteins by reaction with 4-hydroxynonenal. Proc Natl Acad Sci U S A 1992;89:4544–8.

50. Butterfield DA. Amyloid β-peptide(1–42)–induced oxidative stress and neurotoxicity: implications for neurodegeneration in Alzheimer's disease brain [Review]. Free Radic Res 2002;36: 1307–13.

51. Butterfield DA, Castegna A, Lauderback CM, Drake J. Evidence that amyloid β-peptide–induced lipid peroxidation and its sequelae in Alzheimer's disease brain contribute to neuronal death. Neurobiol Aging 2002;23:655–64.

52. Montine TJ, Neely MD, Quinn JF, Beal MF, Markesbery WR, Roberts LJ, et al. Lipid peroxidation in aging brain and Alzheimer's disease [Review]. Free Radic Biol Med 2002;33:620–6.

53. Williams TI, Lynn BC, Markesbery WR, Lovell MA. Increased levels of 4-hydroxynonenal and acrolein, neurotoxic markers of lipid peroxidation, in the brain in mild cognitive impairment and early Alzheimer's disease. Neurobiol Aging June 30, 2005 [Epub ahead of print].

54. Wataya T, Nunomura A, Smith MA, Siedlak SL, Harris PL, Shimohama S, et al. High molecular weight neurofilament proteins are physiological substrates of adduction by the lipid peroxidation product hydroxynonenal. J Biol Chem 2002;277:4644–8.

55. Liu Q, Smith MA, Avila J, DeBernardis J, Kansal M, Takeda A, et al. Alzheimer-specific epitopes of τ represent lipid peroxidation-induced conformations. Free Radic Biol Med 2005;38:746–54.

56. Jenner P. Oxidative stress in Parkinson's disease [Review]. Ann Neurol 2003;53:S26–36.

57. Uchida K. Role of reactive aldehyde in cardiovascular diseases [Review]. Free Radic Biol Med 2000;28:1685–96.

58. Shao B, O'brien KD, McDonald TO, Fu X, Oram JF, Uchida K, et al. Acrolein modifies apolipoprotein α–I in the human artery wall. Ann N Y Acad Sci 2005;1043:396–403.

59. Rahman I, van Schadewijk AA, CrowtherAJ, Hiemstra PS, Stolk J, MacNee W, et al. 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;166:490–5.

60. Barreiro E, de la Puente B, Minguella J, Corominas JM, Serrano S, Hussain SN, et al. Oxidative stress and respiratory muscle dysfunction in severe chronic obstructive pulmonary disease. Am J Respir Crit Care Med 2005;171:1116–24.

61. Klein T, Neuhaus K, Reutter F, Nusing RM. Generation of 8-epi-prostaglandin F(2α) in isolated rat kidney glomeruli by a radical-independent mechanism. Br J Pharmacol 2001;133:643–50.

62. Tsikas D, Schwedhelm E, Suchy MT, Niemann J, Gutzki FM, Erpenbeck VJ, et al. Divergence in urinary 8-iso-PGF(2α) (iPF(2α)-III, 15-F(2t)-IsoP) levels from gas chromatography-tandem mass spectrometry quantification after thin-layer chromatography and immunoaffinity column chromatography reveals heterogeneity of 8-iso-PGF(2α). Possible methodological, mechanistic and clinical implications. J Chromatogr B 2003;794:237–55.

63. Roberts LJ, Morrow JD. Measurement of F_2-isoprostanes as an index of oxidative stress in vivo [Review]. Free Radic Biol Med 2000;28:505–13.

64. Basu S. Isoprostanes: novel bioactive products of lipid peroxidation [Review]. Free Radic Res 2004;38:105–22.

65. Basu S, Helmersson J. Factors regulating isoprostane formation in vivo [Review]. Antioxid Redox Signal 2005;7:221–35.

66. Roberts LJ, Morrow JD. Products of the isoprostane pathway: unique bioactive compounds and markers of lipid peroxidation [Review]. Cell Mol Life Sci 2002;59:808–20.

67. Janssen LJ. Isoprostanes: an overview and putative roles in pulmonary pathophysiology [Review]. Am J Physiol Lung Cell Mol Physiol 2001;280:L1067–82.

68. Montine TJ, Montine KS, McMahan W, Markesbery WR, Quinn JF, Morrow JD. F_2-Isoprostanes in Alzheimer and other neurodegenerative diseases [Review]. Antioxid Redox Signal 2005;7:269–75.

69. Schwedhelm E, Boger RH. Application of gas chromatography-mass spectrometry for analysis of isoprostanes: their role in cardiovascular disease [Review]. Clin Chem Lab Med 2003;4:1552–61.

70. Moore KP, Holt SG, Patel RP, Svistunenko DA, Zackert W, Goodier D, et al. A causative role for redox cycling of myoglobin and its inhibition by alkalinization in the pathogenesis and treatment of rhabdomyolysis-induced renal failure. J Biol Chem 1998;273:31731–7.

71. Morrow JD. Quantification of isoprostanes as indices of oxidant stress and the risk of atherosclerosis in humans [Review]. Arterioscler Thromb Vasc Biol 2005;25:279–86.

72. Kadiiska MB, Gladen BC, Bard DD, Germolec D, Graham LB, Parker CE, et al. Biomarkers of Oxidative Stress Study II. Are oxidation products of lipids, proteins, and DNA markers of CCl_4 poisoning? Free Radic Biol Med 2005;38:698–710.

73. Wang Z, Ciabattoni G, Creminon C, Lawson J, FitzGerald GA, Patrono C, et al. Immunological characterization of urinary 8-epi-prostaglandin $F_{2\alpha}$ excretion in man. J Pharmacol Exp Ther 1995;275:94–100.

74. Pratico D, Lawson JA, Rokach J, FitzGerald GA. The isoprostanes in biology and medicine [Review]. Trends Endocrinol Metab 2001;12:243–7.

75. Pratico D, Rokach J, Lawson J, FitzGerald GA. F_2-Isoprostanes as indices of lipid peroxidation in inflammatory diseases [Review]. Chem Phys Lipids 2004;128:165–71.

76. 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;459:259–62.

77. 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;72:714–22.

78. Montuschi P, Kharitonov SA, Ciabattoni G, Corradi M, van Rensen L, Geddes DM, et al. Exhaled 8-isoprostane as a new non-invasive biomarker of oxidative stress in cystic fibrosis. Thorax 2000;55:205–9.

79. Montuschi P, Collins JV, Ciabattoni G, Lazzeri N, Corradi M, Kharitonov SA, et al. 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;162:1175–7.

80. 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;20:1215–21.

81. Schwedhelm E, Bartling A, Lenzen H, Tsikas D, Maas R, Brummer J, et al. Urinary 8-iso-prostaglandin F2α as a risk marker in patients with coronary heart disease: a matched case–control study. Circulation 2004;109:843–8.

82. Tsikas D, Schwedhelm E, Fauler J, Gutzki FM, Mayatepek E, Frolich JC. Specific and rapid quantification of 8-iso-prostaglandin F2α in urine of healthy humans and patients with Zellweger syndrome by gas chromatography-tandem mass spectrometry. J Chromatogr B 1998;716:7–17.

83. Montine TJ, Markesbery WR, Morrow JD, Roberts LJ. Cerebrospinal fluid F2-isoprostane levels are increased in Alzheimer's disease. Ann Neurol 1998;44:410–3.

84. Pratico D, Clark CM, Lee VM, Trojanowski JQ, Rokach J, Fitzgerald GA. Increased 8,12-iso-iP$F_{2\alpha}$-VI in Alzheimer's disease: correlation of a non-invasive index of lipid peroxidation with disease severity. Ann Neurol 2000;48:809–12.

85. Pratico D, Clark CM, Liun F, Rokach J, Lee VY, Trojanowski JQ. Increase of brain oxidative stress in mild cognitive impairment: a possible predictor of Alzheimer disease. Arch Neurol 2002;59:972–6.

86. Montine TJ, Quinn JF, Milatovic D, Silbert LC, Dang T, Sanchez S, et al. Peripheral F2-isoprostanes and F4-neuroprostanes are not increased in Alzheimer's disease. Ann Neurol 2002;52:175–9.

87. Bohnstedt KC, Karlberg B, Wahlund LO, Jonhagen ME, Basun H, Schmidt S. Determination of isoprostanes in urine samples from Alzheimer patients using porous graphitic carbon liquid chromatography-tandem mass spectrometry. J Chromatogr B 2003;796: 11–9.

88. Troost R, Schwedhelm E, Rojczyk S, Tsikas D, Frolich JC. Nebivolol decreases systemic oxidative stress in healthy volunteers. Br J Clin Pharmacol 2000;50:377–9.

89. Fliser D, Wagner KK, Loos A, Tsikas D, Haller H. Chronic angiotensin II receptor blockade reduces (intra)renal vascular resistance in patients with type 2 diabetes. J Am Soc Nephrol 2005;16:1135–40.

90. Pastore A, Federici G, Bertini E, Piemonte F. Analysis of glutathione: implication in redox and detoxification [Review]. Clin Chim Acta 2003;333:19–39.

91. 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; 732:127–35.

92. 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;798:343–9.

93. Rossi R, Milzani A, Dalle-Donne I, Giustarini D, Lusini L, Colombo R, et al. Blood glutathione disulfide: in vivo factor or in vitro artifact? Clin Chem 2002;48:742–53.

94. Giustarini D, Dalle-Donne I, Colombo R, Milzani A, Rossi R. An improved HPLC measurement for GSH and GSSG in human blood. Free Radic Biol Med 2003;35:1365–72.

95. Giustarini D, Dalle-Donne I, Colombo R, Milzani A, Rossi R. Interference of plasmatic glutathione and hemolysis on glutathione disulfide levels in human blood. Free Radic Res 2004;38: 1101–6.

96. Bharath S, Hsu M, Kaur D, Rajagopalan S, Andersen JK. Glutathione, iron and Parkinson's disease [Review]. Biochem Pharmacol 2002;64:1037–48.

97. Warner DS, Sheng H, Batinić-Haberle I. Oxidants, antioxidants and the ischemic brain [Review]. J Exp Biol 2004;207:3221–31.

98. 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.

99. Navarro J, Obrador E, Carretero J, Petschen I, Avino J, Perez P, et al. Changes in glutathione status and the antioxidant system in blood and in cancer cells associate with tumour growth in vivo. Free Radic Biol Med 1999;26:410–8.

100. 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;27:1080–6.

101. Comhair SA, Erzurum SC. Antioxidant responses to oxidant-mediated lung diseases [Review]. Am J Physiol Lung Cell Mol Physiol 2002;283:L246–55.

102. Lang CA, Mills BJ, Mastropaolo W, Liu MC. Blood glutathione decreases in chronic diseases. J Lab Clin Med 2000;135: 402–5.

103. Lang CA, Mills BJ, Lang HL, Liu MC, Usui WM, Richie J Jr, et al. High blood glutathione levels accompany excellent physical and mental health in women ages 60 to 103 years. J Lab Clin Med 2002;140:413–7.

104. Giustarini D, Rossi R, Milzani A, Colombo R, Dalle-Donne I. S-Glutathionylation: from redox regulation of protein functions to human diseases [Review]. J Cell Mol Med 2004;8:201–12.

105. Dalle-Donne I, Rossi R, Giustarini D, Colombo R, Milzani A. Is there a role for S-glutathionylation of proteins in human disease? IUBMB Life 2005;57:189–92.

106. Pastore A, Tozzi G, Gaeta LM, Bertini E, Serafini V, Di Cesare S, et al. Actin glutathionylation increases in fibroblasts of patients with Friedreich's ataxia: a potential role in the pathogenesis of the disease. J Biol Chem 2003;43:42588–95.

107. 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:L537–46.

108. Brennan ML, Wu W, Fu X, Shen Z, Song W, Frost H, et al. 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: 17415–27.

109. Gaut JP, Yeh GC, Tran HD, Byun J, Henderson JP, Richter GM, et al. Neutrophils employ the myeloperoxidase system to generate antimicrobial brominating and chlorinating oxidants during sepsis. Proc Natl Acad Sci U S A 2001;98:11961–6.

110. Gaut JP, Byun J, Tran HD, Heinecke JW. Artifact-free quantification of free 3-chlorotyrosine, 3-bromotyrosine, and 3-nitrotyrosine in human plasma by electron capture-negative chemical ionisation gas chromatography mass spectrometry and liquid chromatography-electrospray ionization tandem mass spectrometry. Anal Biochem 2002;300:252–9.

111. Duncan MW. A review of approaches to the analysis of 3-nitrotyrosine [Review]. Amino Acids 2003;25:351–61.

112. 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.

113. Tsikas D, Mitschke A, Suchy MT, Gutzki FM, Stichtenoth DO. Determination of 3-nitrotyrosine in human urine at the basal state by gas chromatography-tandem mass spectrometry and evaluation of the excretion after oral intake. J Chromatogr B 2005;827:146–56.

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

115. 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;345:453–8.

116. Yi D, Ingelse BA, Duncan MW, Smythe GA. Quantification of 3-nitrotyrosine in biological tissues and fluids: generating valid results by eliminating artifactual formation. J Am Soc Mass Spectrom 2000;11:578–86.

117. Tsikas D, Schwedhelm E, Stutzer FK, Gutzki FM, Rode I, Mehls C, et al. 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.

118. Kettle AJ, Chan T, Osberg I, Senthilmohan R, Chapman AL, Mocatta TJ, et al. Myeloperoxidase and protein oxidation in the airways of young children with cystic fibrosis. Am J Respir Crit Care Med 2004;170:1317–23.

119. Pattison DI, Davies MJ. Kinetic analysis of the reactions of hypobromous acid with protein components: implications for cellular damage and use of 3-bromotyrosine as a marker of oxidative stress. Biochemistry 2004;43:4799–809.

120. Wu W, Samoszuk MK, Comhair SA, Thomassen MJ, Farver CF, Dweik RA, et al. Eosinophils generate brominating oxidants in allergen-induced asthma. J Clin Invest 2000;105:1455–63.

121. MacPherson JC, Comhair SAA, Erzurum SC, Klein DF, Lipscomb MF, Kavuru MS, et al. 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;166:5763–72.

122. Aldridge RE, Chan T, Van Dalen CJ, Senthilmohan R, Winn M, Venge P, et al. Eosinophil peroxidase produces hypobromous acid in the airways of stable asthmatics. Free Radic Biol Med 2002;33:847–56.

123. Bergt C, Pennathur S, Fu X, Byun J, O'Brien K, McDonald TO, et al. The myeloperoxidase product hypochlorous acid oxidizes HDL in the human artery wall and impairs ABCA1-dependent cholesterol transport. Proc Natl Acad Sci U S A 2004;101:13032–7.

124. Pennathur S, Bergt C, Shao B, Byun J, Kassim SY, Singh P, et al. Human atherosclerotic intima and blood of patients with established coronary artery disease contain high density lipoprotein damaged by reactive nitrogen species. J Biol Chem 2004;279: 42977–83.

125. Zheng L, Nukuna B, Brennan ML, Sun M, Goormastic M, Settle M, et al. Apolipoprotein A-I is a selective target for myeloperoxidase-catalyzed oxidation and functional impairment in subjects with cardiovascular disease. J Clin Invest 2004;114:529–41.

126. Hazen SL, Heinecke JW. 3-Chlorotyrosine, a specific marker of myeloperoxidase-catalyzed oxidation, is markedly elevated in low density lipoprotein isolated from human atherosclerotic intima. J Clin Invest 1997;99:2075–81.

127. Shishehbor MH, Aviles RJ, Brennan ML, Fu X, Goormastic M, Pearce GL, et al. Association of nitrotyrosine levels with cardiovascular disease and modulation by statin therapy. JAMA 2003; 289:1675–80.

128. Shishehbor MH, Brennan ML, Aviles RJ, Fu X, Penn MS, Sprecher DL, et al. Statins promote potent systemic antioxidant effects through specific inflammatory pathways. Circulation 2003;108: 426–31.

129. Gole MD, Souza JM, Choi I, Hertkorn C, Malcolm S, Foust RF III, et al. Plasma proteins modified by tyrosine nitration in acute respiratory distress syndrome. Am J Physiol Lung Cell Mol Physiol 2000;278:L961–7.

130. Pignatelli B, Li CQ, Boffetta P, Chen Q, Ahrens W, Nyberg F, et al. Nitrated and oxidized plasma proteins in smokers and lung cancer patients. Cancer Res 2001;61:778–84.

131. Masri FA, Comhair SAA, Koeck T, Stuehr DJ, Xu W, Janocha A, et al. Abnormalities in nitric oxide and its derivatives in lung cancer. Am J Respir Crit Care Med 2005;172:597–605.

132. Ischiropoulos H, Beckman JS. Oxidative stress and nitration in neurodegeneration: cause, effect, or association? [Review]. J Clin Invest 2003;111:163–9.

133. 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;325:1180–6.

134. Greenacre SA, Ischiropoulos H. Tyrosine nitration: localisation, quantification, consequences for protein function and signal transduction [Review]. Free Radic Res 2001;34:541–81.

135. Turko IV, Murad F. Protein nitration in cardiovascular diseases [Review]. Pharmacol Rev 2002;54:619–34.

136. Bhattacharjee S, Pennathur S, Byun J, Crowley J, Mueller D, Gischler J, et al. NADPH oxidase of neutrophils elevates *o,o'*-dityrosine cross-links in proteins and urine during inflammation. Arch Biochem Biophys 2001;395:69–77.

137. Manary MJ, Leeuwenburgh C, Heinecke JW. Increased oxidative stress in kwashiorkor. J Pediatr 2000;137:421–4.

138. Giulivi C, Traaseth NJ, Davies KJ. Tyrosine oxidation products: analysis and biological relevance [Review]. Amino Acids 2003; 25:227–32.

139. Malencik DA, Anderson SR. Dityrosine as a product of oxidative stress and fluorescent probe. Amino Acids 2003;25:233–47.

140. Orhan H, Coolen S, Meerman JH. Quantification of urinary *o,o'*-dityrosine, a biomarker for oxidative damage to proteins, by high performance liquid chromatography triple quadrupole tandem mass spectrometry: a comparison with ion-trap tandem mass spectrometry. J Chromatogr B 2005;827:104–8.

141. Orhan H, Vermeulen NP, Tump C, Zappey H, Meerman JH. Simultaneous determination of tyrosine, phenylalanine and deoxyguanosine oxidation products by liquid chromatography-tandem mass spectrometry as non-invasive biomarkers for oxidative damage. J Chromatogr B 2004;799:245–54.

142. 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;40:946–54.

143. Dalle-Donne I, Rossi R, Giustarini D, Milzani A, Colombo R. Protein carbonyl groups as a biomarkers of oxidative stress. Clin Chim Acta 2003;329:23–8.

144. Levine RL. Carbonyl modified proteins in cellular regulation, aging, and disease [Review]. Free Radic Biol Med 2002;32: 790–6.

145. Butterfield DA, Castegna A. Proteomics for the identification of specifically oxidized proteins in brain: technology and application to the study of neurodegenerative disorders [Review]. Amino Acids 2003;25:419–25.

146. 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;29:101–4.

147. Oberg BP, McMenamin E, Lucas FL, McMonagle E, Morrow J, Ikizler TA, et al. Increased prevalence of oxidant stress and inflammation in patients with moderate to severe chronic kidney disease. Kidney Int 2004;65:1009–16.

148. 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;3:375–82.

149. Dizdaroglu M, Jaruga P, Birincioglu M, Rodriguez H. Free radical-induced damage to DNA: mechanisms and measurement. Free Radic Biol Med 2002;32:1102–15.

150. Halliwell B. Effect of diet on cancer development: is oxidative DNA damage a biomarker? Free Radic Biol Med 2002;32:968–74.

151. Valko M, Izakovic M, Mazur M, Rhodes CJ, Telser J. Role of oxygen radicals in DNA damage and cancer incidence [Review]. Mol Cell Biochem 2004;266:37–56.

152. 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.

153. Alary J, Gueraud F, Cravedi JP. Fate of 4-hydroxynonenal in vivo: disposition and metabolic pathways [Review]. Mol Aspects Med 2003;24:177–87.

154. Okada K, Wangpoengtrakul C, Osawa T, Toyokuni S, Tanaka K, Uchida K. 4-Hydroxy-2-nonenal-mediated impairment of intracellular proteolysis during oxidative stress: identification of proteasomes as target molecules. J Biol Chem 1999;274:23787–93.

155. Meagher EA, Barry OP, Burke A, Lucey MR, Lawson JA, Rokach J, et al. Alcohol-induced generation of lipid peroxidation products in humans. J Clin Invest 1999;104:805–13.

156. Burke A, Lawson JA, Meagher EA, Rokach J, FitzGerald GA. Specific analysis in plasma and urine of 2,3-dinor-5,6-dihydro-isoprostane $F_{2\alpha}$-III, a metabolite of isoprostane $F_{2\alpha}$-III and an oxidation product of γ-linolenic acid. J Biol Chem 2000;275:2499–504.

157. Souza JM, Choi I, Chen Q, Weisse M, Daikhin E, Yudkoff M, et al. Proteolytic degradation of tyrosine nitrated proteins. Arch Biochem Biophys 2000;380:360–6.

158. Mani AR, Pannala AS, Orie NN, Ollosson R, Harry D, Rice-Evans CA, et al. Nitration of endogenous *para*-hydroxyphenylacetic acid and the metabolism of nitrotyrosine. Biochem J 2003;374: 521–7.

159. Pannala AS, Mani AR, Spencer JPE, Skinner V, Bruckdorfer KR, Moore KP, et al. The effect of dietary nitrate on salivary, plasma, and urinary nitrate metabolism in humans. Free Radic Biol Med 2003;34:576–84.

160. Leeuwenburgh C, Hansen PA, Holloszy JO, Heinecke JW. Oxidized amino acids in the urine of aging rats: potential markers for assessing oxidative stress in vivo. Am J Physiol Regul Integr Comp Physiol 1999;276:128–35.

161. Leeuwenburgh C, Hansen PA, Holloszy JO, Heinecke JW. Hydroxyl radical generation during exercise increases mitochondrial protein oxidation and levels of urinary dityrosine. Free Radic Biol Med 1999;27:186–92.

162. Hazen SL, Crowley JR, Mueller DM, Heinecke JW. Mass spectrometric quantification of 3-chlorotyrosine in human tissues with attomole sensitivity: a sensitive and specific marker for myeloperoxidase-catalyzed chlorination at sites of inflammation. Free Radic Biol Med 1997;23:909–16.

163. Gilgun-Sherki Y, Rosenbaum Z, Melamed E, Offen D. Antioxidant therapy in acute central nervous system injury: current state [Review]. Pharmacol Rev 2002;54:271–84.

164. Gilgun-Sherki Y, Melamed E, Offen D. Antioxidant treatment in Alzheimer's disease: current state [Review]. J Mol Neurosci 2003;21:1–11.

165. Lonn E, Bosch J, Yusuf S, Sheridan P, Pogue J, Arnold JM, et al. HOPE and HOPE-TOO Trial Investigators. Effects of long-term vitamin E supplementation on cardiovascular events and cancer: a randomized controlled trial. JAMA 2005;293:1338–47.

166. Shigenaga MK, Lee HH, Blount BC, Christen S, Shigeno ET, Yip H, et al. Inflammation and NO_x-induced nitration: assay for 3-nitrotyrosine by HPLC with electrochemical detection. Proc Natl Acad Sci U S A 1997;94:3211–6.

167. Söderling AS, Ryberg H, Gabrielson A, Lärstad M, Torén K, Niari S, et al. A derivatization assay using gas chromatography/negative chemical ionization tandem mass spectrometry to quantify 3-nitrotyrosine in human plasma. J Mass Spectrom 2003;38:1187–96.

168. Aulak KS, Miyagi M, Yan L, West KA, Massillon D, Crabb JW, et al. Proteomic method identifies proteins nitrated in vivo during inflammatory challenge. Proc Natl Acad Sci U S A 2001;98:12056–61.

169. Goen T, Muller-Lux A, Dewes P, Musiol A, Kraus T. Sensitive and accurate analyses of free 3-nitrotyrosine in exhaled breath condensate by LC-MS/MS. J Chromatogr B 2005;826:261–6.

170. Kadiiska MB, Gladen BC, Baird DD, Graham LB, Parker CE, Ames BN, et al. Biomarkers of oxidative stress study III. Effects of the nonsteroidal anti-inflammatory agents indomethacin and meclofenamic acid on measurements of oxidative products of lipids in CCl_4 poisoning. Free Radic Biol Med 2005;38:711–8.

171. Kharitonov SA, Barnes PJ. Biomarkers of some pulmonary diseases in exhaled breath [Review]. Biomarkers 2002;7:1–32.

172. Rahman I, Kelly F. Biomarkers in breath condensate: a promising new non-invasive technique in free radical research [Review]. Free Radic Res 2003;37:1253–66.

173. Granger CB, Van Eyk JE, Mockrin SC, Anderson NL. National Heart, Lung, and Blood Institute Clinical Proteomics Working Group report. Circulation 2004;109:1697–703.

174. Vivekananthan DP, Penn MS, Sapp SK, Hsu A, Topol EJ. Use of antioxidant vitamins for the prevention of cardiovascular disease: meta-analysis of randomised trials. Lancet 2003;361:2017–23 [Erratum published in: Lancet 2004;363:662].

175. Tousoulis D, Antoniades C, Stefanadis C. Nitric oxide in coronary artery disease: effects of antioxidants [Review]. Eur J Clin Pharmacol Oct 11, 2005:1–7 [Epub ahead of print].

176. Kocyigit A, Keles H, Selek S, Guzel S, Celik H, Erel O. Increased DNA damage and oxidative stress in patients with cutaneous leishmaniasis. Mutat Res 2005;585:71–8.

177. Craghill J, Cronshaw AD, Harding JJ. The identification of a reaction site of glutathione mixed-disulphide formation on γS-crystallin in human lens. Biochem J 2004;379:595–600.

178. Ghezzi P, Romines B, Fratelli M, Eberini I, Gianazza E, Casagrande S, et al. Protein glutathionylation: coupling and uncoupling of glutathione to protein thiol groups in lymphocytes under oxidative stress and HIV infection. Mol Immunol 2002;38:773–80.

179. Hermo R, Mier C, Mazzotta M, Tsuji M, Kimura S, Gugliucci A. Circulating levels of nitrated apolipoprotein A-I are increased in type 2 diabetic patients. Clin Chem Lab Med 2005;43:601–6.

180. Ahmed N, Babaei-Jadidi R, Howell SK, Beisswenger PJ, Thornalley PJ. Degradation products of proteins damaged by glycation, oxidation and nitration in clinical type 1 diabetes. Diabetologia 2005;48:1590–603.

181. Lorch SA, Banks BA, Christie J, Merrill JD, Althaus J, Schmidt K, et al. Plasma 3-nitrotyrosine and outcome in neonates with severe bronchopulmonary dysplasia after inhaled nitric oxide. Free Radic Biol Med 2003;34:1146–52.

182. Woods AA, Linton SM, Davies MJ. Detection of HOCl-mediated protein oxidation products in the extracellular matrix of human atherosclerotic plaques. Biochem J 2003;370:729–35.

183. Yuan Z, Shioji K, Nimata M, Kishimoto C. Cardioprotective effects of carvedilol on acute autoimmune myocarditis. Mol Cell Biochem 2004;259:223–7.

184. Smirnova IV, Pall ML. Elevated levels of protein carbonyls in sera of chronic fatigue syndrome patients. Mol Cell Biochem 2003;248:93–5.

185. Rottoli P, Magi B, Cianti R, Bargagli E, Vagaggini C, Nikiforakis N, et al. Carbonylated proteins in bronchoalveolar lavage of patients with sarcoidosis, pulmonary fibrosis associated with systemic sclerosis and idiopathic pulmonary fibrosis. Proteomics 2005;5:2612–8.

186. Schaper M, Gergely S, Lykkesfeldt J, Zbaren J, Leib SL, Tauber MG, et al. Cerebral vasculature is the major target of oxidative protein alterations in bacterial meningitis. J Neuropathol Exp Neurol 2002;61:605–13.