Clinical relevance of biomarkers of oxidative stress

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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.

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Introduction

Redox signaling operates through discrete, reversible, and site-specific modifications of certain proteins (184). Reversible modifications of cysteine residues include S-nitrosylation/S-nitrosation, selenylation, 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 \textit{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.](image)

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, *NO and ONOO*. Besides causing post-translational modifications of proteins, these species may also cause nitrative 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
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 (DNPH). 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 fructoseamine 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
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

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

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

*aReferences 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 F2-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 peroxyl radicals, with bisallylic hydrogen of PUFAs initiates the autocatalytic chain reaction of lipid peroxidation (Reactions 1–3) during which lipid peroxyl radicals act as chain-carrying radicals and lipid hydroperoxides are formed as the primary end products.

\[
\text{Initiation: } LH + R^* \rightarrow L^* + RH \tag{1}
\]

\[
\text{Propagation: } L^* + O_2 \rightarrow \text{LOO}^* \tag{2}
\]

\[
\text{Termination: } \text{LOO}^* + \text{RH} \rightarrow \text{LOOH} + \text{L}^* \tag{3}
\]

\[
\text{Degradation: } \text{LOO}^* + \text{x-TOH} \rightarrow \text{LOOH} + \text{x-TO}^* \tag{4a}
\]

\[
\text{Termination: } \text{LOOH} + \text{x-TO}^* \rightarrow \text{NRP} \tag{4b}
\]

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

\[
\text{LOOH} + \text{Fe}^{3+} \rightarrow \text{LOO}^* + \text{Fe}^{2+} + \text{H}^+ \tag{5}
\]

\[
\text{LOOH} + \text{Fe}^{2+} \rightarrow \text{LO}^* + \text{Fe}^{3+} + \text{OH} \tag{6}
\]

In the presence of transition metals, lipid hydroperoxides (LOOHs) give rise to LOO\* (Reaction 5) or lipid alkoxyl radicals (LO\*) (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 lipoperoxidation 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, triglycerols, or cholesterylesters). 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 Spiteller (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 F₂-Isoprostane Levels in Different Diseases**

<table>
<thead>
<tr>
<th>Disease</th>
<th>Marker</th>
<th>Sample</th>
<th>Method</th>
<th>Conclusions</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cardiovascular</td>
<td>HNE</td>
<td>Plasma</td>
<td>ELISA</td>
<td>HNE increased in IS and higher in patients with rs671 A allele (ALDH2).</td>
<td>(112)</td>
</tr>
<tr>
<td>Ischemic stroke (IS), post-stroke epilepsy (PSE)</td>
<td>HNE, MDA, lipid hydroperoxides</td>
<td>Plasma</td>
<td>HPLC</td>
<td>Slight increase in infarction.</td>
<td>(54)</td>
</tr>
<tr>
<td>Acute myocardial infarction</td>
<td>HNE</td>
<td>Plasma</td>
<td>HPLC</td>
<td>Hypertension correlates with RBC MDA and plasma IsoP levels.</td>
<td>(80)</td>
</tr>
<tr>
<td>Hypertension</td>
<td>MDA, IsoP</td>
<td>Plasma, RBC</td>
<td>HPLC</td>
<td>Hypertension correlates with RBC MDA and plasma IsoP levels.</td>
<td>(80)</td>
</tr>
<tr>
<td>Cardiopulmonary bypass</td>
<td>F₂-IsoP</td>
<td>Plasma</td>
<td>GC-MS</td>
<td>Correlation with decreased dynamic lung compliance.</td>
<td>(5)</td>
</tr>
<tr>
<td>Coronary heart disease</td>
<td>F₂-IsoP</td>
<td>Urine</td>
<td>GC-MS</td>
<td>Independent risk marker for CHD.</td>
<td>(59, 87, 102, 105)</td>
</tr>
<tr>
<td>Hypercholesterolemia</td>
<td>F₂-IsoP</td>
<td>Plasma, urine</td>
<td>GC-MS, ELISA</td>
<td>Higher compared with controls. Lowered by statins.</td>
<td>(33, 36, 58)</td>
</tr>
<tr>
<td>Hypertension</td>
<td>F₂-IsoP</td>
<td>Plasma, urine</td>
<td>GC-MS, ELISA</td>
<td>Higher in pulmonary and essential hypertension.</td>
<td>(107)</td>
</tr>
<tr>
<td>Gastrointestinal system</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Duodenal peptic ulcer</td>
<td>HNE</td>
<td>Tissue</td>
<td>IHC</td>
<td>HNE associated with chronic mucosae alterations due to H. Pylori.</td>
<td>(27, 113)</td>
</tr>
<tr>
<td>Crohn’s disease</td>
<td>F₂-IsoP</td>
<td>Urine</td>
<td>GC-MS</td>
<td>Higher in patients.</td>
<td>(30)</td>
</tr>
<tr>
<td>Colon</td>
<td>MDA, HNE</td>
<td>Tissue</td>
<td>HPLC, IHC</td>
<td>HNE and MDA higher in tumor tissue. IHC can distinguish healthy and tumor tissue.</td>
<td>(11, 94)</td>
</tr>
<tr>
<td>Acrolein</td>
<td>F₂-IsoP</td>
<td>Tissue</td>
<td>HPLC</td>
<td>Indicative of transformation benign to malignant phenotype.</td>
<td>(115)</td>
</tr>
<tr>
<td>Breast</td>
<td>MDA</td>
<td>Plasma</td>
<td>HPLC</td>
<td>No correlation with occurrence of colorectal adenomatous polyps.</td>
<td>(92)</td>
</tr>
<tr>
<td></td>
<td>HNE</td>
<td>Plasma</td>
<td>ELISA</td>
<td>Presurgery MDA prognostic for tumor recurrence.</td>
<td>(39, 49)</td>
</tr>
<tr>
<td></td>
<td>F₂-IsoP</td>
<td>Urine</td>
<td>GC-MS</td>
<td>HNE-modified PDGF was altered in plasma samples HNE modification of specific proteins.</td>
<td>(52)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>(32)</td>
</tr>
</tbody>
</table>

(continued)
<table>
<thead>
<tr>
<th>Disease</th>
<th>Marker</th>
<th>Sample</th>
<th>Method</th>
<th>Conclusions</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Prostate</td>
<td>Acrolein</td>
<td>Tissue</td>
<td>IHC</td>
<td>Predictive of tumor relapse in combination with clinical parameters.</td>
<td>(31)</td>
</tr>
<tr>
<td></td>
<td>F₂-IsoP</td>
<td>Urine</td>
<td>Radioimmunoassay; GC-MS</td>
<td>Conflicting results (either no correlation or positive correlation with risk of prostate cancer.</td>
<td>(9, 18, 44)</td>
</tr>
<tr>
<td>Brain</td>
<td>HNE</td>
<td>Tissue</td>
<td>IHC</td>
<td>In astrocytic and ependymal glial tumors, HNE is associated with the level of tumor malignancy.</td>
<td>(53, 114)</td>
</tr>
<tr>
<td>Lung</td>
<td>F₂-IsoP</td>
<td>Urine</td>
<td>ELISA</td>
<td>Association with lung cancer risk in men.</td>
<td>(41)</td>
</tr>
<tr>
<td>Liver</td>
<td>F₂-IsoP</td>
<td>Urine</td>
<td>ELISA</td>
<td>Association with risk of HCC.</td>
<td>(110)</td>
</tr>
<tr>
<td>Neurodegenerative diseases</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Parkinson’s disease</td>
<td>MDA</td>
<td>Plasma</td>
<td>Fluorometric assay</td>
<td>Increased compared with healthy controls.</td>
<td>(90)</td>
</tr>
<tr>
<td>Alzheimer’s disease</td>
<td>HNE</td>
<td>Plasma, CSF</td>
<td>Capillary GC/MS</td>
<td>Increased compared with healthy controls.</td>
<td>(88)</td>
</tr>
<tr>
<td></td>
<td>HNE, MDA</td>
<td>Plasma</td>
<td>Colorimetric assay</td>
<td>HNE, but not MDA, is increased compared with healthy controls.</td>
<td>(65)</td>
</tr>
<tr>
<td></td>
<td>Acrolein, HNE</td>
<td>Tissue</td>
<td>IHC</td>
<td>HNE and acrolein increased compared with healthy controls.</td>
<td>(20)</td>
</tr>
<tr>
<td>Amyotrophic lateral sclerosis</td>
<td>HNE</td>
<td>Serum</td>
<td>HPLC</td>
<td>HNE increased compared with healthy controls.</td>
<td>(93)</td>
</tr>
<tr>
<td></td>
<td>MDA</td>
<td>Plasma</td>
<td>Fluorometric assay</td>
<td>MDA increased compared with healthy controls.</td>
<td>(15)</td>
</tr>
<tr>
<td>Mental</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Age-related cognitive impairment</td>
<td>F₂-IsoP</td>
<td>CSF</td>
<td>GC-MS</td>
<td>Associated with poorer executive function.</td>
<td>(60)</td>
</tr>
<tr>
<td>Depression</td>
<td>F₂-IsoP</td>
<td>Plasma, urine</td>
<td>GC-MS, ELISA</td>
<td>Treatment with bupropion or sertraline decreases symptoms, but increases F₂-IsoPs.</td>
<td>(29, 40)</td>
</tr>
<tr>
<td>Multiple</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Down syndrome</td>
<td>F₂-IsoP</td>
<td>Amniotic fluid</td>
<td>ELISA</td>
<td>Increased in pregnancies with Down syndrome fetuses.</td>
<td>(76)</td>
</tr>
<tr>
<td>Metabolic diseases</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Diabetes type 2</td>
<td>MDA</td>
<td>Serum</td>
<td>Colorimetric assay</td>
<td>High variability due to age.</td>
<td>(73)</td>
</tr>
<tr>
<td>Obesity</td>
<td>HNE</td>
<td>Plasma</td>
<td>HNE-His ELISA</td>
<td>HNE-His increased in obese men.</td>
<td>(108)</td>
</tr>
<tr>
<td>Otosclerosis</td>
<td>HNE</td>
<td>Tissue</td>
<td>IHC</td>
<td>HNE associated with the onset of otosclerosis.</td>
<td>(81)</td>
</tr>
</tbody>
</table>

*aReferences 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; F₂-IsoPs, F₂-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 thio-barbituric 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).

**F₂-isoprostanes**

Several thorough reviews of the biochemistry and utility of F₂-isoprostanes (F₂-IsopPs) 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 H₂-isoprostanes (140). Nonenzymatic rearrangement of these H₂-isoprostanes forms both stable F₂-IsopPs and highly reactive γ-ketoaldehydes termed isolevuglandins (IsoLGs, also known as isoketals) (115). Because of their chemical stability and sensitivity to changes in oxidative stress, F₂-IsopPs are often considered the most reliable markers for monitoring oxidative stress in vivo (89). Elevated concentrations of F₂-IsopPs are found in CVD, correlate with extent of disease, and predict the outcome (39). Elevated F₂-IsopPs 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 F₂-IsopPs 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 F₂-IsopPs, IsoLGs are products derived from the oxidation of AA and are sensitive to changes in oxidative stress. While F₂-IsopPs 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 glaucomatos 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-NO₂) is often described as a stable marker of oxidative/nitrative stress in inflammatory diseases (71). Tyrosine nitration involves the replacement of C₆ hydrogen atom of the tyrosine aromatic ring with a nitro group (R-NO₂) (11) (Fig. 5). This modification can occur within a polypeptide sequence (protein-associated Tyr-NO₂) or to free tyrosine amino acids (free Tyr-NO₂). 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 (NO₂). It is possible for the tyrosine radical to react with nitric oxide (NO), followed by further oxidation to yield Tyr-NO₂, but this pathway has not been well studied (11).
One widely studied pathway for nitration is the production of the RNS peroxynitrite (ONOO\textsuperscript{−}) (Fig. 6, pathway 1) (132). Initially, Tyr-NO\textsubscript{2} 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\textsubscript{2} 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\textsubscript{2} in the high-throughput analysis of clinical samples needs 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\textsubscript{2}, protein-associated Tyr-NO\textsubscript{2}, or total Tyr-NO\textsubscript{2} 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\textsubscript{2} 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\textsubscript{2} as a clinical biomarker, but findings so far are encouraging, with some studies showing that plasma Tyr-NO\textsubscript{2} levels correlate with disease activity and decrease following successful therapeutic interventions. Yet, it is still unclear whether Tyr-NO\textsubscript{2} 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. 4. Regioisomers of isolevuglandins.** Specific IsoLG regioisomers differ by the relative orientation of their keto- and aldehyde moieties (D\textsubscript{2}-IsoLG vs. E\textsubscript{2}-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\textsubscript{2}-IsoLG and 15-E\textsubscript{2}-IsoLG is chemically identical to levuglandin D\textsubscript{2} and E\textsubscript{2}, respectively, which are generated nonenzymatically from prostaglandin H\textsubscript{2} (149, 150). IsoLG, isolevuglandins.
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$_2$ 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$_2$ 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$_2$ ELISAs may be prone to interference; and (iii) the sensitivity of some Tyr-NO$_2$ ELISAs may be too low to detect Tyr-NO$_2$ 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$_2$ 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 proteins (of which Tyr-NO$_2$ 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$_2$ under inflammatory conditions as a marker of oxidative stress and have found plasma Tyr-NO$_2$ 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$_2$ levels with higher mortality in sepsis and a fall in plasma Tyr-NO$_2$ 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$_2$ concentrations upon drug treatment (statins in coronary artery disease and rosuvastatin in hypercholesterolemia). Tyr-NO$_2$ 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 (111a). In asthmatic patients, Tyr-NO$_2$ staining in bronchial biopsies and measurement of Tyr-NO$_2$ by ELISA in exhaled breath condensates both showed a decrease of Tyr-NO$_2$ upon corticosteroid treatment. Conflicting results were obtained in neurodegenerative disorders; although Tyr-NO$_2$ has been reported at increased levels within brain tissue from Parkinson’s (60) and Alzheimer’s disease patients (167), free Tyr-NO$_2$ is not increased in the cerebrospinal fluid of Alzheimer’s disease patients (143). Likewise, protein-bound Tyr-NO$_2$ is not increased in plasma of patients with dementia (44). In contrast, nitrated α-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>
<thead>
<tr>
<th>Disease/Condition</th>
<th>Sample</th>
<th>Method</th>
<th>Observation</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>CAD</td>
<td>Plasma</td>
<td>LC-MS/MS</td>
<td>Reduction in protein-associated Tyr-NO₂ following rosuvastatin treatment.</td>
<td>(91)</td>
</tr>
<tr>
<td>CAD</td>
<td>Plasma</td>
<td>ELISA</td>
<td>Protein-associated Tyr-NO₂ is a robust predictor of CAD in patients with abnormal glucose tolerance.</td>
<td>(28)</td>
</tr>
<tr>
<td>Hypercholesterolemia</td>
<td>Plasma</td>
<td>ELISA</td>
<td>Reduction in protein-associated Tyr-NO₂ following statin treatment; this lowering effect was independent of the fall in lipid parameters and CRP.</td>
<td>(77)</td>
</tr>
<tr>
<td>Asthma</td>
<td>Bronchial biopsies</td>
<td>Immunohistochemical staining</td>
<td>Reduction of Tyr-NO₂ staining after treatment with inhaled glucocorticoid compared with before treatment.</td>
<td>(82)</td>
</tr>
<tr>
<td>Asthma</td>
<td>Exhaled breath condensates</td>
<td>ELISA</td>
<td>Protein-associated Tyr-NO₂ levels decrease following steroid treatment and increase again following a washout period.</td>
<td>(13)</td>
</tr>
<tr>
<td>Asthma</td>
<td>Exhaled breath condensates</td>
<td>ELISA</td>
<td>Protein-associated Tyr-NO₂ levels are higher in mild asthmatics who were not receiving steroid treatment than in patients with moderate/severe asthma who were receiving treatment.</td>
<td>(46)</td>
</tr>
<tr>
<td>Atherosclerosis</td>
<td>Coronary artery; plasma</td>
<td>Immunohistochemical staining; Western blot</td>
<td>Apo-AI nitration at tyrosine-166 leads to functional impairment and is increased in atherosclerotic coronary arteries compared with normal coronary arteries.</td>
<td>(38)</td>
</tr>
<tr>
<td>Pulmonary hypertension</td>
<td>Lung tissue</td>
<td>Immunohistochemical staining</td>
<td>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.</td>
<td>(4)</td>
</tr>
<tr>
<td>Septic shock</td>
<td>Plasma</td>
<td>Amino acid analyzer</td>
<td>Concentrations of free Tyr-NO₂ are associated with prognosis in patients with septic shock, with higher levels observed in those who did not survive than those who did.</td>
<td>(72)</td>
</tr>
<tr>
<td>OA, RA</td>
<td>Serum, synovial fluid</td>
<td>HPLC</td>
<td>RA patients have detectable free Tyr-NO₂ in plasma and synovial fluid, but not healthy subjects or OA patients. Patients with lower RA disease stage had lower free Tyr-NO₂.</td>
<td>(55)</td>
</tr>
<tr>
<td>OA, RA</td>
<td>Plasma, synovial fluid</td>
<td>LC-MS/MS</td>
<td>Increased levels of total Tyr-NO₂ and higher ratio of synovial/serum total Tyr-NO₂ in OA compared with healthy controls. Lowering of total Tyr-NO₂ concentrations after 6 months of anti-TNF treatment, which was associated with a change in the RA disease activity score.</td>
<td>(66)</td>
</tr>
<tr>
<td>OA</td>
<td>Serum</td>
<td>ELISA</td>
<td>Nitrated collagen concentrations are higher in patients with OA compared with healthy control participants. Concentration of collagen-associated Tyr-NO₂ correlated with the serum concentration of CRP.</td>
<td>(34, 79)</td>
</tr>
<tr>
<td>Celiac disease</td>
<td>Plasma</td>
<td>ELISA</td>
<td>Reduction in protein-associated Tyr-NO₂ concentrations following a gluten-free diet.</td>
<td>(96)</td>
</tr>
<tr>
<td>SLE</td>
<td>Serum</td>
<td>ELISA</td>
<td>Significantly higher levels of protein-associated Tyr-NO₂ in patients with a disease activity score greater than six (SLE Disease Activity Index).</td>
<td>(106)</td>
</tr>
<tr>
<td>Parkinson’s disease</td>
<td>Peripheral blood mononuclear cells</td>
<td>ELISA</td>
<td>Observation of nitrated α-synuclein, the concentration of which was inversely correlated with daily dosage of levodopa.</td>
<td>(78)</td>
</tr>
</tbody>
</table>

*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 alkylation 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 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 μM) and its disulfide, cystine (~40–50 μM), 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

![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 sulfonic acids) to irreversible sulfonic acids. *Reversibility of protein cysteine sulfenic acids has so far been demonstrated only for some sulfenylated peroxiredoxins and requires the enzymatic activity of sulfiredoxin.](image-url)
Deoxyguanosine and 8-oxo-guanosine, respectively.

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

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'-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 microosomal 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$_2$O$_2$ 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$_2$•− (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$_2$O$_2$) 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$_2$O$_2$ 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 compiles 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-γ (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$_2$O$_2$ because it is highly abundant and reacts with H$_2$O$_2$ 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>
<thead>
<tr>
<th>Disease/Condition</th>
<th>Sample</th>
<th>Method</th>
<th>Observation</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cardiovascular disease</td>
<td>Serum</td>
<td>High-sensitivity sandwich ELISA</td>
<td>MPO independently predicted endothelial dysfunction better than CRP.</td>
<td>(103)</td>
</tr>
<tr>
<td>Acute coronary syndrome</td>
<td>Serum</td>
<td>ELISA</td>
<td>Increased risk for future cardiac events in patients with elevated serum MPO.</td>
<td>(7)</td>
</tr>
<tr>
<td>Acute chest pain, incident MACE</td>
<td>Plasma</td>
<td>Commercial immuno-based assay</td>
<td>MPO higher in MACE patients at hospitalization. MPO predictive for MACE in patients with normal cardiac TnT.</td>
<td>(71)</td>
</tr>
<tr>
<td>Cardiovascular disease (LURIC study)</td>
<td>Plasma ELISA (MPO) and PCR (MPO polymorphisms)</td>
<td>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.</td>
<td>(84)</td>
<td></td>
</tr>
<tr>
<td>Major adverse cardiac events</td>
<td>Plasma</td>
<td>Commercial immuno-based assay</td>
<td>MPO included in CBS used to predict future MACEs. High CBS predicted increased risk of MACEs at 3 years.</td>
<td>(98)</td>
</tr>
<tr>
<td>Major adverse cardiac events</td>
<td>Plasma</td>
<td>Commercial immuno-based assay</td>
<td>Patient with MPO &gt;322 pmol/L had increased risk of developing future MACE.</td>
<td>(100)</td>
</tr>
<tr>
<td>Chest pain</td>
<td>Plasma</td>
<td>ELISA</td>
<td>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.</td>
<td>(16)</td>
</tr>
<tr>
<td>Myocardial infarction</td>
<td>Plasma</td>
<td>ELISA</td>
<td>MPO increased and independently predicted development of myocardial infarction in the ensuing 24 months.</td>
<td>(21)</td>
</tr>
<tr>
<td>Myocardial infarction</td>
<td>Plasma</td>
<td>ELISA</td>
<td>MPO higher in patients than controls and a predictor of death or nonfatal MI.</td>
<td>(56)</td>
</tr>
<tr>
<td>Heart failure</td>
<td>Plasma</td>
<td>Cardio MPO test</td>
<td>High plasma MPO independently predicted the development of heart failure in apparently healthy elderly subjects.</td>
<td>(99)</td>
</tr>
<tr>
<td>Peripheral artery disease</td>
<td>Serum</td>
<td>Solid-phase sandwich ELISA</td>
<td>High MPO associated with low ankle-brachial index and PAD independent of CRP.</td>
<td>(6)</td>
</tr>
<tr>
<td>Obesity, cardiovascular disease</td>
<td>Plasma</td>
<td>Particle-enhanced turbidimetric immunoassay</td>
<td>MPO, IL-6, and TNF-α were higher in obese than control children and associated with higher cardiovascular risk compared with control.</td>
<td>(74)</td>
</tr>
<tr>
<td>Chronic obstructive pulmonary disease</td>
<td>Serum ELISA</td>
<td></td>
<td>MPO increased in patients during acute exacerbations and persisted for months following acute illness.</td>
<td>(67)</td>
</tr>
<tr>
<td>Alzheimer’s disease</td>
<td>Plasma</td>
<td>Sandwich ELISA</td>
<td>MPO positively associated with the presence of Alzheimer’s disease, correlated with Aβ1-42/1-40 ratio, and may potentially be an ideal biomarker for Alzheimer’s disease.</td>
<td>(101)</td>
</tr>
<tr>
<td>Cognitive decline</td>
<td>Plasma</td>
<td>Sandwich ELISA</td>
<td>Biomarkers of low-grade inflammation, including MPO, and endothelial dysfunction correlated with increased vascular risk and reduced cognitive ability in an older population.</td>
<td>(48)</td>
</tr>
</tbody>
</table>

*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.
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.](image-url)
<table>
<thead>
<tr>
<th>Evidence level category (A–D)</th>
<th>Biomarker</th>
<th>Gold standard</th>
<th>Sample</th>
<th>Stability</th>
<th>Basis for evidence level</th>
<th>Disease</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>Protein carbonyls</td>
<td>ELISA/HPLC</td>
<td>Plasma</td>
<td>Months to years (-80°C)</td>
<td>MCI</td>
<td>(86)</td>
<td></td>
</tr>
<tr>
<td>C</td>
<td>AGE</td>
<td>None</td>
<td>Plasma, skin</td>
<td>Not known</td>
<td>PCOS, T2D, CKD</td>
<td>(37, 57, 62)</td>
<td></td>
</tr>
<tr>
<td>A</td>
<td>Ox-LDL</td>
<td>ELISA</td>
<td>Plasma</td>
<td>Not known</td>
<td>AD</td>
<td>(86)</td>
<td></td>
</tr>
<tr>
<td>C</td>
<td>4-HNE</td>
<td>IHC/HIS-ELISA</td>
<td>Tissue, plasma</td>
<td>Years, months (-80°C)</td>
<td>Cancer (brain, breast), obesity</td>
<td>(53, 108, 114)</td>
<td></td>
</tr>
<tr>
<td>C</td>
<td>Acrolein</td>
<td>IHC</td>
<td>Formalin-fixed tissue</td>
<td>Years</td>
<td>Prostate carcinoma</td>
<td>(31)</td>
<td></td>
</tr>
<tr>
<td>A</td>
<td>MDA</td>
<td>HPLC</td>
<td>EDTA plasma</td>
<td>Months (-80°C)</td>
<td>PCOS, AD, MCI</td>
<td>(68, 86)</td>
<td></td>
</tr>
<tr>
<td>A</td>
<td>F2-IsoP</td>
<td>GC-MS, LC-MS/MS</td>
<td>Plasma, urine, tissue</td>
<td>Months (-80°C)</td>
<td>Depression</td>
<td>(12)</td>
<td></td>
</tr>
<tr>
<td>A</td>
<td>IsoLGs</td>
<td>LC-MS/MS</td>
<td>Tissue, plasma</td>
<td>Months (-80°C)</td>
<td>Several preclinical</td>
<td>Multiple, see main text</td>
<td></td>
</tr>
<tr>
<td>B</td>
<td>Protein-Tyr-NO2</td>
<td>LC-MS/MS, GC-MS/MS</td>
<td>Tissue, plasma</td>
<td>Weeks to months (-80°C)</td>
<td>Diabetes</td>
<td>(22–25)</td>
<td></td>
</tr>
<tr>
<td>A</td>
<td>GSH</td>
<td>LC-MS/MS, HPLC</td>
<td>Tissue, plasma?</td>
<td>Short</td>
<td>ASD; PCOS</td>
<td>(42, 68)</td>
<td></td>
</tr>
<tr>
<td>C</td>
<td>P-SSG (hemoglobin)</td>
<td>LC-MS/MS</td>
<td>Tissue, plasma?</td>
<td>Short</td>
<td>T2D, CRF</td>
<td>(63, 83)</td>
<td></td>
</tr>
<tr>
<td>C</td>
<td>Thioredoxin</td>
<td>ELISA</td>
<td>Plasma</td>
<td>Years (-80°C)</td>
<td>CPB, AIDS</td>
<td>(69, 70)</td>
<td></td>
</tr>
<tr>
<td>A</td>
<td>Peroxiredoxin</td>
<td>ELISA</td>
<td>Plasma</td>
<td>Years (-80°C)</td>
<td>CHD, T2D</td>
<td>(1, 2)</td>
<td></td>
</tr>
<tr>
<td>A</td>
<td>oxodG</td>
<td>UPLC-MS/MS</td>
<td>Urine</td>
<td>Years</td>
<td>Depression</td>
<td>(12)</td>
<td></td>
</tr>
<tr>
<td>A</td>
<td>oxoGuo</td>
<td>UPLC-MS/MS</td>
<td>Urine</td>
<td>Years</td>
<td>T2D, AD, MCI</td>
<td>(17, 86)</td>
<td></td>
</tr>
<tr>
<td>B</td>
<td>MPO</td>
<td>ELISA</td>
<td>Plasma</td>
<td>Months</td>
<td>AMI</td>
<td>(26)</td>
<td></td>
</tr>
<tr>
<td>B</td>
<td>3-Ch-Tyr</td>
<td>LC-MS/MS</td>
<td>Many</td>
<td>Months</td>
<td>Atherosclerosis, RA</td>
<td>(47, 89, 95, 104)</td>
<td></td>
</tr>
<tr>
<td>C</td>
<td>NRF2</td>
<td>IHC/qRT-PCR</td>
<td>Tissue</td>
<td>Years (IHC), months (PCR)</td>
<td>Various cancers</td>
<td>Multiple, see main text</td>
<td></td>
</tr>
<tr>
<td>A</td>
<td>P-VASP</td>
<td>Immunolabeling with FC</td>
<td>Platelets</td>
<td>24–48 h</td>
<td>PCI/clopidogrel resistance</td>
<td>(14)</td>
<td></td>
</tr>
<tr>
<td>A</td>
<td>ADMA</td>
<td>LC-MS/MS</td>
<td>Plasma</td>
<td>Months (-20°C)</td>
<td>PCOS, CVD</td>
<td>(68, 109)</td>
<td></td>
</tr>
</tbody>
</table>

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 N²,N¹-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
Biomarkers of oxidative stress 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 theraonistic 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
8oxoGdG = 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
CO2 = carbon dioxide
CRF = chronic renal failure
CRP = C-reactive protein
CSF = cerebrospinal fluid
CVD = cardiovascular disease
DAAH = N(3)-N(3)-dimethylarginine dimethylaminohydrolase
dNP = 2,4-dinitrophenylhydrazine
eNOS = endothelial NOS
FC = flow cytometry
GC = gas chromatography
Grx = glutaredoxin
GSH = glutathione
GSSG = oxidized glutathione
H2O2 = hydrogen peroxide
HCC = hepatocellular carcinoma
HDL = high-density lipoprotein
HIV = human immunodeficiency virus
HOCI = 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
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
ONOOCO2 = nitrosoperoxocarbonate
oxLDL = oxidized LDL
OxRed = oxidoreductases
PAD = peripheral artery disease
PC = protein carbonyls
PCI = percutaneous coronary intervention
POCS = 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-NO2 = nitrotyrosine
u-eNOS = uncoupled eNOS
UPLC = ultra performance LC
XDH = xanthine dehydrogenase
XO = xanthine oxidase
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