



## FORUM REVIEW ARTICLE

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# Clinical Relevance of Biomarkers of Oxidative Stress

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### Abstract

**Significance:** Oxidative stress is considered to be an important component of various diseases. A vast number of methods have been developed and used in virtually all diseases to measure the extent and nature of oxidative stress, ranging from oxidation of DNA to proteins, lipids, and free amino acids. **Recent Advances:** An increased understanding of the biology behind diseases and redox biology has led to more specific and sensitive tools to measure oxidative stress markers, which are very diverse and sometimes very low in abundance. **Critical Issues:** The literature is very heterogeneous. It is often difficult to draw general conclusions on the significance of oxidative stress biomarkers, as only in a limited proportion of diseases have a range of different biomarkers been used, and different biomarkers have been used to study different diseases. In addition, biomarkers are often measured using nonspecific methods, while specific methodologies are often too sophisticated or laborious for routine clinical use. **Future Directions:** Several markers of oxidative stress still represent a viable biomarker opportunity for clinical use. However, positive findings with currently used biomarkers still need to be validated in larger sample sizes and compared with current clinical standards to establish them as clinical diagnostics. It is important to realize that oxidative stress is a nuanced phenomenon that is difficult to characterize, and one biomarker is not necessarily better than others. The vast diversity in oxidative stress between diseases and conditions has to be taken into account when selecting the most appropriate biomarker. *Antioxid. Redox Signal.* 23, 1144–1170.

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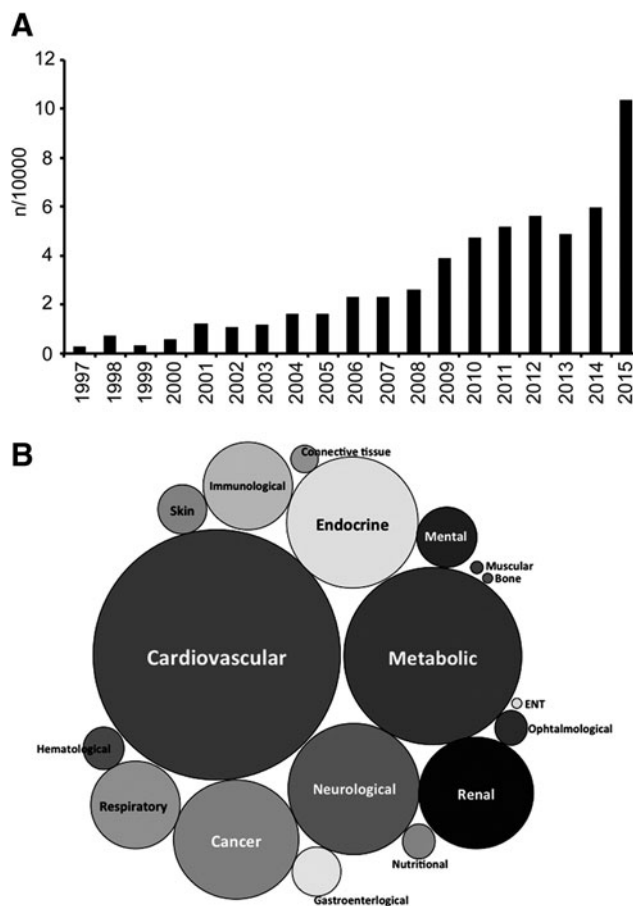
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## Introduction

**R**EDOX SIGNALING OPERATES through discrete, reversible, and site-specific modifications of certain proteins (184). Reversible modifications of cysteine residues include S-nitrosylation/S-nitrosation, sulfenylation, disulfide bonds, and S-glutathionylation (83, 87). These redox signaling-induced changes, performed by reactive oxygen and nitrogen species (ROS and RNS), target protein activities within complex networks of kinases, phosphatases, ion channels, and apoptotic cascades and can cause changes in transcriptional activity (50, 53, 74, 83). Oxidative stress, characterized by an imbalance between oxidants and antioxidants in favor of oxidants, leads to disruption of redox signaling and physiological function (157, 158). Oxidative stress might also lead to irreversible chemical modifications (152, 164). One of the weaknesses of the underpinning science is the lack of validated oxidative stress biomarkers. Analytical issues surrounding the validation of oxidative stress biomarkers have received substantial attention recently (33, 100). However, there remains increasing interest in exploring their potential clinical applications. While the current review will touch upon analytical issues, its focus will be on synthesizing the status—and future potential—of oxidative stress biomarkers as clinical diagnostics from available literature.

The World Health Organization has defined a biomarker as any substance, structure, or process that can be measured in the body or its products and influence or predict the incidence of outcome or disease (192). Markers of oxidative stress often fulfill the first part of the criteria (*i.e.*, they can be measured) and many studies suggest oxidative stress can influence the disease, but to be a *clinically relevant* biomarker, some additional issues must also be addressed. In summary, a clinically useful biomarker must be able to meet one of the following criteria: (i) show specificity for a certain disease (diagnostic), (ii) have prognostic value, and (iii) correlate with disease activity. This then allows treatment efficacy to be assessed. To be clinically useful, a biomarker must also be reasonably stable, present in an easily accessible tissue, and cost-effective to measure reproducibly on a large scale.

An increasing number of studies are published on markers of oxidative stress in a whole range of human diseases (Fig. 1). While a plethora of markers and methods are used, many of these do not correlate well with each other, do not reflect a state of oxidative stress, or are not specific. In this study, we critically review the current state of oxidative stress biomarkers that are used to assess the redox state of the body or specific tissues and cells in health and disease, with a focus on those that can be realistically applied to the clinic (Fig. 2). This approach excludes by definition several commonly used preclinical and *in vitro* methods. Visualization of biomarkers measured in various diseases by cluster analysis (Fig. 3) shows that the majority of studies have used ROS-induced modifications as markers of oxidative stress, which will be discussed first. We then focus on biomarkers assessing two important components whose deregulation can result in oxidative stress, ROS generation, and antioxidant defense. We conclude with two functional markers that are downstream of oxidative stress. From a clinical perspective, what matters is which marker is predictive with respect to risk and therapeutic outcome.



**FIG. 1. Publications on oxidative stress biomarkers in different diseases.** Searches were performed using oxidative stress biomarkers patients and the specific disease MeSH term using Web of Science. (A) Indicates the number of hits of all diseases combined per 10,000, normalized to a search with patients and the diseases in question. (B) Shows the number of hits per disease, which is proportional to the circle size, for the years 2005–2015.

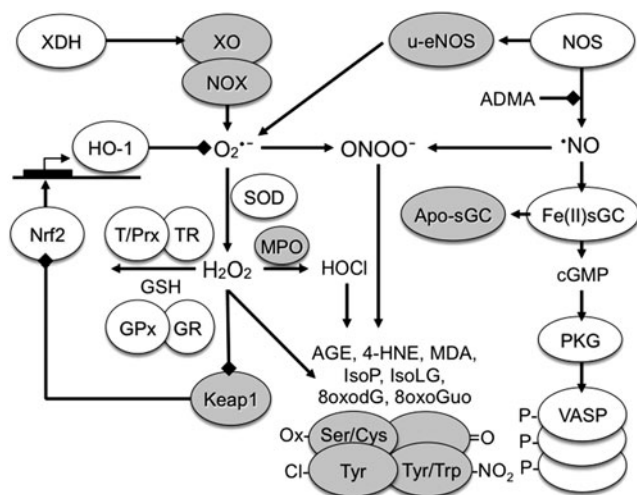
## ROS-Induced Modifications

This category includes biomarkers measuring evidence of direct chemical impact of ROS in biological systems. One of the ROS subsets is also described as RNS, for example,  $\cdot\text{NO}$  and  $\text{ONOO}\cdot$ . Besides causing post-translational modifications of proteins, these species may also cause nitrate stress and RNS-induced modifications, such as tyrosine nitration.

### Protein carbonyls and advanced glycation end products

Protein carbonyls are formed through oxidative cleavage of protein backbones. Oxidative deamination of lysine and glutamic acid also results in protein carbonyls (34). Since carbonyls can arise from different mechanisms, their concentration is commonly higher than that of other biomarkers (40).

Carbonyl groups may also be introduced by binding of aldehydic lipid oxidation products to lysine, cysteine, and histidine residues—a reaction termed Michael addition—resulting in advanced lipoxidation end products. Reactions



**FIG. 2. Redox pathways associated with putative biomarkers of oxidative stress.** The processes that lead to oxidative modifications of proteins, lipids, and nucleotides are highly complex. Enzymes, such as XO, NOX, and NOS, can produce ROS and RNS. These ROS can furthermore serve as substrates for other enzymes to generate additional types of ROS, such as the generation of HOCl from H<sub>2</sub>O<sub>2</sub> by MPO. Cellular systems and enzymes, including the GSH and thioredoxin system, together with peroxiredoxins (T/Prx), counterbalance the production of ROS. In addition, increased levels of ROS activate Nrf2 to transcribe genes that are involved in counteracting these ROS. Oxidative stress affects cGMP signaling through its effects on nitric oxide (\*NO) production, scavenging, and on the \*NO receptor sGC. cGMP, cyclic guanosine monophosphate; GSH, glutathione; H<sub>2</sub>O<sub>2</sub>, hydrogen peroxide; HOCl, hypochlorous acid; MPO, myeloperoxidase; NOS, nitric oxide synthase; NOX, NADPH oxidase; RNS, reactive nitrogen species; ROS, reactive oxygen species; sGC, soluble guanylate cyclase; XO, xanthine oxidase.

between lysine and arginine residues and carbohydrates—a reaction called glycoxidation—result in advanced glycation end products (AGEs).

AGEs are a group of heterogeneous molecules that arise from the nonenzymatic reaction of reducing sugars with amino groups of lipids, DNA, and especially long-lived proteins. This process occurs during normal metabolism, but is even more pronounced under hyperglycemic, hyperlipidemic, and oxidative stress conditions.

The glycation reaction can be accompanied by an oxidation leading to glycoxidation products. Carboxymethyl valine and pentosidine are among the most prominent AGEs resulting from glycoxidation. Glyoxal, generated from metal-catalyzed oxidation of polyunsaturated fatty acids (PUFAs), forms adducts with lysine (resulting in carboxymethyl lysine [CML]), an advanced lipoxidation product (55). About 90% of CML and pentosidine in blood are bound to proteins (116). Due to their relationship to sugars, AGEs have been linked to diabetes mellitus and other diseases, such as obesity (20), atherosclerosis, renal failure (193), and Alzheimer's disease (172). Due to the different possible formation mechanisms and heterogeneity, numerous glycation products exist, of which only some have been characterized so far.

Protein carbonyls (*i.e.*, having aldehyde and ketone moieties) are usually detected after derivatization with

2,4-dinitrophenylhydrazine (DNP). The resulting carbonyl-2,4-dinitrophenylhydrazine adduct (101) can be detected spectrophotometrically or by specific anti-DNP antibodies with ELISA (24), Western blot (91), immunohisto- and cytochemistry, or by high-performance liquid chromatography (HPLC). The results of the ELISA correlate well with the colorimetric assay (24), whereby the ELISA is more convenient to analyze a larger number of samples within one run and requires significantly less sample volume. Concerning clinical settings, the only methods that seem to be applicable are ELISA (kits are available) and HPLC as they enable high throughput, involve internal/external standards, and comparison of samples under constant conditions.

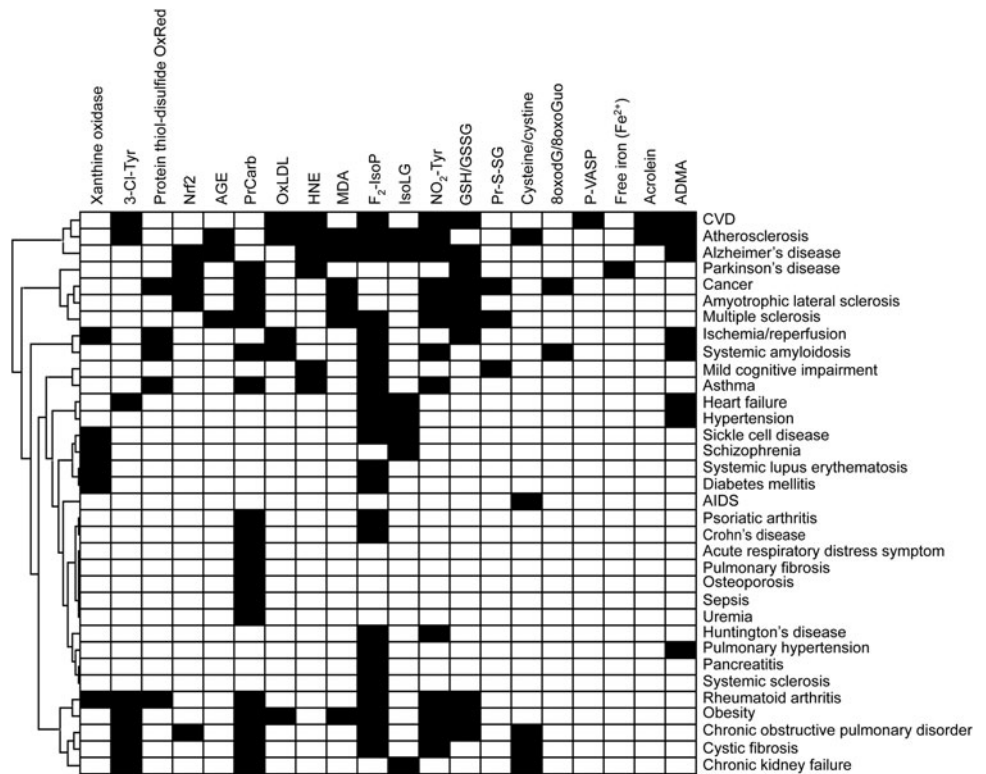
A number of methods have been reported to measure AGEs based on the use of antibodies for immunohistochemistry, immunoblot, and commercial ELISA, as well as special AGE readers that utilize the autofluorescence properties of AGEs in human skin to assess AGE concentrations. Spectrofluorometry can be applied to diluted plasma or serum samples and a fructosamine assay to detect ketoamines (9). HPLC allows the identification and measurement of specific AGEs such as pentosidine (169) and CML (52). Creatinine glycation products can be measured with stable isotope dilution analysis and liquid chromatography (LC)-MS/MS (97). Due to the structural heterogeneity of AGEs, there is no method that can be especially recommended for measuring specific AGEs in a clinical setting. Noninvasive spectrographic autofluorescence readers can be applied in a clinical setting; however, this should be standardized in terms of using the average of three readings, the same body region, avoiding surrounding light and skin areas with tattoos. Elevated skin autofluorescence has been demonstrated in diabetes, kidney disease, and in patients with arterial stiffness.

In humans, elevated protein carbonyl levels have been reported in numerous conditions, including aging (61), neurodegenerative diseases (62), obesity, diabetes mellitus, age-related macular degeneration (174), human immunodeficiency virus (HIV), anemia, sickle cell disease, newborn bronchopulmonary dysplasia, and hepatocellular carcinoma (Table 1).

Protein carbonyls increase with age in healthy women and men (61, 122). With age, AGEs accumulate in the skin and correlate with the glucose exposure dose in patients on peritoneal dialysis (25). In diabetes, ROS are generated through several pathways, and elevated AGE concentrations have been reported. Ischemia/reperfusion is clearly associated with oxidative stress. Following coronary surgery in the reperfused human heart, a 2–3-fold increase in protein carbonyls, as measured by ELISA, was observed in plasma isolated from the venous coronary sinus (130). Protein carbonyls remained increased in blood for up to 18 h and therefore meet one important criterion for being a marker of oxidative stress, which is their stability.

Most methods detect protein carbonyls after derivatization and therefore do not provide a direct measure of these oxidative modifications. While commercial ELISA kits for AGE measurement provide ease of use, many of these do not specify the antibody used, which is just described as polyclonal anti-AGE antibody. This may lead to differences between commercial kits. Nevertheless, protein carbonyls and AGEs have been among the most successful markers of

**FIG. 3. Cluster analysis of ROS biomarkers in disease.** Different diseases were clustered according to described ROS biomarkers in Refs. (33, 100, 181) and studies described in this review. Some disease conditions cluster as might be expected, such as ischemia/reperfusion and heart failure, and amyotrophic lateral sclerosis and multiple sclerosis. A comprehensive analysis of ROS markers and pattern analysis in diseases might uncover common disease mechanisms or new measures of disease progression or treatment outcome. Cluster analysis was performed using Genesis software ([https://genome.tugraz.at/genesisclient/genesisclient\\_description.shtml](https://genome.tugraz.at/genesisclient/genesisclient_description.shtml)) as described in Mengozzi *et al.* (111).



oxidative stress and are associated with disease state and treatment in multiple diseases (Tables 1 and 2).

*Oxidized low-density lipoprotein*

The measurement of oxidized low-density lipoprotein (oxLDL) as a biomarker of oxidative stress has its origin in

the oxidative modification hypothesis of atherosclerosis (165). However, oxLDL does not chemically define a specific form of modified LDL, molecule, or family of molecules (166). As the potential of oxLDL as a biomarker for cardiovascular disease (CVD) has been the subject of previous reviews (175, 177), we will critically assess the clinical utility of oxLDL as a biomarker.

TABLE 1. SELECTED CLINICAL STUDIES OF PROTEIN CARBONYL LEVELS IN DIFFERENT DISEASES

Disease/Condition	Sample	Method	Observation	Reference <sup>a</sup>
HIV-associated cognitive impairment	Cerebrospinal fluid	Commercial Oxyblot kit	No significant differences in PC between treatment and placebo after selegiline transdermal system <i>versus</i> placebo.	(85)
Chronic heart failure, secondary to ischemic or idiopathic cardiomyopathy	Plasma	Spectrophotometry	Significantly decreased PC after treatment with darbepoetin alfa (placebo: no effect).	(75)
Sickle cell disease	Plasma	Spectrophotometry	$\alpha$ -Lipoic acid treatment in healthy subjects reduced PC ( $P < 0.05$ ), no effect in sickle cell disease.	(64)
Newborns (low birth weight), without bronchopulmonary dysplasia	Plasma	ELISA	No significant difference in PC between newborns and controls treated with inhaled nitric oxide. Positive correlation between PC and respiratory severity score.	(8)
Hepatocellular carcinoma	Plasma	ELISA	Significantly higher PC in subjects with hepatocellular carcinoma compared with subjects with or without Hepatitis B virus.	(61)

<sup>a</sup>References are provided as supplementary material (Supplementary Data are available online at [www.liebertpub.com/ars](http://www.liebertpub.com/ars)). HIV, human immunodeficiency virus; PC, protein carbonyl.

TABLE 2. SELECTED CLINICAL STUDIES OF AGE LEVELS IN DIFFERENT DISEASES

<i>Disease/Condition</i>	<i>Sample</i>	<i>Method</i>	<i>Observation</i>	<i>Reference</i> <sup>a</sup>
T2D	Urine, plasma	Stable isotope dilution analysis and LC-MS/MS	Higher basal level in diabetics.	(57)
T2D	Skin	SAF	Short-term therapy with benfotiamine did not significantly influence SAF.	(97)
T1D, T2D	Skin	SAF	Comparable SAF between children with type I diabetes and nondiabetic adults, indicating chronological aging of the skin.	(88a)
Diabetes	EDTA plasma, carotid plaque tissue	ELISA, immunohistochemistry	Plasma sRAGE concentrations higher in symptomatic patients.	(10)
Acute coronary syndrome	Serum	Competitive ELISA	AGE decreased significantly with statin treatment, AGE levels correlated with plaque progression independently of diabetes.	(43)
Chronic kidney disease	Skin	SAF (AGE Reader)	Significantly higher SAF in children with kidney disease in comparison with controls; positive linear correlation between SAF and dialysis treatment duration.	(62)
Dialysis	Skin	SAF (AGE Reader)	Accumulation of tissue AGEs is correlated with glucose exposure dose and independently associated with cardiovascular morbidity in patients on peritoneal dialysis.	(50)
Pancreatic cancer (EPIC study)	Serum	ELISA	Elevated CML was associated with reduced pancreatic cancer risk (the association disappeared after adjustment for HbA1c, BMI, smoking status, and endogenous secreted RAGE).	(45)
Pancreatic cancer (ATBC study)	Serum	AGE CML-ELISA	Higher levels of CML-AGE were not associated with a higher risk of pancreatic cancer.	(51)
PCOS	Serum	Competitive AGE-ELISA	AGEs are elevated in women with PCOS and strongly positively correlated with androgen levels in women with PCOS.	(37)

<sup>a</sup>References are provided as supplementary material.

AGE, advanced glycation end product; BMI, body-mass index; CML, carboxymethyl lysine; LC, liquid chromatography; MS, mass spectrometry; PCOS, polycystic ovary syndrome; SAF, skin autofluorescence; sRAGE, secreted receptor for AGE; T1D, type 1 diabetes; T2D, type 2 diabetes.

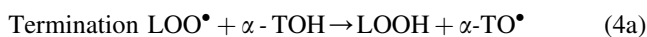
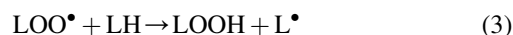
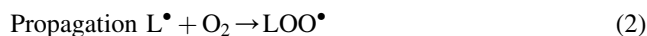
OxLDL is most commonly measured in plasma or isolated LDL by immunological methods using one of three different antibodies that appear most frequently in the literature: 4E6, DLH3, and E06. The monoclonal antibody, 4E6, binds to aldehyde-modified lysine residues on LDL (73) and is the basis of a commercial method. The monoclonal antibodies, DLH3 and E06, recognize oxidized phosphatidylcholine (82) and phosphorylcholine containing short oxidized or nonoxidized side chains, respectively. Plasma oxLDL has been consistently found elevated in patients with CVD, independent of the assay used. However, conflicting results have been reported in studies on the association of oxLDL with atherosclerosis severity and the usefulness of oxLDL for CVD prediction. Contrasting results, depending on the assay, have been reported for plasma oxLDL following pharmacological intervention with statins (175, 177). In addition to CVD, plasma oxLDLs are increased in patients with insulin resistance, diabetes, and obesity (175).

A general limitation of the single most commonly used 4E6-based assay is that native LDL is also detected. As a result, the concentrations of oxLDLs determined closely reflect the concentrations of LDL cholesterol, and the predictive value of the assays is dependent on the levels of apoB (194). This casts serious doubt over the usefulness of oxLDL as a measure of oxidative stress and its clinical utility to predict cardiovascular and associated diseases above that of LDL cholesterol. Another general problem is that results obtained with different antibodies/methods cannot be compared and often do not correlate with each other, which is inconsistent with oxLDL being a quantitative measure of oxidative stress or representing a meaningful tool to predict CVD. The DLH13-based method was developed for isolated LDL, which limits its clinical utility because LDL isolation is time-consuming and isolated LDL is prone to *ex vivo* oxidation when stored at 4°C or after coating on plates. An extension of this assay to plasma has been developed commercially, but its utility is questionable because plasma and isolated LDL data do not match (81). A major problem with E06-based methods to determine oxLDL is that contrary to the common notion (175, 177), the monoclonal antibody is not specific for oxidized (phospho)lipids (51) and most of the recognized antigens in plasma reside in lipoproteins other than LDLs (178).

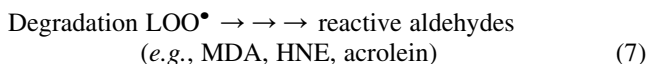
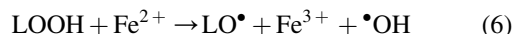
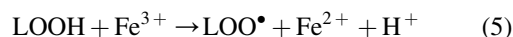
Given the limitations summarized above, oxLDL is unlikely a specific measure of oxidative stress. This is consistent with the majority of human plasma F<sub>2</sub>-isoprostanes (135a) and cholesterylester hydroperoxides (16) being associated with high-density lipoproteins (HDLs) rather than LDLs.

#### Lipid oxidation products

PUFAs, in particular linoleic and arachidonic acid (AA), are important targets of lipid peroxidation. Reaction of ROS, in particular hydroxyl and peroxy radicals, with bisallylic hydrogen of PUFAs initiates the autocatalytic chain reaction of lipid peroxidation (Reactions 1–3) during which lipid peroxy radicals act as chain-carrying radicals and lipid hydroperoxides are formed as the primary end products.



The chain reaction of lipid peroxidation can be terminated by tocopherols, such as  $\alpha$ -tocopherol ( $\alpha$ -TOH), *via* reaction with the chain-propagating lipid peroxy radical ( $\text{LOO}^\bullet$ ) (Reaction 4a). The resulting  $\alpha$ -tocopherol radical ( $\alpha\text{-TO}^\bullet$ ) can be reduced back to  $\alpha$ -TOH by certain reducing agents (*e.g.*, ascorbate) (not shown) or react with a second molecule of  $\text{LOO}^\bullet$  to form a nonradical product (NRP) (Reaction 4b).



In the presence of transition metals, lipid hydroperoxides (LOOHs) give rise to  $\text{LOO}^\bullet$  (Reaction 5) or lipid alkoxy radicals ( $\text{LO}^\bullet$ ) (Reaction 6) that are further cyclized and/or degraded into several different reactive aldehydes (Reaction 7) that participate in various biological processes and signal transduction pathways (200). Depending on the type of PUFAs undergoing lipid oxidation, these include *trans*-4-hydroxy-2-nonenal (4-HNE), malondialdehyde (MDA), and others. Aldehydes derived from lipids are very reactive and therefore readily react with proteins to form Michael adducts, often denoted as advanced lipoxidation end products (49).

Isoprostanes (IsoPs) are formed during free radical-catalyzed oxidation of AA (119). Radical-mediated oxidation of AA occurs independently of whether it is esterified (as in phospholipids, triacylglycerols, or cholesterylestes). The types of IsoPs that will be formed and in what ratio depend on oxygen tension and glutathione concentration (120).

#### 4-HNE and MDA

Different methods are available for the detection of both MDA and 4-HNE. Antibodies against MDA and 4-HNE bound to different amino acids have been developed (179, 186) and used in qualitative and semiquantitative immunocyto- and immunohistochemistry, where their presence and relative abundance are detected in cells and tissues in parallel with evaluation of morphological changes (126). Since Spittler (163) reviewed the involvement of lipid peroxidation in a variety of chronic diseases, lipid oxidation end products emerged as oxidative stress markers, with 4-HNE and MDA being among the most investigated (126, 200) (Table 3).

TABLE 3. SELECTED CLINICAL STUDIES ON 4-HNE, MDA, AND F<sub>2</sub>-ISOPROSTANE LEVELS IN DIFFERENT DISEASES

Disease	Marker	Sample	Method	Conclusions	Reference <sup>a</sup>
Cardiovascular Ischemic stroke (IS), post-stroke epilepsy (PSE)	HNE	Plasma	ELISA	HNE increased in IS and higher in patients with rs671 A allele (ALDH2).	(112)
	HNE, MDA, lipid hydroperoxides	Plasma	HPLC	Slight increase in infarction.	(54)
Acute myocardial infarction	MDA, IsoP	Plasma, RBC	HPLC	Hypertension correlates with RBC MDA and plasma IsoP levels.	(80)
Hypertension	F <sub>2</sub> -IsoP	Plasma	GC-MS	Correlation with decreased dynamic lung compliance.	(5)
Cardiopulmonary bypass	F <sub>2</sub> -IsoP	Urine	GC-MS	Independent risk marker for CHD.	(59, 87, 102, 105)
Coronary heart disease	F <sub>2</sub> -IsoP	Plasma, urine	GC-MS, ELISA	Higher compared with controls. Lowered by statins.	(33, 36, 58)
Hypercholesterolemia	F <sub>2</sub> -IsoP	Plasma, urine	GC-MS, ELISA	Higher in pulmonary and essential hypertension. Lowered by ATIR antagonists.	(107)
Hypertension	F <sub>2</sub> -IsoP	Plasma, urine	GC-MS, ELISA	Higher in pulmonary and essential hypertension. Lowered by ATIR antagonists.	(107)
Gastrointestinal system	HNE	Tissue	IHC	HNE associated with chronic mucosae alterations due to <i>H. Pylori</i> .	(27, 113)
Duodenal peptic ulcer	F <sub>2</sub> -IsoP	Urine	GC-MS	Higher in patients.	(30)
Crohn's disease	MDA, HNE	Tissue	HPLC, IHC	HNE and MDA higher in tumor tissue. IHC can distinguish healthy and tumor tissue.	(11, 94)
	Acrolein	Tissue	HPLC	Indicative of transformation benign to malignant phenotype.	(115)
Cancer	F <sub>2</sub> -IsoP	Urine	LC-MS/MS	No correlation with occurrence of colorectal adenomatous polyps.	(92)
	MDA	Plasma	HPLC	Presurgery MDA prognostic for tumor recurrence.	(39, 49)
Breast	HNE	Plasma	ELISA	HNE-modified PDGF was altered in plasma samples HNE modification of specific proteins.	(52)
	F <sub>2</sub> -IsoP	Urine	GC-MS	F <sub>2</sub> -IsoP not different between patients and controls. In women with BMI >29, high F <sub>2</sub> -IsoPs are associated with a higher risk for breast cancer. For women with BMI <23, F <sub>2</sub> -IsoP levels inversely correlated with risk of breast cancer.	(32)

(continued)

TABLE 3. (CONTINUED)

Disease	Marker	Sample	Method	Conclusions	Reference <sup>a</sup>
Prostate	Acrolein	Tissue	IHC	Predictive of tumor relapse in combination with clinical parameters.	(31)
	F <sub>2</sub> -IsoP	Urine	Radioimmunoassay; GC-MS	Conflicting results (either no correlation or positive correlation with risk of prostate cancer).	(9, 18, 44)
Brain	HNE	Tissue	IHC	In astrocytic and ependymal glial tumors, HNE is associated with the level of tumor malignancy.	(53, 114)
Lung	F <sub>2</sub> -IsoP	Urine	ELISA	Association with lung cancer risk in men.	(41)
Liver	F <sub>2</sub> -IsoP	Urine	ELISA	Association with risk of HCC.	(110)
Neurodegenerative diseases					
Parkinson's disease	MDA	Plasma	Fluorometric assay	Increased compared with healthy controls.	(90)
Alzheimer's disease	HNE	Plasma, CSF	Capillary GC/MS	Increased compared with healthy controls.	(88)
	HNE, MDA	Plasma	Colorimetric assay	HNE, but not MDA, is increased compared with healthy controls.	(65)
	Acrolein, HNE	Tissue	IHC	HNE and acrolein increased compared with healthy controls.	(20)
Amyotrophic lateral sclerosis	HNE	Serum	HPLC	HNE increased compared with healthy controls.	(93)
	MDA	Plasma	Fluorometric assay	MDA increased compared with healthy controls.	(15)
Mental					
Age-related cognitive impairment	F <sub>2</sub> -IsoP	CSF	GC-MS	Associated with poorer executive function.	(60)
Depression	F <sub>2</sub> -IsoP	Plasma, urine	GC-MS, ELISA	Treatment with bupropion or sertraline decreases symptoms, but increases F <sub>2</sub> -IsoPs.	(29, 40)
Multiple					
Down syndrome	F <sub>2</sub> -IsoP	Amniotic fluid	ELISA	Increased in pregnancies with Down syndrome fetuses.	(76)
Metabolic diseases					
Diabetes type 2	MDA	Serum	Colorimetric assay	High variability due to age.	(73)
Obesity	HNE	Plasma	HNE-His ELISA	HNE-His increased in obese men.	(108)
Otosclerosis	HNE	Tissue	IHC	HNE associated with the onset of otosclerosis.	(81)

<sup>a</sup>References are provided as supplementary material.

4-HNE, *trans*-4-hydroxy-2-nonenal; AT1R, angiotensin receptor 1; CHD, coronary heart disease; CSF, cerebrospinal fluid; GC, gas chromatography; F<sub>2</sub>-IsoPs, F<sub>2</sub>-isoprostanes; HCC, hepatocellular carcinoma; HPLC, high-performance liquid chromatography; IHC, immunohistochemistry; IS, ischemic stroke; MDA, malondialdehyde; PDGF, platelet-derived growth factor; PSE, post-stroke epilepsy; RBC, red blood cell.



Despite their widespread use, all methods that detect both MDA and 4-HNE have their pitfalls (162). In the thiobarbituric acid reactive substances (TBARS) assay, up to 98% of the measured MDA can be formed by the high-temperature conditions during the procedure itself (117). When combined with HPLC, MDA-TBA adducts in *in vitro* oxidized human plasma can be determined reproducibly and reliably, although this method requires individual sample processing and its validity as a marker of *in vivo* oxidative stress remains uncertain, making it less applicable for routine clinical use (19). The numerous commercial easy-to-use kits lack specificity and their significance for clinical research is questionable.

In general, direct MDA and 4-HNE measurement is insensitive as the vast majority of these reactive products are bound to proteins and other biomolecules and remain undetected unless released before the assay (49). To measure the presence of 4-HNE in biological samples, including protein-bound aldehydes, protein immunodetection is preferred, either applied as immunohistochemistry or as HNE-His ELISA (187). The specificity of the monoclonal antibodies against HNE-His adducts allows their use in human and animal tissues, tissue homogenates, and in plasma and serum samples (63, 126, 162).

### *F<sub>2</sub>-isoprostanes*

Several thorough reviews of the biochemistry and utility of *F<sub>2</sub>*-isoprostanes (*F<sub>2</sub>*-IsoPs) as biomarkers have been recently published (39, 113, 114), so only the most seminal points will be summarized here. Oxidation of AA forms a family of 64 bicyclic endoperoxide regio- and stereoisomers collectively termed *H<sub>2</sub>*-isoprostanes (140). Nonenzymatic rearrangement of these *H<sub>2</sub>*-isoprostanes forms both stable *F<sub>2</sub>*-IsoPs and highly reactive  $\gamma$ -ketoaldehydes termed isolevuglandins (IsoLGs, also known as isoketals) (115). Because of their chemical stability and sensitivity to changes in oxidative stress, *F<sub>2</sub>*-IsoPs are often considered the most reliable markers for monitoring oxidative stress *in vivo* (89). Elevated concentrations of *F<sub>2</sub>*-IsoPs are found in CVD, correlate with extent of disease, and predict the outcome (39). Elevated *F<sub>2</sub>*-IsoPs are also found in a wide range of human clinical conditions (113).

Despite strong evidence for their utility as biomarkers (Table 3), one challenge to widespread adaptation of *F<sub>2</sub>*-IsoPs in clinical trials is that the most reliable methods for their quantitation, gas chromatography–mass spectrometry (GC-MS) and LC-MS/MS, are labor-intensive and require specialized and expensive instrumentation (7, 114). While commercial immunoassays have been developed as a cheaper and easier alternative to mass spectrometry (MS), the results obtained with these immunoassays often do not correlate well with those obtained with GC-MS (78, 136). Thus, the results from immunoassays, particularly for individual patients, must be used with extreme caution, only with appropriate sample cleanup, and validated by MS whenever possible.

### *Isolevuglandins*

Similar to *F<sub>2</sub>*-IsoPs, IsoLGs are products derived from the oxidation of AA and are sensitive to changes in oxidative stress. While *F<sub>2</sub>*-IsoPs are stable products of lipid oxidation,

IsoLGs (Fig. 4) react rapidly and irreversibly with primary amines (*e.g.*, protein lysyl residues and phosphatidylethanolamine) in the cell to form pyrrole (lactam) and oxidized pyrrole (hydroxylactam) adducts (18, 115, 146). Thus, only IsoLG adducts, and not unreacted IsoLGs, are detected in cells and tissues.

IsoLG adducts may eventually prove to have greater utility as disease biomarkers than more generalized measures of oxidative stress status because they appear to directly participate in pathological processes. The biological effects of exogenous IsoLGs on cultured cells include induction of inflammatory pathways, immune responses, and cell death, as well as inhibiting ion channel function (17, 36, 56, 65, 95). These results, along with the therapeutic effects of administering small-molecule IsoLG scavengers in animal models, suggest that IsoLGs could contribute to disease processes, including inflammation, hypertension, arrhythmia, atherosclerosis, and neurodegeneration (38, 41, 95, 146).

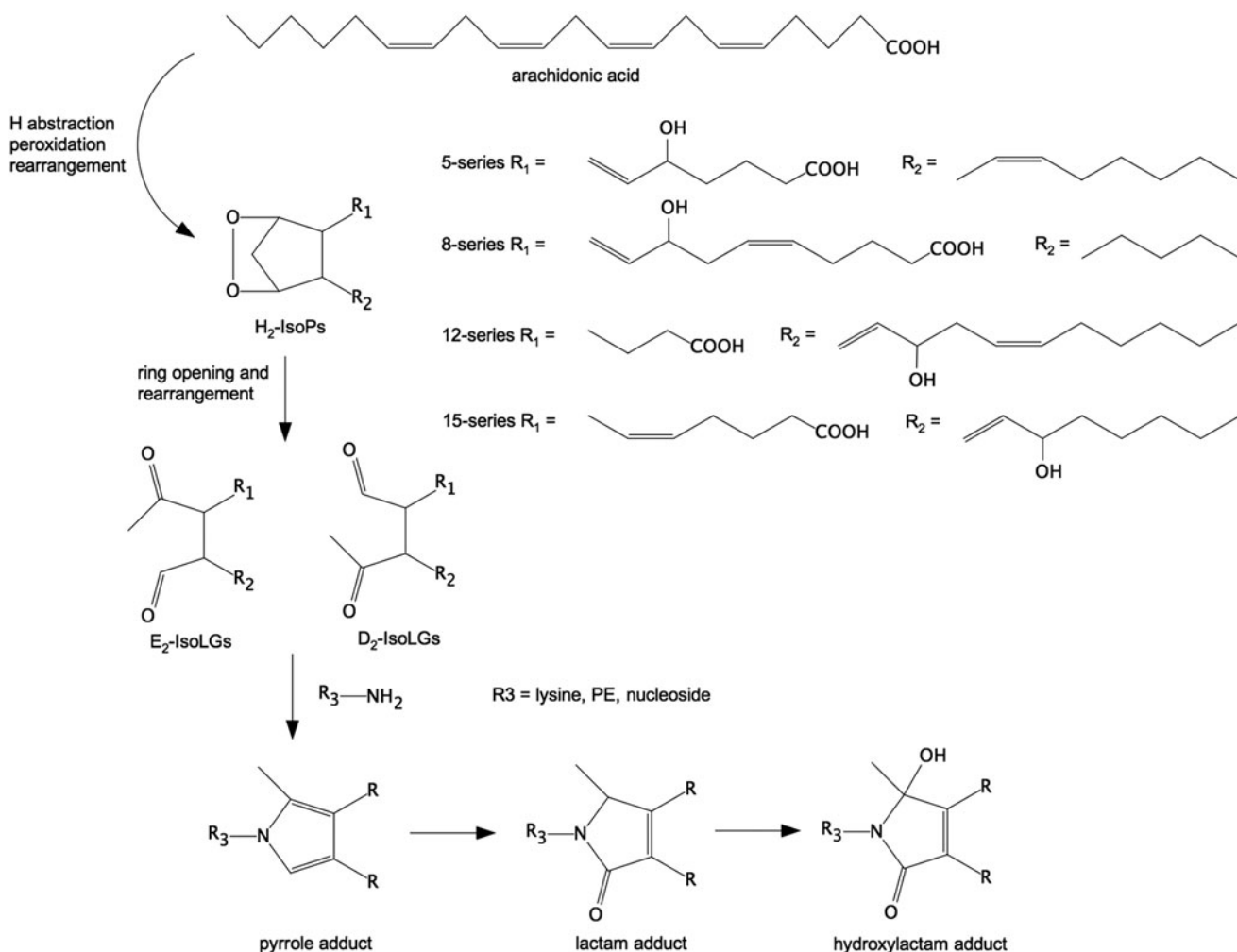
Quantitative immunoassays using polyclonal antibodies against IsoLG-protein adducts detected increased IsoLG-protein adduct formation in plasma from patients with atherosclerosis, in plasma from patients with end-stage renal disease, and in the glaucomatous trabecular meshwork (146). Immunohistochemical staining with the single-chain antibody D11ScFv that selectively recognized IsoLG-protein adducts showed increased adducts in the epicardial border zone of myocardial infarcts (56), in the hippocampus of Alzheimer's disease patients (38), and in heart, aorta, and dendritic cells during hypertension (64).

Mass spectrometric methods have demonstrated increased IsoLG-protein adducts compared with controls in the epicardial border zone of myocardial infarcts (56), in the hippocampus of Alzheimer's patients (38), and dendritic cells during hypertension (64). Using MS, IsoLG-phosphatidylethanolamine adducts have been found to be increased in plasma from patients with macular degeneration (102).

Currently, there are no published studies demonstrating that increased levels of IsoLG adducts predict onset or severity of subsequent disease. Therefore, the utility of measuring IsoLG adducts in urine or plasma as clinical biomarkers remains to be established. Nevertheless, current findings provide strong rationale for further investigation of the potential use of IsoLG adducts as clinical biomarkers, both to identify persons at risk and to determine the efficacy of treatments targeting IsoLGs such as dicarbonyl scavengers.

### *3-Nitrotyrosine*

Nitrotyrosine (Tyr-NO<sub>2</sub>) is often described as a stable marker of oxidative/nitrative stress in inflammatory diseases (71). Tyrosine nitration involves the replacement of C<sub>3</sub> hydrogen atom of the tyrosine aromatic ring with a nitro group (R-NO<sub>2</sub>) (11) (Fig. 5). This modification can occur within a polypeptide sequence (protein-associated Tyr-NO<sub>2</sub>) or to free tyrosine amino acids (free Tyr-NO<sub>2</sub>). Nitration can occur by several pathways *in vivo*, but always involves RNS and is usually a two-step process (161), in which (i) tyrosine is oxidized resulting in a tyrosine radical and (ii) a radical–radical reaction occurs between the tyrosine radical and nitrogen dioxide ( $\bullet$ NO<sub>2</sub>). It is possible for the tyrosine radical to react with nitric oxide ( $\bullet$ NO), followed by further oxidation to yield Tyr-NO<sub>2</sub>, but this pathway has not been well studied (11).



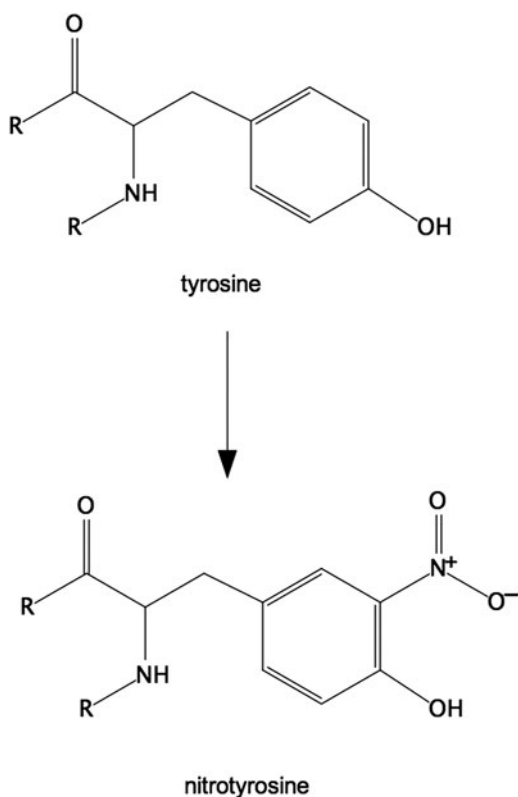
**FIG. 4. Regioisomers of isolevuglandins.** Specific IsoLG regioisomers differ by the relative orientation of their keto- and aldehyde moieties (D<sub>2</sub>-IsoLG vs. E<sub>2</sub>-IsoLG) and the position of the double bonds and hydroxyl group on the side chains (5-, 8-, 12-, or 15-IsoLG) (37, 141, 147, 148). Theoretical considerations from peroxidation chemistry suggest that the 5- and 15-IsoLG series should predominate over the 8- and 12-IsoLG series (198). It is important to recognize that one of the eight stereoisomers of both 15-D<sub>2</sub>-IsoLG and 15-E<sub>2</sub>-IsoLG is chemically identical to levuglandin D<sub>2</sub> and E<sub>2</sub>, respectively, which are generated nonenzymatically from prostaglandin H<sub>2</sub> (149, 150). IsoLG, isolevuglandins.

One widely studied pathway for nitration is the production of the RNS peroxynitrite (ONOO<sup>-</sup>) (Fig. 6, pathway 1) (132). Initially, Tyr-NO<sub>2</sub> was believed to be a specific marker of peroxynitrite-mediated damage, but this has since been disproved, with the most-cited alternate pathway involving myeloperoxidase (MPO), as proposed in 1997 (183) (Fig. 6, pathway 2). When solely measuring Tyr-NO<sub>2</sub> in complex biological samples, it is not possible to tell which of the mechanisms is responsible for any nitration detected *in vivo*. The effect of nitration on protein activity varies with the protein being studied and can be a gain of function (180) or loss of function (195).

The issue of which method to employ for the determination of Tyr-NO<sub>2</sub> in the high-throughput analysis of clinical samples needs to be addressed as MS, while considered the gold standard (176), is not yet feasible for high-throughput analysis and other methods suffer from methodological flaws or cannot be properly assessed due to a lack of detailed methodological information (45, 197). In addition, some authors

fail to clearly state whether free Tyr-NO<sub>2</sub>, protein-associated Tyr-NO<sub>2</sub>, or total Tyr-NO<sub>2</sub> is measured, the concentrations of which may be different. While evidence does suggest that nitration of certain proteins enhances proteolytic degradation (160), a fall in protein-associated Tyr-NO<sub>2</sub> concentration will only be measurable if the nitrated protein is degraded in parallel with a decrease in disease activity.

There is still much work to do in assessing the utility of Tyr-NO<sub>2</sub> as a clinical biomarker, but findings so far are encouraging, with some studies showing that plasma Tyr-NO<sub>2</sub> levels correlate with disease activity and decrease following successful therapeutic interventions. Yet, it is still unclear whether Tyr-NO<sub>2</sub> is any more informative, in clinical terms, than other already available markers, for example, C-reactive protein (CRP). CRP is an acute phase protein synthesized by the liver in response to signaling by upregulated inflammatory cytokines (*e.g.*, IL-6). Serum CRP is widely used clinically as a marker of acute inflammation, but of course an increase in serum CRP concentration is delayed until some hours after the



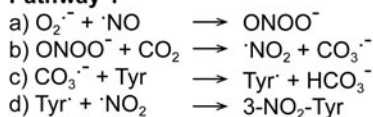
**FIG. 5. Structure of 3-nitrotyrosine.** Tyrosine nitration involves the replacement of the C<sub>3</sub> hydrogen atom of the tyrosine aromatic ring with a nitro group (R-NO<sub>2</sub>). The 3-nitrotyrosine is depicted as part of a polypeptide/protein.

initial inflammatory insult because liver synthesis of CRP protein is required. In contrast, chemical modifications to pre-existing cellular or extracellular proteins may occur more swiftly within an inflammatory environment. One application where the measurement of Tyr-NO<sub>2</sub> might provide a significant enhancement is in the monitoring of clinical responses to therapy in the assessment of the current pipeline of novel drugs targeted at enzymes involved in ROS generation, for example, NADPH oxidase and MPO inhibitors.

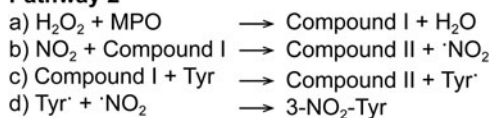
Tyr-NO<sub>2</sub> can be detected in tissues by various methods. Among the quantitative approaches, both ELISAs and LC/GC have been described; however, it is important to note that quantification by ELISAs is limited by several factors: (i) antibodies employed in ELISAs will have different affinities for different nitrated proteins (94), although this is addressed by methods that measure a single nitrated protein (188); (ii) some Tyr-NO<sub>2</sub> ELISAs may be prone to interference; and (iii) the sensitivity of some Tyr-NO<sub>2</sub> ELISAs may be too low to detect Tyr-NO<sub>2</sub> in healthy human plasma. LC/GC has been coupled to various detection systems, for example, UV detection, thermal energy analysis, electrochemical detection, and MS. One explanation for the lack of agreement between reported plasma ELISA- and MS-based Tyr-NO<sub>2</sub> plasma concentrations is that the reported ELISA results are based on a nitrated bovine serum albumin standard, in which the actual concentration of nitrated tyrosine residues is unknown.

The National Institute of Environmental Health Biomarkers of Oxidative Stress Study concluded that oxidized

#### Pathway 1



#### Pathway 2



**FIG. 6. Formation of nitrotyrosine.** In pathway 1, peroxynitrite is formed by the reaction of  $\cdot NO$  with the superoxide anion radical ( $O_2^{\bullet-}$ ). The enzymatic generation of both these radicals is increased during inflammation. Radical-radical combination of the two species occurs exceedingly fast (rate constant  $1 \times 10^{10} \text{ M}^{-1}\text{s}^{-1}$ ), meaning that  $\cdot NO$  can out-compete the dismutation of  $O_2^{\bullet-}$  by SODs (138). Under physiological conditions in which CO<sub>2</sub> is present, nitration *via* peroxynitrite is increased (3) due to the formation of the adduct  $ONOO^-$ . This adduct undergoes homolysis to the secondary free radicals, nitrogen dioxide ( $\cdot NO_2$ ) and carbonate anion radical ( $CO_3^{\bullet-}$ ) (132).  $CO_3^{\bullet-}$  is able to perform *step 1* of the nitration process by oxidizing tyrosine to tyrosine radical, which then reacts with the  $\cdot NO_2$ . In pathway 2, MPO catalyzes, in the presence of H<sub>2</sub>O<sub>2</sub> and nitrite (NO<sub>2</sub><sup>-</sup>), the production of both the tyrosine radical and  $\cdot NO_2$  (11, 132). CO<sub>2</sub>, carbon dioxide; ONOOCO<sub>2</sub><sup>-</sup>, nitrosoperoxocarbonate; SODs, superoxide dismutases.

proteins (of which Tyr-NO<sub>2</sub> was one of the measured biomarkers) are not suitable biomarkers of free radical damage caused by carbon tetrachloride treatment in rats (89). Nevertheless, many studies have investigated Tyr-NO<sub>2</sub> under inflammatory conditions as a marker of oxidative stress and have found plasma Tyr-NO<sub>2</sub> concentrations to be higher in patients in a variety of diseases (summarized in Table 4). Some of the clinically relevant findings include the association of higher Tyr-NO<sub>2</sub> levels with higher mortality in sepsis and a fall in plasma Tyr-NO<sub>2</sub> concentration, as well as a correlation with other markers following treatment with various anti-inflammatory drugs. In addition, two studies using different methodologies reported a lowering of plasma protein-associated Tyr-NO<sub>2</sub> concentrations upon drug treatment (statins in coronary artery disease and rosuvastatin in hypercholesterolemia). Tyr-NO<sub>2</sub> may not only be a potential biomarker of oxidative stress in diabetes patients (28, 29) but may also be a biomarker of disease progression/risk for CVD (11a). In asthmatic patients, Tyr-NO<sub>2</sub> staining in bronchial biopsies and measurement of Tyr-NO<sub>2</sub> by ELISA in exhaled breath condensates both showed a decrease of Tyr-NO<sub>2</sub> upon corticosteroid treatment. Conflicting results were obtained in neurodegenerative disorders; although Tyr-NO<sub>2</sub> has been reported at increased levels within brain tissue from Parkinson's (60) and Alzheimer's disease patients (167), free Tyr-NO<sub>2</sub> is not increased in the cerebrospinal fluid of Alzheimer's disease patients (143). Likewise, protein-bound Tyr-NO<sub>2</sub> is not increased in plasma of patients with dementia (44). In contrast, nitrated  $\alpha$ -synuclein was detected in peripheral blood mononuclear cells from Parkinson's disease patients, the concentrations of which were inversely correlated with the daily dosage of levodopa (Table 4).

TABLE 4. SELECTED CLINICAL STUDIES ON NITROTYROSINE LEVELS IN DIFFERENT DISEASES

<i>Disease/Condition</i>	<i>Sample</i>	<i>Method</i>	<i>Observation</i>	<i>Reference<sup>a</sup></i>
CAD	Plasma	LC-MS/MS	Reduction in protein-associated Tyr-NO <sub>2</sub> following rosuvastatin treatment.	(91)
CAD	Plasma	ELISA	Protein-associated Tyr-NO <sub>2</sub> is a robust predictor of CAD in patients with abnormal glucose tolerance.	(28)
Hypercholesterolemia	Plasma	ELISA	Reduction in protein-associated Tyr-NO <sub>2</sub> following statin treatment; this lowering effect was independent of the fall in lipid parameters and CRP.	(77)
Asthma	Bronchial biopsies	Immunohistochemical staining	Reduction of Tyr-NO <sub>2</sub> staining after treatment with inhaled glucocorticoid compared with before treatment.	(82)
Asthma	Exhaled breath condensates	ELISA	Protein-associated Tyr-NO <sub>2</sub> levels decrease following steroid treatment and increase again following a washout period.	(13)
Asthma	Exhaled breath condensates	ELISA	Protein-associated Tyr-NO <sub>2</sub> levels are higher in mild asthmatics who were not receiving steroid treatment than in patients with moderate/severe asthma who were receiving treatment.	(46)
Atherosclerosis	Coronary artery; plasma	Immunohistochemical staining; Western blot	Apo-AI nitration at tyrosine-166 leads to functional impairment and is increased in atherosclerotic coronary arteries compared with normal coronary arteries.	(38)
Pulmonary hypertension	Lung tissue	Immunohistochemical staining	Nitration of protein kinase G at tyrosine-247 inhibits its activity and is increased in the lung in patients with pulmonary hypertension compared with healthy controls.	(4)
Septic shock	Plasma	Amino acid analyzer	Concentrations of free Tyr-NO <sub>2</sub> are associated with prognosis in patients with septic shock, with higher levels observed in those who did not survive than those who did.	(72)
OA, RA	Serum, synovial fluid	HPLC	RA patients have detectable free Tyr-NO <sub>2</sub> in plasma and synovial fluid, but not healthy subjects or OA patients. Patients with lower RA disease stage had lower free Tyr-NO <sub>2</sub> .	(55)
OA, RA	Plasma, synovial fluid	LC-MS/MS	Increased levels of total Tyr-NO <sub>2</sub> and higher ratio of synovial/serum total Tyr-NO <sub>2</sub> in OA compared with healthy controls. Lowering of total Tyr-NO <sub>2</sub> concentrations after 6 months of anti-TNF treatment, which was associated with a change in the RA disease activity score.	(66)
OA	Serum	ELISA	Nitrated collagen concentrations are higher in patients with OA compared with healthy control participants. Concentration of collagen-associated Tyr-NO <sub>2</sub> correlated with the serum concentration of CRP.	(34, 79)
Celiac disease	Plasma	ELISA	Reduction in protein-associated Tyr-NO <sub>2</sub> concentrations following a gluten-free diet.	(96)
SLE	Serum	ELISA	Significantly higher levels of protein-associated Tyr-NO <sub>2</sub> in patients with a disease activity score greater than six (SLE Disease Activity Index).	(106)
Parkinson's disease	Peripheral blood mononuclear cells	ELISA	Observation of nitrated $\alpha$ -synuclein, the concentration of which was inversely correlated with daily dosage of levodopa.	(78)

<sup>a</sup>References are provided as supplementary material.

CAD, coronary artery disease; CRP, C-reactive protein; OA, osteoarthritis; RA, rheumatoid arthritis; SLE, systemic lupus erythematosus; TNF, tumor necrosis factor.

### Thiols

The main thiol compound in the body is the amino acid cysteine, which is susceptible to oxidation. Oxidation of a cysteine residue can change a protein's function. Thus, measuring the thiol status may represent a mechanism-based biomarker. Biologically occurring thiols include low-molecular-weight thiols (cysteine, GSH) and protein thiols.

The use of thiols as biomarkers is affected by a number of factors. In addition to specific limitations for those thiols that are only present intracellularly, a general problem is that oxidation products of protein cysteine residues are unstable. They can be easily reduced by other thiols and thus require immediate treatment with an alkylating agent to prevent further redox changes. A change in GSH levels might not be due to oxidative stress, but might reflect a nutritional/metabolic imbalance. Plasma GSH levels may also be affected by GSH transporters, while cellular mechanisms, such as nuclear factor (erythroid-derived 2)-like 2 (NRF2), counteract oxidative stress by increasing GSH synthesis. Finally, oxidized glutathione (GSSG) concentrations are very low and difficult to measure unless sensitive HPLC methods are used.

### Glutathione

The main nonprotein thiol is the tripeptide GSH. Since intracellular concentrations of GSH are high, in the millimolar range, it is an important component of antioxidant defense systems to scavenge ROS, which leads to GSSG. Oxidation of GSH is reversible as GSSG reductase and NADPH reduce GSSG back to two molecules of GSH.

In general, any condition associated with excessive ROS will decrease GSH levels or decrease the GSH/GSSG ratio. Within cells, GSH is present at millimolar concentrations, resulting in high GSH/GSSG ratios (>30) (76). The GSH/GSSG ratio in serum is substantially lower (~3). Whether this meaningfully reflects a cellular redox state is questionable (90) and it may not be a good indicator of oxidative stress (86). Thus, most studies measure erythrocyte GSH where GSH concentrations are high, but not necessarily a good indicator of oxidative stress across tissues. Furthermore, lower GSH levels may not necessarily be due to oxidation, but rather due to a consequence of lower cysteine levels (cysteine is the rate-limiting GSH precursor) due to nutritional deficiency. Nevertheless, many studies have measured plasma GSH/GSSG. Three meta-analyses confirmed a decrease in plasma GSH and an increase in plasma GSSG in patients with autism spectrum disorders (54) and lower plasma GSH levels in polycystic ovary syndrome (121), two conditions in which oxidative stress has been implicated (127, 144). It should be noted that these meta-analyses are based on studies where GSH/GSSG are measured by a variety of techniques, including enzymatic methods, HPLC with fluorometric, UV, or electrochemical detectors, and LC-MS/MS.

Several pathological conditions are associated with decreased GSH levels (6). In particular, studies on GSH in acquired immunodeficiency syndrome (AIDS) and other conditions have shown very clearly that rather than in plasma, GSH should be measured within cells by fluorescence activated cell sorting (72). GSH measurement is important to identify patients who may benefit from GSH repletion by GSH derivatives or precursors, for example, in clinical trials (6).

### Cysteine

Free cysteine is the main nonprotein thiol in plasma (86, 118). Studies have measured plasma cysteine (~10  $\mu$ M) and its disulfide, cystine (~40–50  $\mu$ M), in CVDs with varying results (43, 112). Cysteine is a semiessential amino acid and its requirement may increase following oxidative stress due to the consumption of GSH (6). Historically, one important condition associated with lower plasma cysteine is AIDS, originally reported by the group of Droge (46).

### Protein thiols and mixed disulfides

Protein cysteine residues can exist in many oxidation states (Fig. 7). Protein glutathionylation (mixed disulfides with GSH) received particular attention. Significant amounts of glutathionylated proteins are detected under normal conditions or following exposure to oxidants (58, 156). Most of the glutathionylated proteins are intracellular because GSH is predominantly present in the cytoplasm and intracellular proteins have cysteine residues predominantly in the chemically reduced state and thus are available to form mixed disulfides, in contrast to extracellular proteins where most cysteine residues are engaged in disulfide bridges.

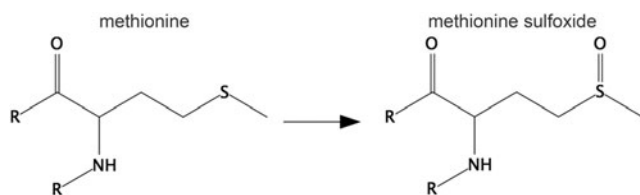
The only plasma protein identified as glutathionylated is transthyretin (58). Many studies propose glutathionylated hemoglobin, measured in red blood cells by MS, as a biomarker of oxidative stress in diabetes, hyperlipidemia, hemodialysis, and chronic renal failure (31, 58). An increase in plasma cysteinylated albumin, measured by MS, has also been reported in chronic liver and kidney diseases and diabetes (124).

### Surface thiols

The plasma membrane is the interface between the reducing intracellular and the oxidizing extracellular environments. While one might expect the extracellular (exofacial) membrane thiols to be oxidized, they are in fact not, and

-2	-SH	Free thiol (sulfhydryl)	
-1	-SS-	Disulfide	Protein-Protein (P-SS-P) Protein-Glutathione (P-SSG) Protein-Cysteine (P-SSC) <b>Reversible oxidation</b>
0	-SNO -SOH	S-nitrosothiol Sulfenic acid	<b>Reversible oxidation</b>
+2	-SO <sub>2</sub> H	Sulfinic acid	<b>Reversible oxidation*</b>
+4	-SO <sub>3</sub> H	Sulfonic acid	<b>Irreversible oxidation</b>

**FIG. 7. Protein cysteine oxidation states.** Cysteine residues in proteins can exist in different oxidation states, ranging from reduced free thiols to reversible oxidized forms (disulfides, S-nitrosothiols, sulfenic acids, and sulfinic acids) to irreversible sulfonic acids. \*Reversibility of protein cysteine sulfinic acids has so far been demonstrated only for some sulfinylated peroxiredoxins and requires the enzymatic activity of sulfiredoxin.



**FIG. 8. Structure of methionine sulfoxide.** Methionine contains a sulfur atom that is also susceptible to oxidation and can give rise to methionine sulfoxide. The methionine sulfoxide is depicted as part of a polypeptide/protein.

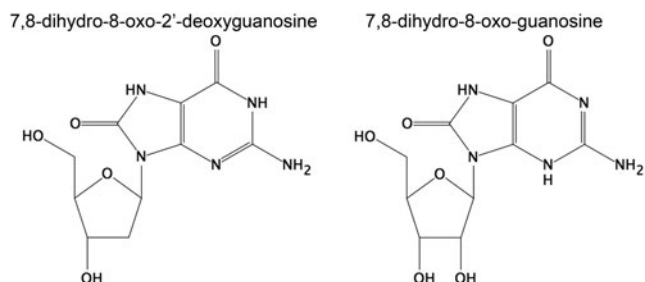
active mechanisms maintain specific surface thiols (98), with surface thiols lower in rheumatoid arthritis (RA) (131). Their measurement may provide additional information on the redox state of a patient (145).

#### Methionine sulfoxide

Methionine is the other sulfur-containing amino acid beside cysteine (Fig. 8). Sulfur in methionine can be reversibly oxidized by ROS to a sulfoxide. Oxidation of an essential methionine in the abundant serum protein  $\alpha$ -1-proteinase inhibitor leads to its inactivation (85). Elevated levels of this sulfoxidized form have been detected in the bronchoalveolar lavage of smokers, contributing to the pathogenesis of emphysema (27). The presence of methionine sulfoxide in plasma proteins and in HDL is increased in sepsis and diabetes (5, 21). Although not as widely studied as a form of thiol oxidation, methionine sulfoxide has potential advantages as a biomarker: it is easily measured with a conventional amino acid analyzer and is more stable than thiol oxidation products.

#### DNA/RNA oxidation

Oxidative stress induces oxidation of DNA and RNA (Fig. 9), particularly in the guanine moiety. The oxidized nucleosides are excreted into the urine and their measurement can be interpreted as the cumulative total body oxidative stress, that is, number of hits to the nucleic acids in a defined time period, meaning the global rate of DNA and RNA oxidation. Therefore, as urinary biomarkers, they are most relevant to conditions where oxidative stress occurs in all tissues in the body and less to high oxidative stress in minor organs without assumed systemic oxidative stress. Several commercial assays are available to measure 7,8-dihydro-8-oxo-2'-



**FIG. 9. Structure of 8-oxo-2'-deoxyguanosine and 8-oxo-guanosine.** Oxidation of DNA and RNA commonly occurs in the guanosine moiety, leading to 8-oxo-2'-deoxyguanosine and 8-oxo-guanosine, respectively.

deoxyguanosine (8oxodG) with ELISA. However, the clinical significance of these methods has been questioned (10). While chromatography coupled to MS may not be readily available clinically, biomarkers for oxidative stress measured by oxidation of nucleic acids are among—if not the best—biomarkers that have been examined. Nucleic acid oxidation products have also been demonstrated to be predictive of the development of disease (22, 23).

The oxidative modification in DNA can cause mispair and thereby lead to mutations, particularly GC-TA transversion mutations, and therefore relates to cancer (104, 134). Oxidative lesions in DNA are recognized by repair enzymes; the nucleotide pool can be oxidized, but is sanitized by other enzyme systems (133). There is some debate as to whether the lesions in DNA relate to incorporation from the nucleotide pool or direct oxidation in DNA (70). Chronically high oxidation of DNA, measured as urinary excretion of the nucleoside 8oxodG, is associated with risk of lung and breast cancer (103, 105).

Recently, RNA oxidation, measured as 7,8-dihydro-8-oxoguanosine (8oxoGuo), has been introduced as a marker in relation to diseases, particularly neurodegenerative diseases and diabetes (22, 23, 88). Because of the single strand nature of RNA, repair is not possible. Remarkably, relatively little is known about how RNA integrity is maintained, but it is assumed to rely on quality control and degradation (133). The cellular effects of RNA oxidation also remain largely obscure, although formation of truncated or mutated proteins has been suggested (133, 135). There are indications of formation of mutated proteins (170) and of microsomal stalling induced by oxidized RNAs (159). Very recently, advanced methodology has demonstrated that the effects of RNA lesions fall into two categories, one that includes ribosomal stalling and one that leads to a mixture of full length and truncated translational products (26). It thus appears that nucleic acid oxidation/modification has much more diverse and multifaceted biological effects, exemplified both with different effects on translation stalling and also in the target molecule, for example, in diabetes where RNA oxidation is not only more pronounced than DNA oxidation but also has a very different prognostic value.

Extensive DNA oxidation is predictive for the risk of breast and lung cancer (103, 105). Elevated RNA oxidation is predictive for development of complications and death in type 2 diabetes, and there are indications that high RNA oxidation is associated with breast cancer development in type 2 diabetic females (22). Thus, screening for urinary DNA/RNA oxidation could help to identify such people and patients at risk and help to implement a treatment plan to minimize it.

For measurement of 8oxodG and 8oxoGuo in urine, the most reliable methodology is chromatography coupled with MS (189–191). 8oxodG can also be measured by HPLC-electrochemical detection, which is rarely used presently.

#### Markers of ROS Generation

Some ROS-forming enzymes that are normally present intracellularly can also be found in the circulation, independently of the mechanism responsible for their release. For this reason, we will only describe xanthine oxidase (XO) and MPO. Higher circulating levels of XO and MPO

could potentially result in increased ROS production, although this depends on other factors such as availability of the substrate (xanthine for XO and  $H_2O_2$  for MPO) and whether ROS produced by these enzymes overcome the antioxidant defense. In some cases, a better indicator of the enzyme activity *in vivo* is the formation of the metabolite or reaction product.

#### Xanthine oxidase

XO catalyzes the oxidation of xanthine to uric acid. While the product is a known antioxidant (4), the enzyme is also a well-known source of  $O_2^{\cdot-}$  (109). Inflammatory agents and interferon increase XO activity and its plasma levels (59). However, the most important translational breakthrough was the hypothesis of the role of XO in ischemia–reperfusion injury (108). This led to several, ongoing clinical trials with XO inhibitors in CVD and prompted many studies to measure circulating XO (12). It should be mentioned that XO inhibition has other effects than inhibiting ROS production. In particular, by decreasing uric acid, it may improve CVD by lowering hyperuricemia (14), and uric acid is not only an antioxidant (4) but also proinflammatory through activation of the NALP3 inflammasome (107).

While we list XO among the ROS-generating enzymes, it could also be an indicator of oxidative stress. In fact, the protein exists in two forms, an oxidase (that oxidizes xanthine to uric acid using oxygen as the electron acceptor and produces  $H_2O_2$ ) and a dehydrogenase (that carries out the same reaction, but uses  $NAD^+$  and generates NADH). The dehydrogenase form can be converted into XO by, among other things, thiol oxidation (48). Thus, oxidative stress will increase XO activity by increasing dehydrogenase-to-oxidase conversion.

#### Myeloperoxidase

MPO is a heme peroxidase that catalyzes the reaction between  $H_2O_2$  and chloride ions to produce HOCl as the primary oxidant. These are not only important in the innate immune system's antimicrobial activities but also contribute to inflammatory diseases, such as atherosclerosis and related vascular diseases (35) and Parkinson's and Alzheimer's disease (32, 123, 139), *via* multiple mechanisms (166).

The suitability of MPO as a potential independent prognostic biomarker of inflammation is summarized in Table 5. A limitation of MPO as a biomarker is that current methods are not standardized between laboratories and do not provide direct information on MPO activity. The methods used to directly measure MPO activity as well as biomarkers specific for MPO have been reviewed recently (92). MPO-derived oxidants generate a footprint of specific and nonspecific oxidation products. 3-Chlorotyrosine (3-Cl-Tyr) and chlorinated lipids, as well as glutathione sulfonamide, are specific products for MPO/HOCl (92). Nonspecific oxidation products include protein carbonyls and 3-nitrotyrosine modifications. Of the specific biomarkers, 3-Cl-Tyr has received the most attention and has been detected in a wide variety of diseases (Table 6).

Chlorinated lipids have been detected in human atherosclerotic plaque (173). 2-Chloradipic acid (185) is excreted in urine and hence offers the potential as a biomarker. Glutathione sulfonamide is present in the airways of preterm

infants with respiratory disease as well as in children suffering from cystic fibrosis (93).

A general limitation of the specific biomarkers of MPO activity is the requirement for expensive equipment and time-consuming sample workup and analysis. Often, concentration of these biomarkers in biological samples is low, which complicates accurate measurement. As a result, investigators have fractionated plasma and observed that HDL can be the major carrier of 3-Cl-Tyr in CVD (15). However, the extensive preparation procedures for HDL analysis limit its clinical use. Glutathione sulfonamide is a relatively minor oxidation product derived from the reaction of reduced glutathione (GSH) with HOCl. This limits its application to biological samples that contain significant amounts of GSH. Plasma, which has very little GSH, is therefore not a suitable source to analyze glutathione sulfonamide.

Within these limitations, the determination of MPO protein is a reasonable approach to at least initially assess a potential contribution of MPO-mediated oxidative damage to a disease, and in most studies, MPO and specific MPO activity biomarkers with different specificities provide similar results (Tables 5 and 6).

#### Markers of Antioxidant Defense

In principle, oxidative stress can also derive from an impaired antioxidant defense. We focus here not only on protein thiol-disulfide oxidoreductases that can be measured in serum or plasma but also the transcription factor NRF2 that drives the transcription of several antioxidant genes. NRF2 is activated in response to oxidative stress and its activation could therefore be used as an indicator of ROS generation that exceeded the existing antioxidant defense systems.

#### Protein thiol-disulfide oxidoreductases

These enzymes include, among others, thioredoxin (Trx) and peroxiredoxins (Prxs; Fig. 10). Trx main function is to keep protein thiols in the reduced state. For this reason, Trx is often regarded as an antioxidant enzyme. Secretion of Trx was originally discovered in leukemia cells by virologist Junji Yodoi, who initially thought he had identified interleukin-1- $\gamma$  (199). While it cannot be excluded that Trx secretion may be induced by other factors, it is thought that its secretion is induced by oxidative stress. Its secretion is inhibited by antioxidants *in vitro* (96) and its plasma levels are elevated in cancer patients (8) and AIDS patients, where it negatively correlates with survival (125). Various reports have suggested that plasma/serum Trx concentrations are diagnostically relevant in a range of diseases. For instance, Qi *et al.* found that serum Trx concentrations reflected disease severity in acute ischemic stroke (137), and the addition of Trx to an established disease severity score provided a significant improvement in diagnostic capacity.

The Prx family has a key role in the elimination of  $H_2O_2$  because it is highly abundant and reacts with  $H_2O_2$  at high rates. Prxs are often identified in proteomics studies and several studies identified Prxs in the secretome under various disease conditions (80). In particular, oxidized Prx6 in the cerebrospinal fluid has been proposed as a biomarker of oxidative stress in brain injury, where it is a good predictor of the outcome (106). The importance of the redox state of Prxs as biomarkers is also demonstrated by studies showing that

TABLE 5. SELECTED CLINICAL STUDIES ON MPO LEVELS IN DIFFERENT DISEASES

<i>Disease/Condition</i>	<i>Sample</i>	<i>Method</i>	<i>Observation</i>	<i>Reference<sup>a</sup></i>
Cardiovascular disease	Serum	High-sensitivity sandwich ELISA	MPO independently predicted endothelial dysfunction better than CRP.	(103)
Acute coronary syndrome	Serum	ELISA	Increased risk for future cardiac events in patients with elevated serum MPO. MPO, soluble CD40 ligand, and TnT are independent predictors of adverse outcome.	(7)
Acute chest pain, incident MACE	Plasma	Commercial immuno-based assay	MPO higher in MACE patients at hospitalization. MPO predictive for MACE in patients with normal cardiac TnT.	(71)
Cardiovascular disease (LURIC study)	Plasma	ELISA (MPO) and PCR (MPO polymorphisms)	High MPO predicted mortality independent of established cardiovascular risk factors. Five of eight MPO polymorphisms associated with increased MPO. No association of MPO genotype with mortality.	(84)
Major adverse cardiac events	Plasma	Commercial immuno-based assay	MPO included in CBS used to predict future MACEs. High CBS predicted increased risk of MACEs at 3 years.	(98)
Major adverse cardiac events	Plasma	Commercial immuno-based assay	Patient with MPO >322 pmol/L had increased risk of developing future MACE.	(100)
Chest pain	Plasma	ELISA	MPO elevated at baseline and 2 h after onset of symptoms, correlated with TnT concentration, and is an independent predictor of MI and CVD risk.	(16)
Myocardial infarction	Plasma	ELISA	MPO increased and independently predicted development of myocardial infarction in the ensuing 24 months.	(21)
Myocardial infarction	Plasma	ELISA	MPO higher in patients than controls and a predictor of death or nonfatal MI.	(56)
Heart failure	Plasma	Cardio MPO test	High plasma MPO independently predicted the development of heart failure in apparently healthy elderly subjects.	(99)
Peripheral artery disease	Serum	Solid-phase sandwich ELISA	High MPO associated with low ankle-brachial index and PAD independent of CRP.	(6)
Obesity, cardiovascular disease	Plasma	Particle-enhanced turbidimetric immunoassay	MPO, IL-6, and TNF- $\alpha$ were higher in obese than control children and associated with higher cardiovascular risk compared with control.	(74)
Chronic obstructive pulmonary disease	Serum	ELISA	MPO increased in patients during acute exacerbations and persisted for months following acute illness.	(67)
Alzheimer's disease	Plasma	Sandwich ELISA	MPO positively associated with the presence of Alzheimer's disease, correlated with A $\beta_{1-42/1-40}$ ratio, and may potentially be an ideal biomarker for Alzheimer's disease.	(101)
Cognitive decline	Plasma	Sandwich ELISA	Biomarkers of low-grade inflammation, including MPO, and endothelial dysfunction correlated with increased vascular risk and reduced cognitive ability in an older population.	(48)

<sup>a</sup>References are provided as supplementary information.

CBS, cardiac biomarker score; CVD, cardiovascular disease; IL-6, interleukin-6; MACE, major adverse cardiac event; MI, myocardial infarct; MPO, myeloperoxidase; PAD, peripheral artery disease; PCR, polymerase chain reaction; TnT, troponin T.



TABLE 6. SELECTED CLINICAL STUDIES ON SPECIFIC MARKERS OF MPO ACTIVITY IN DIFFERENT DISEASES

Disease/Condition	Sample	Method	Observation	Reference <sup>a</sup>
Acute myocardial infarction	Plasma	ELISA (MPO), HPLC (3-Cl-Tyr)	Plasma MPO and 3 Cl-Tyr increased in AMI, and AMI incidence increased with higher MPO and 3-Cl-Tyr.	(26)
Coronary artery disease, Acute coronary syndrome	Plasma, HDL	Turbidimetric immunoassay (MPO) LC-MS/MS (3 Cl-Tyr)	No difference in plasma MPO, but increased 3-Cl-Tyr in HDL of subjects with CAD/ACS. HDL-associated 3-Cl-Tyr may be a better biomarker of CAD/ACS than plasma MPO.	(89)
CVD	apoB-100	LC-MS/MS	Different apoB-100-derived peptides with modifications characteristic of active MPO are present in humans with increased risk for CVD.	(35)
Rheumatoid arthritis	Synovial fluid	ELISA (MPO), LC-MS/MS (3 Cl-Tyr)	MPO higher in patients than controls. MPO protein 20-fold higher in synovial fluid compared with plasma in RA. 3-Cl-Tyr detected in synovial fluid of patients with rheumatoid arthritis.	(95)
Rheumatoid arthritis	HDL	MS/MS	Subjects with RA have increased 3-Cl-Tyr and Tyr-NO <sub>2</sub> in HDL. MPO-mediated HDL oxidation is regiospecific in RA and further exacerbated with cardiovascular disease.	(104)
Rheumatoid arthritis, Osteoarthritis	Synovial fluid	Spectrophotometer ( $\alpha_2$ M) and GC-MS (3 Cl-Tyr)	$\alpha_2$ M more oxidized and inactivated in RA, and 3-Cl-Tyr elevated in RA compared with OA.	(111)
Preterm infants	Tracheal aspirate	LC-MS/MS	3-Cl-Tyr concentration was elevated in infants who developed chronic lung disease or had lung infection.	(19)

<sup>a</sup>References are provided as supplementary material.

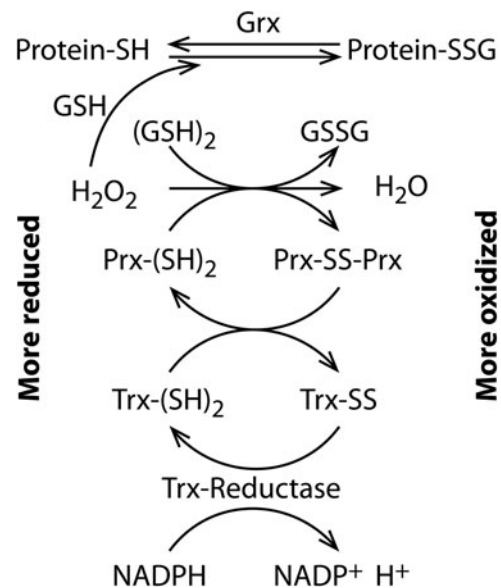
AMI, acute myocardial infarction; ACS, acute coronary syndrome; HDL, high-density lipoprotein.

Prx2 is secreted *via* a nonclassical pathway by inflammatory cells (151) independently of cell death (30) and is present in the serum and synovial fluid of RA patients (168). Bayer *et al.* (13) have shown *in vivo* that erythrocyte Prx2 undergoes a transient overoxidation following lipopolysaccharide exposure, and released Prx1/2 is present in the oxidized form (30, 151), suggesting that its redox state, not just its levels, can be an early marker of oxidative stress.

Human serum Prx4 has also been proposed as a diagnostic marker of oxidative stress in diabetes (1).

#### Nuclear factor (erythroid-derived 2)-like 2

The transcription factor NRF2 is considered a prognostic biomarker in cancer. It is found constitutively and at very high levels in biopsies of malignant tumors with high proliferation rates and poor response to anticancer drugs. NRF2 is a master regulator of the antioxidant response that controls the expression of about 250 genes. Some of these genes encode antioxidant enzymes, including those involved in synthesis and use of GSH and Trx, or participate in metabolic reactions that generate reductive power in the form of NADPH. Others participate in phase II biotransformation reactions and phase III external transport of toxins. Thus, high and constitutive activity of NRF2 provides transformed cells with a defense against the ROS that are generated during active proliferation, while at the same time reducing the effective dose of certain anticancer drugs. To date, a prognostic



**FIG. 10. Protein thiol-disulfide oxidoreductases regulate the redox state of protein thiols.** Different thiol compounds can be present under different redox states, depending on the overall redox state of the cell, from the more reduced (*left*) to more oxidized (*right*), and the significance of a specific biomarker will depend on its intracellular localization, tissue expression, and redox potential.

TABLE 7. OVERVIEW OF HIGHEST EVIDENCE LEVELS FOR ALL DISCUSSED MARKERS

Evidence level category (A–D)	Biomarker	Gold standard	Sample	Stability	Basis for evidence level	
					Disease	Reference <sup>a</sup>
A	Protein carbonyls	ELISA/HPLC	Plasma	Months to years (-80C)	MCI	(86)
C	AGE	None	Plasma, skin	Not known	PCOS, T2D, CKD	(37, 57, 62)
A	Ox-LDL	ELISA	Plasma	Not known	AD	(86)
C	4-HNE	IHC/HIS-ELISA	Tissue, plasma	Years, months (-80C)	Cancer (brain, breast), obesity	(53, 108, 114)
C	Acrolein	IHC	Formalin-fixed tissue	Years	Prostate carcinoma	(31)
A	MDA	HPLC	EDTA plasma	Months (-80C)	PCOS, AD, MCI	(68, 86)
A	F <sub>2</sub> -IsoP	GC-MS, LC-MS/MS	Plasma, urine, tissue	Months (-80C)	Depression	(12)
D	IsoLGs	LC-MS/MS	Tissue, plasma	Months (-80C)	Several preclinical	Multiple, see main text
B	Protein-Tyr-NO <sub>2</sub>	LC-MS/MS, GC-MS/MS	Tissue, plasma	Weeks to months (-80C)	Diabetes	(22–25)
A	GSH	LC-MS/MS, HPLC	Tissue, plasma?	Short	ASD; PCOS	(42, 68)
C	P-SSG (hemoglobin)	LC-MS/MS	Tissue, plasma?	Short	T2D, CRF	(63, 83)
C	Thioredoxin	ELISA	Tissue, plasma?	Years (-80C)	CPB, AIDS	(69, 70)
A	Peroxiredoxin	ELISA	Plasma	Years (-80C)	CHD, T2D	(1, 2)
A	8oxodG	UPLC-MS/MS	Urine	Years	Depression	(12)
A	8oxoGuo	UPLC-MS/MS	Urine	Years	T2D, AD, MCI	(17, 86)
B	MPO	ELISA	Plasma	Months	AMI	(26)
B	3-Cl-Tyr	LC-MS/MS	Many	Months	Atherosclerosis, RA	(47, 89, 95, 104)
C	NRF2	IHC/qRT-PCR	Tissue	Years (IHC), months (PCR)	Various cancers	Multiple, see main text
A	P-VASP	Immunolabeling with FC	Platelets	24–48 h	PCI/clopidogrel resistance	(14)
A	ADMA	LC-MS/MS	Plasma	Months (-20C)	PCOS, CVD	(68, 109)

<sup>a</sup>References are provided as supplementary information.

Categories are based on the following evidence levels: (A) Meta-analysis or large prospective studies, (B) Prospective studies, (C) Retrospective studies, and (D) Case reports or preclinical studies. AD, Alzheimer's disease; ADMA, asymmetric dimethyl L-arginine; AIDS, acquired immunodeficiency syndrome; ASD, autism spectrum disorders; CKD, chronic kidney disease; CPB, cardiopulmonary bypass; CRF, chronic renal failure; FC, flow cytometry; IsoLG, isolevuglandins; MCI, myocardial infarction; ox-LDL, oxidized LDL; PCI, percutaneous coronary intervention; qRT-PCR, quantitative real time polymerase chain reaction; UPLC, ultra performance LC.

value of NRF2 has been reported for adenocarcinoma (79), squamous cell carcinoma (75), colon (84), lung (196), and breast cancer (129), among many others. NRF2 does not initiate carcinogenesis, but potentiates tumor promotion or metastasis. Somatic mutations in the NRF2 gene and in its repressor, KEAP1, have been found in a large percentage of human tumors, leading to constitutively increased NRF2 levels (68). Moreover, malignant transformation elicited by some oncogenes such as KRAS (42) or loss of tumor suppressor PTEN (142) leads to abnormally high levels of NRF2 protein and activity. Thus, analysis of NRF2 levels by either immunoblot or qRT-PCR in tumor biopsies is becoming a tool to clinically assess tumor malignancy.

NRF2 activity declines with age as well as in degenerative disorders. Considering that NRF2 is being validated as an antioxidant and immunomodulator target for diseases with an autoimmune component or with low-grade chronic inflammation, it will be necessary to develop reliable predictive markers that help to monitor effective targeting. The most significant advancement in development of these prospective biomarkers has been in patients with chronic obstructive pulmonary disorder. In a small cohort of these patients, it was reported that dietary administration of sulforaphane-rich broccoli sprout extract for two weeks enhanced the mRNA levels of several NRF2-regulated genes in peripheral blood mononuclear cells (67).

#### Downstream Functional Markers of ROS-Induced Damage

The biomarkers described above are indicative of increased ROS levels, either by increased formation or decreased removal. An alternative would be markers that reflect oxidative stress downstream of the ROS-induced damage. Ideally, this marker would be a direct risk factor so that its modulation by therapeutic interventions would predict a positive outcome. Two markers appear to qualify for this, asymmetric dimethyl L-arginine (ADMA) and phosphorylated vasodilator-stimulated phosphoprotein (P-VASP).

##### *Asymmetric dimethyl L-arginine*

ADMA is a ubiquitous metabolite derived from protein modification and degradation. Upon accumulation, it can interfere with arginine metabolism and •NO formation by endothelial •NO synthase (NOS) eNOS/NOS3 (182), and plasma ADMA concentrations correlate with endothelial, kidney, and erectile dysfunction (100), as well as heart failure (66). Plasma ADMA concentrations are significantly associated with every disease of the cardiovascular system, showing an independent, strong prognostic value for mortality and future cardiovascular events. However, non-CVDs with a possible deregulation of NOS have not been studied in great detail. ADMA is either excreted by cationic amino acid transporters that supply intracellular NOS with its substrate, L-arginine, and then eliminated by the kidney or metabolized to L-citrulline by  $N^G$ - $N^G$ -dimethylarginine dimethylaminohydrolase (DDAH) (171). DDAH has an active site cysteine residue that can be a direct target of oxidative or nitrosative modification (99), resulting in the inhibition of ADMA degradation. Increased intracellular ADMA levels may be the reason for the observed therapeutic effects of L-arginine (153, 154) (see the accompanying ARS FORUM review on Therapeutics).

##### *Phosphorylated vasodilator-stimulated phosphoprotein*

The best-established marker for physiological cyclic guanosine monophosphate (cGMP) signaling is probably P-VASP. It is phosphorylated mainly by cGMP-dependent protein kinases (25), and lowered P-VASP levels are indicative of pathological signaling (77, 110). This pathological signaling can be brought about by oxidative stress, for example, through scavenging of •NO by superoxide or direct oxidation of soluble guanylate cyclase (sGC), both of which limit the level of sGC activation and cGMP production (Fig. 2). However, in human blood samples, for example, in platelets, P-VASP levels can be utilized to establish the efficacy of (or detect nonresponders to) antiplatelet drugs (57), to detect physiological responses to NO donors and hence the presence of sGC (155), or to identify pathological responses to sGC activators as an indirect assay of increased oxidized/apo-sGC levels (2) (see the accompanying ARS Forum review on Targets).

#### Conclusion

The markers discussed here have been studied in different disease settings and with different rigor, ranging from meta-analyses of several clinical studies to promising evidence in preclinical studies (Table 7). However, even when the highest evidence level is available, their specificity as a biomarker of oxidative stress could be questionable, as in the case of oxLDL. Oxidative stress likely plays a role in several diseases, yet very few oxidative stress markers have made it into routine clinical use, which may have several reasons. The properties of the oxidative modifications, such as the labile nature of cysteine modifications, or their low abundance poses significant challenges to translate them into a high-throughput, cost-effective clinical diagnostic. Stable oxidative modifications, such as protein carbonyls, certain lipid oxidation products, DNA/RNA oxidation, and 3-nitrotyrosine, certainly circumvent the first issue, which likely contributes to some of their positive clinical findings. Another limitation is methodology. While MS provides sensitivity and specificity and has become more accessible, antibody-based methods remain, for now, the clinical standard. However, as we have seen, some of these methods fall short on specificity, such as antibodies specific for oxLDL, and any new antibody-based marker requires rigorous testing for specificity and sensitivity. Other antibody-based methods, such as immunohistochemistry, require tissues that are not commonly accessible. Circulating cell harvesting methods may provide a future solution to this. For a new biomarker to be established for clinical use, it would also require additional benefit over established clinical markers. Paradoxically, this additional value of oxidative stress biomarkers may come from being indicators of a disease mechanism common to several pathologies rather than diagnostic for a specific disease. Oxidative stress biomarkers may help in identifying patient populations that benefit from certain treatments, allowing patient stratification based on pathogenic mechanisms rather than just disease severity, thus responding to a specific request from regulatory agencies (47). On the other hand, protein-specific modifications such as nitrotyrosine could be disease-specific biomarkers of oxidative stress (Table 4).

#### Outlook

One way forward may be the analysis of oxidative stress markers for specific proteins. Such markers might better

represent an underlying specific disease mechanism and a means for therapeutic monitoring and outcome prediction. In addition, as many of the markers have been measured in similar diseases, a combination of them in large-scale panels and pattern analysis could provide an additional approach to measure disease progression or therapeutic outcome (Fig. 3). This will help overcome the problem of the fragmentation of the literature in the field as different markers of oxidative stress are measured in different diseases. Measurement of larger panels of biomarkers in key conditions will help give a more comprehensive picture of their significance. In parallel with the exciting developments on ROS-validated targets and clinical indications, those markers and patterns that correlate best with treatment efficacy or mortality will eventually advance the field of ROS biomarkers, for example, in the form of theranostic couples of a new drug comarketed with a diagnostic marker.

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#### Abbreviations Used

3-Cl-Tyr = 3-chlorotyrosine  
 4-HNE = *trans*-4-hydroxy-2-nonenal  
 8oxodG = 7,8-dihydro-8-oxo-2'-deoxyguanosine  
 8oxoGuo = 7,8-dihydro-8-oxo-guanosine  
 AA = arachidonic acid  
 ACS = acute coronary syndrome  
 AD = Alzheimer's disease  
 ADMA = asymmetric dimethyl L-arginine  
 AGEs = advanced glycation end products  
 AIDS = acquired immunodeficiency syndrome  
 AMI = acute myocardial infarction  
 ASD = autism spectrum disorders  
 AT1R = angiotensin receptor 1  
 BMI = body-mass index  
 CAD = coronary artery disease  
 CBS = cardiac biomarker score  
 CPB = cardiopulmonary bypass  
 cGMP = cyclic guanosine monophosphate  
 CHD = coronary heart disease  
 CKD = chronic kidney disease;  
 CML = carboxymethyl lysine  
 CO<sub>2</sub> = carbon dioxide  
 CRF = chronic renal failure  
 CRP = C-reactive protein  
 CSF = cerebrospinal fluid  
 CVD = cardiovascular disease  
 DDAH = *N*<sup>G</sup>-*N*<sup>G</sup>-dimethylarginine  
     dimethylaminohydrolase  
 DNP = 2,4-dinitrophenylhydrazine  
 eNOS = endothelial NOS  
 FC = flow cytometry  
 GC = gas chromatography  
 Grx = glutaredoxin  
 GSH = glutathione  
 GSSG = oxidized glutathione  
 H<sub>2</sub>O<sub>2</sub> = hydrogen peroxide  
 HCC = hepatocellular carcinoma  
 HDL = high-density lipoprotein  
 HIV = human immunodeficiency virus

HOCl = hypochlorous acid  
 HPLC = high-performance liquid chromatography  
 IHC = immunohistochemistry  
 IL-6 = interleukin-6  
 IS = ischemic stroke  
 IsoLG = isolevuglandins  
 IsoPs = isoprostanes  
 LC = liquid chromatography  
 LDL = low-density lipoprotein  
 MACE = major adverse cardiac events  
 MCI = myocardial infarction  
 MDA = malondialdehyde  
 MI = myocardial infarct  
 MPO = myeloperoxidase  
 MS = mass spectrometry  
 NO = nitric oxide  
 NOS = NO synthase  
 NOX = NADPH oxidase  
 NRF2 = nuclear factor (erythroid-derived  
     2)-like 2  
 NRP = nonradical product  
 OA = osteoarthritis  
 ONOOCO<sub>2</sub> = nitrosoperoxocarbonate  
 oxLDL = oxidized LDL  
 OxRed = oxidoreductases  
 PAD = peripheral artery disease  
 PC = protein carbonyls  
 PCI = percutaneous coronary intervention  
 PCOS = polycystic ovary syndrome  
 PCR = polymerase chain reaction  
 PDGF = platelet-derived growth factor  
 PSE = post-stroke epilepsy  
 P-VASP = phosphorylated vasodilator-stimulated  
     phosphoprotein  
 Pr-SS-G = protein mixed disulfide  
 PrCarb = protein carbonyls  
 Prx = peroxiredoxin  
 PUFA = polyunsaturated fatty acids  
 qRT-PCR = quantitative real time polymerase  
     chain reaction  
 RA = rheumatoid arthritis  
 RBC = red blood cell  
 RNS = reactive nitrogen species  
 ROS = reactive oxygen species  
 SAF = skin autofluorescence  
 sGC = soluble guanylate cyclase  
 SLE = systemic lupus erythematosus  
 SODs = superoxide dismutases  
 sRAGE = secreted receptor for AGE  
 T1D = type 1 diabetes  
 T2D = type 2 diabetes  
 TBARS = thiobarbituric acid reactive substances  
 TNF = tumor necrosis factor  
 TnT = troponin T  
 Trx = thioredoxin  
 Tyr-NO<sub>2</sub> = nitrotyrosine  
 u-eNOS = uncoupled eNOS  
 UPLC = ultra performance LC  
 XDH = xanthine dehydrogenase  
 XO = xanthine oxidase