Biomarkers of Oxidative Stress

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Abstract

Biological processes can lead to reduction–oxidation reactions that generate potentially harmful reactive oxygen species (ROS), such as peroxides and free radicals. Exposure to xenobiotics and/or microbial organisms, for example, can lead to an imbalance between the concentrations of ROS and the antioxidant buffer in the target tissue, which if sustained can result in oxidative stress. Oxidative stress and inflammation have been implicated in various disorders, such as cancer, atherosclerotic cardiovascular disease, lung disease (including asthma and chronic obstructive pulmonary disease), chronic inflammatory diseases, and diabetes mellitus. The pathways and metabolic processes of disease development are complex and involve many components.

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The processes often overlap and the balance between stressors and protectors is not always clear. Thus, the identification of mechanistic biomarkers to improve understanding of disease pathogenesis, aid in the diagnosis of disease, develop therapeutic strategies, and monitor treatment compatibility, performance, and complications is vitally important. This chapter discusses various biomarkers of oxidative stress that have been previously identified and possible routes to discovering further useful biomarkers and potential applications.

Keywords
Antioxidants • Biomarkers • Cancer • Chronic disease • Inflammation • Oxidative stress

Oxidative Stress

As part of normal cellular metabolic processes, aerobic organisms produce endogenous free radicals, such as reactive oxygen species (ROS), through enzymatic or nonenzymatic chemical processes. Initially, ROS were thought always to be harmful, leading to mutagenesis, aging, and cancer (Commoner 1954). The study of enzyme antioxidants, such as superoxide dismutase and glutathione peroxidase, however, began to clarify the mechanisms of ROS metabolism and reveal positive biological effects (Gregory and Fridovich 1973). At moderate concentrations, therefore, ROS by-products help to maintain the balance between reduction and oxidation – the redox state – and act as signaling molecules for various physiological functions (Droge 2002; Genestra 2007).

In humans, oxygen from aerobic respiration undergoes reduction by mitochondrial electron transport. This process does not yield any free radical intermediates. The remaining molecular oxygen (around 5 %) undergoes univalent reduction (Ferrari 1991). The univalent reduction process is mediated by enzymes, such as NAD(P)H oxidases and xanthine oxidase, or nonenzymatically by redox-reactive compounds, such as glutathione (Droge 2002). Electron transport chain complexes all possess the potential to add electrons to oxygen, which results in superoxide formation (Evans 1997). The superoxide anion ($O_2^{\bullet -}$), which is formed when oxygen accepts an electron, is used by the immune system in the defense against invading pathogens. Due to its toxicity, most organisms living in an oxygenated environment utilize an isoform of superoxide dismutase to reduce superoxide levels. Superoxide undergoes enzymatic conversion by superoxide dismutases to hydrogen peroxide, which is then subsequently detoxified by catalases and glutathione peroxidases present in the cytosol. However, while hydrogen peroxide itself is capable of damaging cellular components, it can be a major source of further endogenous radical species such as hydroxyl radicals, by interactions with reduced transitional metal ions such as ferrous or cuprous ions, which are capable of generating further peroxides by interactions with cellular lipids, for example (Droge 2002).

Further information on how reactive oxygen species interact with cellular biology is well described in the review by Droge (2002). ROS may also be generated by ionizing or ultraviolet radiation (Cooke 2003). Irrespective of their origin, ROS may interact with and modify cellular biomolecules, such as DNA, which could have potentially serious consequences for the cell, that is, mutation.

ROS act directly by damaging DNA, lipids, and proteins or indirectly through the recruitment of inflammatory mediators that trigger a secondary oxidative response. This inflammatory response in turn generates the release of further oxidative species and leads to an overall imbalance in the redox state of the tissue. Extensive DNA damage can also lead to genomic instability. For example, endogenously formed $OH^{\bullet -}$ can lead to alterations in DNA bases (purines and pyrimidines) and cause DNA strand breaks, which in turn affect gene integrity (Balasubramanian 1998). Oxidative stress is, therefore, thought to play an important part in the initiation of various chronic diseases, such as cancer, cardiovascular disease, lung disorders (e.g., chronic obstructive pulmonary disease [COPD]), inflammatory conditions, and metabolic disorders. The mechanisms of oxidative stress are complex. Owing to the presence of many different oxidative species in the cellular environment, specific identification of which of these species is directly responsible for a given biological effect can be difficult to determine. Detection of measurable biomarkers associated with oxidative processes and pathways involved in disease development would, therefore, be useful. The complexity also makes it likely that testing multiple biomarkers concurrently will yield the most useful results.

Selection of Biomarkers

Oxidative DNA Damage

In a situation where repeated and sustained intranuclear ROS are generated, DNA repair mechanisms become overwhelmed and DNA damage may become extensive and generate genomic instability, which contributes to carcinogenesis (Hanahan 2000; Charames 2003). It is unlikely that highly reactive species such as $^{\bullet}OH$ generated in a remote cell compartment can diffuse into the cell nucleus, due to its extreme reactivity. Hence, it has been proposed that H_2O_2 serves as a diffusible latent form of $^{\bullet}OH$ that reacts with a metal ion in the vicinity of a DNA molecule to generate the oxidant species (Marnett 2000). Others have suggested that lipid peroxidation products may also function as intermediates between endogenous metabolic products or xenobiotic agent-induced alterations and DNA effects (Voulgaridou et al. 2011). Any oxidative lesion that is not repaired is likely to become a fixed mutation in a cell with replicative capability, which increases the risk of carcinogenesis (Clayson 1994).

More than 20 base lesions have been identified, although only a few, such as 8-oxo-2′ deoxyguanosine, have been studied to any notable degree (Cooke 2003). Oxidative DNA damage has been associated with various diseases,

such as neurological brain disorders (e.g., Alzheimer's disease and Parkinson's disease) (Jeppesen 2011); chronic inflammatory and autoimmune disorders (e.g., rheumatoid arthritis; Hitchon and El-Gabalawy 2004) and systemic lupus erythematosus (Kurien 2006); liver disease (Sastre 2007); disorders of metabolic dysregulation, such as obesity, metabolic syndrome, and diabetes (Whaley-Connell 2011); and ischemia–perfusion injury after transplantation (Evans et al. 1997; Thier 1999). The strongest link, however, is with carcinogenesis (Toyokuni 1995). Products of DNA repair might, therefore, be useful as biomarkers of cancer risk.

The most frequently studied product of DNA lesion repair is 8-hydroxydeoxyguanosine (8OHdG) (Halliwell 2004). This product is a major repair marker for free radical-induced oxidative lesions (Valko 2004; Valavanidis 2009). The presence of 8OHdG in the DNA strand can induce G → T and A → C transversions, which are among the most frequent mutations in human cancers (Cheng 1992; Henderson 2002; Pilger 2006). Additionally, elevated concentrations of 8OHdG have been seen in patients with chronic hepatitis B virus or hepatitis C virus infections, which are important causes of hepatocellular carcinoma (Shimoda 1994; Farinati 1999). The link between disease and elevated concentrations of 8OHdG is well documented (Vineis and Husgafvel-Pursiainen 2005). Cooke and colleagues (2006) reported elevated levels of 8OHdG in high proportions of cases of several precancerous and cancerous conditions. DNA repair is achieved mainly by excision of 8OHdG, which is excreted as an intact molecule. Thus, 8OHdG is measurable in urine, which makes it suitable for use as a biomarker in clinical practice and in trials, due to the relative ease of sample collection. The biological relevance of elevated levels of urinary 8OHdG however has been open to debate. On one hand, elevated 8OHdG levels could imply a greater exposure to ROS, whereas on the other, it could indicate the presence of a fully functioning DNA repair system which has been able to mitigate potential damage. Thus, interpretation of biomarker data is critical to understanding the complex processes taking place within a cell/tissue and often involves further mechanistic studies to aid with the interpretation.

Other DNA repair products are found in urine and could have roles as biomarkers (Lowe 2009). For instance, the oxidation of thymidine by $OH^{\bullet -}$ generates 5,6-dihydroxy-5,6-dihydrothymidine (thymidine glycol). Most thymidine products are not generally potent pre-mutagenic lesions, however thymidine glycol notably distorts the DNA molecule, which leads to a lethal lesion (Wallace 2002). As thymidine glycol has been found to be "inefficient as a pre-mutagenic lesion" (Evans et al. 1993), the link between levels of urinary thymidine glycol and malignant disease is questionable. That said, the concentration of urinary thymidine glycol correlates well with exposure to dimethylated arsenic compounds (Yamanaka et al. 2003), increased excretion was reported in kidney transplant recipients with ischaemia–reperfusion-induced oxidative DNA damage (Makropoulos et al. 2000; Thier et al. 1999), and elevated levels have been observed in smokers compared to nonsmokers (Lowe 2009). Thymidine glycol therefore, might be useful as a generic biomarker of oxidative DNA damage to complement other biomarkers such as 8OHdG.

Antioxidant Status

Antioxidants protect the body from the harmful effects of free radical damage. Thus, measurement of antioxidant levels in target tissues or biofluids has been widely used to assess the extent of oxidant exposure, and, in turn, oxidative stress. Total antioxidant capacity (TAC) indicates the oxidant-buffering potential of a tissue or biofluid. Some specific compounds (which can be absorbed in the diet or synthesized in vivo), precursors or metabolites, such as ascorbic acid, that scavenge free radicals have been used previously (Lykkesfeldt 2007). Additionally, enzyme activity, such as that of superoxide dismutase (SOD), can reflect conversion of free radicals into less toxic entities and might be a useful approach to assess the reduction of disease risk by oxidative stress.

The TAC system involves two major component groups: enzymatic components (SOD, catalase and glutathione peroxidase, and other enzymes) and low-molecular-weight antioxidants, comprising endogenous small macromolecules (bilirubin, albumin, ceruloplasmin, and ferritin) and molecules of dietary origin (ascorbic acid, α-tocopherol, β-carotene, and polyphenols) (Kohen and Nyska 2002; Grune 2005), and is generally decreased when oxidative stress is increased (Ghiselli 2000; Young 2001). Depletion of even one antioxidant molecule can cause changes in the overall level of antioxidant capacity. Importantly, variation in antioxidant levels has been associated with an elevated risk of a number of conditions, including obesity, metabolic syndrome and diabetes mellitus, cardiovascular disease, and cancer (Serafini 2006; Stephens 2009; Tinahones 2009). The use of TAC as a biomarker could be advantageous, because measurements can be made in tissues and biofluids that can be easily obtained. Nevertheless, the usefulness of TAC has been criticized because in vitro and in vivo results are not always in agreement (Somogyi 2007). The results vary across different TAC assays (Cao and Prior 1998), as do oxidation sources, targets, and measurements (Somogyi 2007). Hence, TAC should be assessed in parallel with other biomarkers of oxidative damage (Young 2001).

Ascorbic acid (and less frequently its oxidized form, dehydroascorbic acid) have been used as biomarkers of oxidative stress for several years. Ascorbic acid is a major radical scavenger in vivo as well as a regenerator of other antioxidants and is thought to offer a general indication of the antioxidant status. Upon oxidation via the semidehydroascorbyl radical, ascorbic acid is converted to dehydroascorbic acid and subsequently regenerated intracellularly to ascorbic acid upon combining with reduced glutathione. During oxidative stress, concentrations of dehydroascorbic acid increase as the intracellular availability of reduced glutathione, required to regenerate ascorbic acid, is limited. Therefore, elevated dehydroascorbic acid in biofluids has been used to characterize oxidative stress (Lykkesfeldt 2007). Comstock et al. (1997) indicated a link between various antioxidant compounds in 258 patients with lung cancer and 515 matched healthy controls in the USA. They measured ascorbic acid in plasma and α-carotene, β-carotene, cryptoxanthin, lutein/zeaxanthin, lycopene, α-tocopherol, selenium, and peroxyl radical absorption capacity in serum or plasma. Concentrations of

cryptoxanthin, β-carotene, and lutein/zeaxanthin were significantly lower in lung cancer patients than in controls. Small differences in a protective direction were noted for α-carotene and ascorbic acid, but these were nonsignificant.

Superoxide dismutases (SOD), glutathione peroxidases, and catalases are among the most widely studied enzymes involved in oxidative stress. The discovery of SOD greatly improved the understanding of antioxidant defense systems, since it led to the postulation of oxygen-related toxic effects (Gregory and Fridovich 1973). SOD catalyzes the dismutation of superoxide into oxygen and H_2O_2 as part of the antioxidant defense system (Maier and Chan 2002). Glutathione peroxidases are the main enzymes involved in removal of peroxides in human tissue. This family of enzymes utilizes reduced glutathione as a substrate to convert peroxides such as H_2O_2 and fatty acid hydroperoxides into alcohols, water, and a dimer of oxidized glutathione (GSSG) (Chance 1979; Gaber 2001). GSSG is then recycled by glutathione reductases back into reduced glutathione utilizing NADPH as a substrate (Meister 1988). In addition to glutathione peroxidases, catalases can mediate the decomposition of H_2O_2 into water and oxygen (Gaetani 1996). These enzymes work in conjunction and therefore, measurement of all three together could be useful to indicate the antioxidant status of a tissue/biofluid. Changes in activity of these catalytic enzymes have been associated with various disorders, but especially neurodegenerative diseases (Cookson and Shaw 1999; Halliwell 2001; Golden and Patel 2009)

The glutathione S-transferases (GSTs) are a family of enzymes responsible for the metabolism of a broad range of xenobiotics and carcinogens and are found predominantly in the liver (Mannervik 1985). GSTs bind and conjugate electrophiles to reduced glutathione to, in effect, neutralize them and protect the cell from electrophilic deleterious effects. Some GSTs also have glutathione peroxidase activities, for example, MGST1 (Schaffert 2011). Glutathione *S*-transferase has been proposed as a plasma marker for oxidative stress in cancer (Khan 2010).

Owing to the number of individual antioxidants and the intricacy of their interactions, measurement of individual compounds and molecules might miss some important effects (Prior 1999; Kohen 2003). Likewise, the wide variations in enzyme activity can make it difficult to draw meaningful conclusions on antioxidant status. A useful approach, therefore, might be to measure concentrations of low-molecular-weight antioxidants together, as they are thought to work in concert (Berry and Kohen 1999). Enzyme activity might also benefit from simultaneous assessment with other biomarkers, such as F_2-isoprostanes.

Lipid Peroxidation

Among the mechanisms of damage caused by ROS, lipid peroxidation is probably the most extensively investigated. Oxidation of cell membrane phospholipids results in the formation of unstable lipid hydroperoxides and secondary carbonyl compounds, such as endogenous aldehydes (Liebler 1999). Highly reactive endogenous aldehydes react directly with DNA, form aldehyde-derived DNA adducts,

and lead to DNA damage (Voulgaridou 2011). Some of the most reactive aldehydes are 4-hydroxy-2-nonenal, malondialdehyde, acrolein, crotonaldehyde, and methylglyoxal. The mutagenic and carcinogenic effects are attributed to the ability of these aldehydes to directly modify DNA bases or yield promutagenic exocyclic adducts (Voulgaridou 2011). These compounds might be useful as biomarkers because they are measureable in biofluids, although complex processes might be required to prepare them for analysis (Michel 2008).

Prostaglandin F_2-like compounds, termed F_2-isoprostanes, are endogenous prostaglandin-like compounds formed by a nonenzymatic mechanism (Morrow 1990a, b). These eicosanoid molecules are derived from the peroxidation of arachidonic acid found in biological membranes (Janssen 2001). Most F_2-isoprostanes are initially esterified on production in phospholipids and undergo hydrolysis into their free form by platelet-activating factor acetylhydrolase (Stafforini 2006) and possibly other phospholipases. Free isoprostanes are released from tissues into the circulation, where they are partly metabolized. F_2-isoprostanes are well suited to be biomarkers of oxidative stress because they can be measured accurately to picomolar concentrations in fresh or stored biofluids, such as urine and plasma (Morrow 1990a; Awad 1993) or breath condensate (Carpenter 1998) and in specific organs (Morrow 1992). Total body production of F_2-isoprostanes may be assessed by measurement of concentrations of unmetabolized free F_2-isoprostanes in plasma and of their metabolites in urine (Davies 2011). Concentrations are not affected by diurnal variation but do vary strikingly across clinical and experimental pathological conditions. Thus, F_2-isoprostanes have been proposed to be useful biomarkers for many diseases and diverse disease states (Janssen 2001) and the assessment of commercial products such as cigarettes (Hatsukami 2006).

F_2-isprostanes are also thought to mediate the underlying biological mechanisms of several diseases and, therefore, show promise not only as biomarkers of the presence of ongoing disease processes, but also of severity. The most widely studied F_2-isoprostane is 8-isoprostane. In patients with coronary artery disease, 8-isoprostane concentrations were significantly elevated and were correlated with a number of risk factors for heart disease, stenotic segments, and with extent scores for coronary stenosis (Vassalle 2004; Wang 2006). The presence of 8-isoprostane in samples from vulnerable plaques strongly supports the role for lipid peroxidation by free radicals in coronary artery disease (Nishibe 2008). Multiple other cardiovascular conditions (Cracowski 2001), especially ischemic conditions, such as myocardial ischemia–reperfusion injury (Reilly 1997), acute ischemic stroke (van Kooten 1997), and atherothrombosis (Patrono 2005), are marked by elevated F_2-isoprostane levels.

Isoprostanes have been reported to affect many lung cell types and multiple conditions have been shown to elicit changes in F_2-isprostane profiles (Janssen 2001). For example, elevated concentrations have been reported after exposure to allergens (Brussino 2010), cigarette smoke (Morrow 1995; Reilly 1996; Praticò 1998a), and hyperoxia (Carpagnano 2004). Associations have also been made with multiple respiratory disorders, such as asthma (Louhelainen 2008) and

COPD (Praticò 1998b), where it might also indicate disease progression (Makris 2008). Measurements of F_2-isoprostane to assess lung disease can be done from breath condensate (Montuschi 2010), which increases the attraction of these compounds as biomarkers, due to the relative ease of sample collection.

The most widely used technique for analysis is high-performance liquid chromatography and gas chromatography–mass spectrometry (Andreoli 2003; Milne 2007; Liu 2009). This approach, however, requires extensive preparation and expensive instrumentation (Morrow and Roberts 1994), but does display excellent sensitivity and specificity even for low picomolar concentrations (Milne 2007, 2008). Commercial immunoassays are available for F_2-isoprostanes, which improve the throughput and ease of analysis, but specificity of the antibody is problematic where precise quantification is required.

Identification of New Biomarkers

'Omics Studies

The 'omics technologies reveal changes in DNA, RNA, epigenetics, proteins, and metabolism that might be associated with disease. Where oxidative stress is known to contribute to disease development, the identification of specific disease mutations might yield genomic markers for this imbalance.

An accumulation of ROS owing to oxidative stress can lead to genomic instability, deregulation of transcription, induction of mitogenic signal transduction pathways, and replication errors and adversely affect genetic and epigenetic cascades (Ziech et al. 2011). Broadly, genomic instability is classified into microsatellite instability associated with mutator phenotype (Imai and Yamamoto 2008) and chromosome instability, which is recognized by gross chromosomal abnormalities (Nowak 2002). Loss of heterozygosity, which is the loss of normal function of one allele of a gene in which the other allele was already inactivated, and copy number abnormality, which arises from alterations in the number of copies of a gene or chromosome in a cell, are important features of genomic instability. Abnormal activation of telomerase, which affects cellular longevity, has also been shown to contribute towards genomic instability (Steiling et al. 2008). Single nucleotide polymorphisms of antioxidant defense genes, such as the *SOD* and *GPX* families of genes, which encode SOD and glutathione peroxidase, respectively, could lead to oxidative stress (Yuzhalin and Kutikhin 2012), and, as such, might be suitable biomarkers to investigate in genomic studies.

Genomic instability is a key factor in various diseases. Microsatellite instability classification includes three intracellular mechanisms involved in DNA damage repair that lead to mutation: nucleotide excision repair, base excision repair, and mismatch repair. The chromosome instability pathway is typically associated with the accumulation of mutations in tumor suppressor genes and oncogenes and, therefore, has a crucial role in the development of cancer (Charames and Bapat 2003; Pino and Chung 2010). In Alzheimer's disease, aneuploidy

in chromosomes 14, 17, and 21, where the *APP*, *PSEN1*, and *TAU* genes are located, could be indicative of oxidative stress (Taupin 2010); chromosome 17 is particularly susceptible to aneuploidy in the presence of oxidative insult (Ramírez 2000).

DNA Methylation Analysis

DNA methylation is a crucial part of normal organism development and cellular differentiation in higher organisms. The process occurs by covalent addition of a methyl group at the five' carbon of the cytosine ring, which produces 5-methylcytosine. Promoter regions of mammalian genes, which control the expression of the associated gene, typically have cytosine–guanine-rich regions (CpG islands) in their genetic sequence. Methylation at specific sites can prevent the binding of transcriptional machinery or ubiquitous transcription factors to regulatory sites on the DNA double helix (Jenuwein and Allis 2001). The subsequent change in chromatin structure renders the promoter sequence of the target DNA inaccessible to activating transcription factors and prevents gene expression. Although methylation controls gene activity, it is insufficient to repress gene activity without histone deacetylation, chromatin-binding proteins, and changes in chromatin structure. The subsequent modulation of gene expression can lead to the development of various disease processes in which oxidative stress is also known to be involved.

Promoter hypermethylation plays a major part in the development of various diseases, but the most widely studied is cancer. DNA methylation contributes to carcinogenesis through transcriptional silencing of critical cell growth regulators, such as tumor suppressor genes. In normal tissues, the genetic coding region is methylated but CpG-poor, whereas the promoter region is unmethylated but CpG-rich. In neoplastic cells, widespread genomic hypomethylation and localized hypermethylation by targeting of the CpG islands, including in the promoter region, are observed. The result is coding region mutations that lead to the inactivation of tumor suppressor genes (Baylin 1998). At the same time, aberrant methylation might affect the regulation of cell proliferation, leading to uncontrolled cell division and proliferation in cancerous cells; aberrant methylation of genes controlling the cell cycle, proliferation, apoptosis, metastasis, drug resistance, and intracellular signaling has been identified in multiple cancer types (Cheung 2009). ROS-induced oxidative stress has been implicated in the altered methylation patterns seen in neoplasms and, therefore, they might be useful biomarkers in genetic and epigenetic studies of cancer. 5-methylcytosine is a target for oxidative damage by ROS, such as $^{\bullet}OH$ (Hori 2003; Kamiya 2002), which can lead to mutation and, in turn, hypomethylation or demethylation. Thus, intact 5-methylcytosine might serve as a useful biomarker in the diagnosis of cancer (Zukiel 2004). Improved understanding of how epigenetic mechanisms affect and are affected by methylation is needed (Shenker and Flanagan 2012).

Individual or multiple sites of aberrant methylation in promoter regions of genes related to oxidative stress could serve as biomarkers for oxidative stress. For example, studies of prostate cancer development have shown that glutathione S-transferase P1 (GSTP1) is the most frequently methylated gene (Donkena 2010). GSTP1 catalyzes conjugation reactions between potentially damaging oxidants, electrophiles, and reduced glutathione (Hayes 2005). The expression of GSTP1 in prostate cancer lesions is significantly reduced compared to healthy tissue, and this phenomenon is tightly regulated by hypermethylation of the promoter CpG Island. Thus, the study of GSTP1 methylation has improved the standard histological diagnosis in sextant biopsies and quantitative assessment of GSTP1 methylation may be of prognostic significance (Donkena 2010). Furthermore, in vitro studies utilizing H_2O_2 to generate oxidative stress have shown that exposure of hepatocellular carcinoma cells to H_2O_2 induced hypermethylation of the E-cadherin gene promoter (a protein linked to malignant transformation). H_2O_2 exposure caused an increase in the expression of Snail (a transcription factor that downregulates the expression of E-cadherin) (Lim 2008). A study by Kang et al. (2012) investigated the mechanisms involved in ROS-induced silencing of RUNX3 (a tumor suppressor gene) by hypermethylation of its promoter region. RUNX3 mRNA and protein expression was downregulated in response to H_2O_2 exposure in the human colorectal cancer cell line SNU-407. This downregulation was abolished with pretreatment of the ROS scavenger, N-acetylcysteine (NAC). In the same study, PCR data revealed that H_2O_2 treatment increased RUNX3 promoter methylation, whereas NAC and the cytosine methylation inhibitor, 5-aza-2-deoxycytidine, decreased it. The authors suggested that an epigenetic regulatory mechanism by ROS-induced methylation may be involved in RUNX3 silencing. Further studies highlighting the link between oxidative stress and DNA methylation are described in the review by Ziech et al. (2011).

Methylation patterns have been detected for various cancers in cultured cell lines (Gitan 2002; Meissner 2008), paraffin-embedded solid tumors (Umetani 2006), blood (serum and plasma) (Goessl 2002; Langevin 2012; Baccarelli 2010), sputum (Varella-Garcia 2010), and exhaled breath condensate (Han 2009), although substantial further assessment and validation are required if these patterns are to be used in a diagnostic/prognostic capacity (Shi 2007). Measurement of DNA methylation has several advantages over other genomic approaches. DNA is more inherently stable than RNA and various relevant proteins, and the localized and consistent position of CpG island methylation enables simpler detection than for common mutations, which may differ in location within the same gene from subject to subject.

In conclusion, ROS-induced DNA hypo- and/or hypermethylation alterations have been implicated with antioxidant defense modulation and redox-sensitive mechanisms leading to tumor formation. Sites of DNA methylation in promoter regions could potentially serve as biomarkers of oxidative stress in relevant tissues given their sensitivity to known oxidants. Since quantification of DNA methylation is possible in a target promoter region, and crucially, reductions in methylation have been demonstrated using known antioxidants, the extent of oxidative

stress in a given tissue can be characterized. By utilizing panels of redox-sensitive genes in relevant tissues, more information can be gathered with respect to how associated pathways are affected by oxidant exposure.

Transcriptomic Analysis

Understanding of the transcriptional output of the human genome is thought to be a key part of understanding many processes, including the development of disease. Transcriptomics enables gene expression profiling through microarray analysis of an entire messenger RNA (mRNA) population (transcriptome) in a tissue sample at a given time point (Mäder 2011; Saha 2002). Large-scale, data-rich detection of biological responses can be achieved. For instance, RNA from cells can be captured at various time points following the administration of a drug or xenobiotic and, therefore, cellular responses can be identified in their entirety. For instance, if a drug or xenobiotic has a particular mechanism of action, the relevant cellular responses can be tracked at the transcriptomic level. This approach could lead to the discovery of previously unknown pathways, which might enable the creation of biomarker panels of differentially expressed genes and/or the distinction of diseased tissue from normal tissue and could be used to monitor the effects of treatments in drug development. The complexity of the human genome, and therefore the transcriptome, remains a notable challenge and the biological importance of unannotated transcripts remains unknown (Kapronov et al. 2005). Other issues are that transcriptomic expression does not necessarily mean an equal proportion of protein will be synthesized because of the action of regulatory systems, such as microRNAs (Lewin 1997). Furthermore, complex transcriptions might lead to subtle effects at the cellular level, which could make the transcriptional controls difficult to trace (Kapronov et al. 2005), especially due to limitations in the technology, differential expression of genes frequently needs to be confirmed by targeted assays, such as polymerase chain reaction. Next-generation sequencing technologies (Davey 2011) and new approaches to analysis (Bussemaker 2007) are, however, making more comprehensive and accurate transcriptome analysis feasible.

Proteomic Analysis

Proteomic analysis is the logical next step after gene expression analysis to determine whether changes in gene expression alter protein expression, but substantial further work is needed in this area to ensure changes related to disease are measurable and to confirm predictive value. Little overlap of differentially expressed genes and proteins has been seen across studies so far, which highlights a major difficulty in the proteomic approach. The human proteome is vast and contains considerably more potential targets than the human genome. Additionally, posttranslational modification of proteins might change their mode of action and increase the number of targets even further. How these proteins then interact

mechanistically to contribute to disease development is likely to be highly complex and might differ from person to person. Thus a global proteomic approach for biomarker discovery must be backed up by mechanistic studies to understand how the candidate biomarkers interact physiologically and how interactions change in a pre-disease state or following exposure to a drug or xenobiotic.

While cancer has been a substantial area of study with 'omics technology, other diseases have also been the subject of investigation, especially the neurodegenerative diseases. For instance, oxidative stress has been highlighted by different 'omics technologies as a common pathway in the development and progression of Parkinson's disease (Smith 2009; Caudle 2010). Likewise, for Alzheimer's disease, 'omics studies have identified roles for various genes in susceptibility, including NOS2 and NOS3, which are involved in oxidative stress (Serretti 2007). Metabolic approaches, such as metabolomics, could be suitable for assessment of airway diseases, such as COPD (Sofia 2011), and proteomic and metabolomic studies are showing some promise in the study of cardiovascular disease (Martinez-Pinna 2010).

MicroRNAs

The study of microRNAs (miRNAs) began after their discovery in the early 1990s (Lee 1993). They comprise a family of small, endogenous, noncoding, functional RNA molecules that regulate gene expression by inhibition of the translation of protein from mRNA or by promotion of mRNA degradation. miRNAs are involved in the regulation of gene expression in cells, specifically by silencing or promoting gene expression (Staszel 2011). Aberrant expression of miRNAs has been linked to various human diseases, such as inflammatory disorders, cardiovascular disease, and cancer. miRNAs are also involved in many preclinical conditions associated with oxidative stress, such as chronic inflammation (Alam and O'Neill 2011), obesity and atherosclerosis (Hulsmans et al. 2011), and the metabolic syndrome (Sookoian and Pirola 2011) and, therefore, might be useful biomarkers of pre-disease changes. Interactions have been seen between key pathways, such as oxidative stress, apoptosis, cell cycle regulation, and tumor suppression (Yang 2011). Genomic, epigenomic, and gene–environment studies should be done to elucidate these relations further.

Expression of miRNAs is highly tissue specific, measurement can distinguish between diseased and normal tissues and show reversal of effects, and assessment can be done with noninvasive isolation techniques. These features and the shared roles across diseases make miRNAs excellent candidates for biomarkers of oxidative stress (Gilad 2008; Lu et al. 2005).

Application of Biomarkers of Oxidative Stress

The identification of biomarkers related to oxidative stress can be applied in a wide set of circumstances. Some of the key areas are in the research, development, and

assessment of beneficial effects of and harm-reduction strategies for foods, medicines, and other consumable products; the identification of novel treatment targets for drug development; and the monitoring of treatment response and adverse effects.

As oxidative stress has a key role in multiple and varied diseases and disorders, related biomarkers could be highly relevant to the development and use of many treatments. Drug research and development can be a long and complicated process in which the study of a huge number of candidate compounds, mechanisms, and targets is necessary to yield a low number of feasible prospects to take forward into more substantial clinical trials. The identification of biomarkers that indicate disease and pre-disease states, can show reversal of signs and symptoms, and that can be assessed quickly after treatment to avoid lengthy trials of conditions that are slow to manifest is greatly desired.

Biomarker "qualification" is of utmost importance during the development of any biomarker to test whether a biomarker is fit for purpose within a defined context of use. The context should be set early in the development process to ensure that studies are well designed and will yield relevant supporting evidence for regulators and scientists alike with respect to biomarker performance and biological application (Matheis 2011). Biomarkers may also be prioritized according to likelihood of positive impact. Although the initial cost may be inhibitive, careful selection of biomarkers will ultimately lead to safer and better products and therapeutics (Goodsaid 2008). In the USA, a pilot process for biomarker qualification has been introduced that outlines the route to submission and is expected to expedite development of successful marketing applications for drug development (US Department of Health 2011). Further information is, however, needed on the type of data and studies required to fulfill the voluntary exploratory data submission for the biomarker qualification review team (Goodsaid 2008).

Recently, the US Institute of Medicine (IOM) published the proceedings of a workshop entitled "Perspectives on biomarker and surrogate endpoint evaluation." The panel elaborated on the concept of biomarker "qualification," in that they proposed a 3-stage iterative evaluation process, which could help scientists fully assess biomarkers for their proposed use. The stages proposed were "Analytical Validation," that is, an assessment of robustness of the biomarker measurement system; "Qualification," that is, assessment of the available evidence that links a biomarker with a biological process, such as a disease state, a clinical outcome, or an intervention; and "Utilization," that is, a subjective and contextual analysis based on the specific use proposed and the applicability of available evidence to this use. This includes a determination of whether the validation and qualification conducted provide sufficient support for the use proposed as contrasted with the objective analyses in the analytical validation and qualification steps and is specific to the use of a given biomarker or surrogate endpoint (IOM 2011). To give a hypothetical example to highlight the importance of the "Utilization" stage, a company who manufactures an immunosuppressant drug has successfully used serum immunoglobulin levels to demonstrate the efficacy of their drug. The health foods division of that same company wishes to make a claim that their latest

product "helps boost the immune system" and has utilized serum immunoglobulin levels as a biomarker to demonstrate this, based upon data from the drug intervention study. One would question whether modulated immunoglobulin levels actually represent a "boost" to the immune system. Thus, the context of use of immunoglobulins is different for the health food claim compared to characterizing the therapeutic effects of the immunosuppressant drug, as the former is trying to demonstrate an undefined benefit and the latter is showing a clear effect of the drug. In order to back up their health claim, the company would need to clearly define what is meant by "boosts the immune system" and then determine if the biomarker is indicative of that with the use of relevant studies.

For therapeutics available on the market, it is possible that biomarkers will help to target treatment by the identification through prescreening of patients who will be sensitive to the drug's effects (Tan 2008). This approach might be particularly useful where genetic polymorphisms can influence pharmacokinetics. Screening might also prevent the use of drugs in patients at risk of cellular-stress-related adverse effects (Robaey 2008). Panels of biomarkers that could be rapidly and cheaply tested could help with early diagnosis, personalized treatment decisions on the basis of a patient's specific molecular, genetic, and other characteristics, and monitoring of treatment responses. The European Food Safety Authority Scientific Panel on Dietetic Products, Nutrition and Allergies has released new guidance on the use of data to substantiate claims related to foodstuffs with antioxidant properties (EFSA 2011). They pay particular attention to claims of beneficial effects and how to design human studies to assess such effects. To be able to make health-benefit or harm-reduction claims, trials should have clearly tested the effects of specific constituents in vivo, in well-designed studies done with robust methods and in a representative population. As mentioned above, however, to adequately test the effects of one constituent in oxidative stress can be extremely difficult. Thus, to show prevention of cellular and molecular oxidative damage, the guidelines recommend the study of biomarkers, and support the use of multiple markers.

A crucial part of the testing of food and drug products before and after introduction to the market relates to their safety, efficacy, benefits, and harm in long-term studies in the general population. Biomarkers may be applied in such studies as surrogate measures of outcomes. For instance, rather than waiting for disease to manifest or treatment benefits to be seen, altered biomarker profiles might be able to indicate relevant changes quickly and easily (Ohshima 2002; Milbury 2003). Biomarkers used in such a manner however, are not without their limitations. The IOM recently commented on ways that candidate surrogate biomarkers can fail to predict clinical outcomes (reproduced from Fleming and DeMets 1996).

1. The surrogate endpoint does not involve the same pathophysiologic process that results in the clinical outcome.
2. The intervention affects only one pathway mediated through the surrogate, of several possible causal pathways of the disease.

3. The surrogate is not part of the causal pathway of the intervention's effect or is insensitive to its effect.
4. The intervention has mechanisms of action independent of the disease process.

They further commented that the most promising setting in which to qualify a surrogate biomarker occurs when the surrogate is on the only causal pathway of the disease, and the intervention's entire effect on the clinical outcome is mediated through its effect on the surrogate biomarker (IOM 2010; Fleming and DeMets 1996). A good example of such a "failed" biomarker in the area of oxidative stress is beta-carotene levels, as a biomarker for cardiovascular disease and cancer risk. Furthermore, beta-carotene was proposed as a therapeutic intervention to prevent these diseases. Beta-carotene was reported to have antioxidant effects in vitro, and hence, dietary supplementation with beta-carotene was proposed to help reduce the risk of cardiovascular disease and cancer (as both diseases have elements of oxidative stress as a driving factor of the disease process). However, upon conducting large population studies to check for efficacy, with mortality as an endpoint, beta-carotene supplementation had no effect on cardiovascular disease risk and actually increased cancer incidence (IOM 2010; Omenn 1996; Peto 1981).

Concluding Remarks

Identification and context-specific development of biomarkers of oxidative stress will be important, as this process is crucial to multiple human diseases, such as cancer, heart disease, and respiratory diseases. Oxidative injury may also be seen in precursor disease states, such as the metabolic syndrome, diabetes, hepatitis B virus and hepatitis C virus infections, and alcohol-related liver disease. Thus, to find biomarkers that can help with the identification of pre-disease changes and with early diagnosis might have beneficial effects for drug development and other therapeutic research. Likewise, biomarkers that show reversibility of harmful effects in response to treatment or product exposure could help to detect treatment/product failure promptly. While complexity makes oxidative stress a difficult area to study, the use of panels of context-specific biomarkers should help to give a greater understanding of the underlying biology, and it is hoped that the intricate systems will in turn, offer up a wealth of novel and useful biomarkers.

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