Protein carbonylation in human diseases

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Oxidative modifications of enzymes and structural proteins play a significant role in the aetiology and/or progression of several human diseases. Protein carbonyl content is the most general and well-used biomarker of severe oxidative protein damage. Human diseases associated with protein carbonylation include Alzheimer’s disease, chronic lung disease, chronic renal failure, diabetes and sepsis. Rapid recent progress in the identification of carbonylated proteins should provide new diagnostic (possibly pre-symptomatic) biomarkers for oxidative damage, and yield basic information to aid the establishment of efficacious antioxidant therapy.

There is a large body of evidence revealing the role of reactive oxygen species (ROS) in several pathologies [1]. ROS are generated as by-products of cellular metabolism, primarily in the mitochondria. Small (physiological) amounts of ROS are a cellular requirement, because they are involved in signalling pathways (inducing and regulating a variety of cellular activities, including cytokine secretion, growth, differentiation and gene expression [1,2]) and in the defence against invading pathogens. However, ROS have the potential to induce significant biological damage and, hence, cells possess many antioxidant systems for scavenging or otherwise eliminating them. Under physiological conditions, there is a well-managed balance between formation and neutralization of ROS by these systems. Oxidative stress can occur when ROS production is accelerated (e.g. inappropriate activation of phagocytes in chronic inflammatory diseases) or when the mechanisms involved in maintaining the normal reductive cellular milieu are impaired [e.g. mutations affecting antioxidant defence enzymes – as in 15–20% of patients with familial amyotrophic lateral sclerosis, who carry mutations in the gene encoding the Cu/Zn-superoxide dismutase (SOD) [1] – or a depletion of antioxidants] (Fig. 1). Increased production of ROS is thought to occur more frequently than diminished antioxidant defence, and has been postulated to play a role in the pathogenesis of several diseases [1]. However, in many human diseases, it is not clear whether oxidative stress is the cause or the consequence of the primary disease process.

ROS can damage all types of biomolecules, and oxidative damage to DNA, lipids and proteins can be deleterious and concomitant. The primary cellular target of oxidative stress depends upon the cell type, the nature of the stress imposed (radical or non-radical oxidant), the site of generation (intra- or extra-cellular), the proximity of ROS to a specific target, and the severity of the stress [1].

Protein oxidation as a biomarker of oxidative stress
Many different types of protein oxidative modification (Table 1) can be induced directly by ROS or indirectly by reactions of secondary by-products of oxidative stress [3]. Cysteine and methionine are particularly prone to oxidative attack by almost all ROS. Protein modifications

Fig. 1. Origins and consequences of oxidative stress in disease. Reactive oxygen species (ROS) are constantly generated inside cells by oxidase enzymes and by dismutation of the superoxide anion, and their intended functions range from host defence to signal transduction. There are several cellular systems that eliminate ROS, thereby balancing the ratio between generation and detoxification of ROS, including antioxidant enzymes such as catalase, superoxide dismutases, peroxidases and glutathione peroxidases, and low-molecular-weight compounds such as vitamin E (α-tocopherol), vitamin C (ascorbic acid) and glutathione. However, endogenous and exogenous triggers can cause the overproduction of ROS or the impairment of antioxidant defence systems, leading to a deleterious condition known as ‘oxidative stress’. Adaptive upregulation of defence systems can protect against damage, either completely or partially, but oxidative-stress-mediated damage to all types of biological macromolecules often leads to tissue injury, and eventually to cell death by necrosis or apoptosis.
oxidative stress [6,9]. However, the tissue levels of such as the conversion of tyrosine residues to 3-chlorotyrosine, 3-nitrotyrosine or dityrosine, are better markers of specific, it has been argued that other protein modifications, such as the conversion of tyrosine residues to 3-chlorotyrosine, 3-nitrotyrosine or dityrosine, are better markers of oxidative stress [6,9]. However, the tissue levels of such markers are orders of magnitude lower than the overall carbonyl content and, hence, their measurement often requires highly sensitive and expensive methods [9].

<table>
<thead>
<tr>
<th>Amino acid</th>
<th>Products</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cysteine</td>
<td>Oxidation of a sulphydryl group (Cys-SH) to form sulphenic (Cys-SOH), sulphinic (Cys-SO₂H) or sulphonic (Cys-SO₃H) derivatives</td>
</tr>
<tr>
<td>Lysine, arginine, proline, threonine</td>
<td>Formation of a disulphide bond (Cys-S-S-Cys) between two nearby Cys residues within a protein (intramolecular cross-linking) or between two proteins (intermolecular cross-linking)</td>
</tr>
<tr>
<td>Histidine</td>
<td>Formation of a mixed disulphide (Cys-S-S-glutathione) between a sulphydryl group and glutathione (S-glutathionylation)</td>
</tr>
<tr>
<td>Glutamic acid, tyrosine, lysine, leucine, valine, proline, isoleucine</td>
<td>Hydperoxide</td>
</tr>
<tr>
<td>Lysine, cysteine, histidine</td>
<td>2-oxo-histidine</td>
</tr>
<tr>
<td>Histidine</td>
<td>Formation of carbonyl derivatives by direct oxidative attack on amino-acid side chains (α-aminoacidic semialdehyde from Lys, glutamic semialdehyde from Arg, 2-pyrrolidone from Pro, and 2-amino-3-ketobutyric acid from Thr)</td>
</tr>
<tr>
<td>Methionine</td>
<td>Formation of carbonyl derivatives by secondary reaction with reactive carbonyl compounds derived from oxidation of carbohydrates (glycoxidation products), lipids (malondialdehyde, 4-hydroxynonenal, acrolein) and advanced glycation and lipoxidation end products</td>
</tr>
<tr>
<td>Phenylalanine</td>
<td>Methionine sulphoxide</td>
</tr>
<tr>
<td>Lysine, arginine, proline, threonine</td>
<td>o-tirosine, m-tirosine</td>
</tr>
<tr>
<td>Tryptophan</td>
<td>N-formylykynureine, kynureine, 5-hydroxytryptophan, 7-hydroxytryptophan</td>
</tr>
<tr>
<td>Tyrosine</td>
<td>3,4-dihydroxyphenylalanine, 3-chlorotyrosine, 3-nitrotirosine, ditryosine (Tyr-Tyr cross-links)</td>
</tr>
</tbody>
</table>

elicited by direct oxidative attack on Lys, Arg, Pro or Thr, or by secondary reaction of Cys, His or Lys residues with reactive carbonyl compounds (RCOs), can lead to the formation of protein carbonyl (PCO) derivatives (aldehydes and ketones) (Fig. 2) [3]. Studies of the formation of PCOs cannot differentiate between those produced through direct protein oxidation and those formed by the addition of previously oxidized molecules, and hence PCOs must be considered as a broad marker of oxidation. Carbonyls are relatively difficult to induce compared with methionine sulfoxide and cysteinyl derivatives, and might therefore indicate a more severe oxidative stress. Indeed, elevated levels of PCOs are generally a sign not only of oxidative stress but also of disease-derived protein dysfunction.

PCOs have a major advantage over lipid peroxidation products as markers of oxidative stress: oxidized proteins are generally more stable. PCOs form early and circulate in the blood for longer periods (their elevation in serum is stable for at least four hours [4]), compared with other parameters of oxidative stress, such as glutathione disulphide and malondialdehyde. The formation of PCOs seems to be a common phenomenon during oxidation, and their quantification can be used to measure the extent of oxidative modification. This has prompted the development of various sensitive biochemical (spectrophotometric and fluorometric) and immunological [western blot, enzyme-linked immunosorbent assay (ELISA) and proteomics] methods for the detection and measurement of PCOs in human tissues and body fluids (for discussion see Refs [5,7,8]). PCO content is the most general indicator and by far the most commonly used marker of protein oxidation [3,5,6].

Because the mechanisms of PCO generation are non-specific, it has been argued that other protein modifications, such as the conversion of tyrosine residues to 3-chlorotyrosine, 3-nitrotirosine or ditryosine, are better markers of oxidative stress [6,9]. However, the tissue levels of such markers are orders of magnitude lower than the overall carbonyl content and, hence, their measurement often requires highly sensitive and expensive methods [9].

![Fig. 2. The production of protein carbonyls (aldehydes and ketones).](http://tmm.trends.com)
Human diseases associated with carbonylated proteins

Human diseases associated with carbonylated proteins are listed in Table 2. In this review, we examine selected diseases in which elevated levels of PCOs have been reported recently. In some cases, these elevations correlate well with the progression or severity of the disease.

Alzheimer’s disease

Alzheimer’s disease (AD) is the most common form of adult dementia, with aging being its chief risk factor. The neuropathological hallmarks of AD are the presence, in cerebral cortex and hippocampus, of intracellular neurofibrillary tangles (NFT) of hyperphosphorylated microtubule-associated protein tau, and of extracellular deposits of amyloid β-peptides (senile plaques) derived from larger transmembrane proteins (amyloid precursor proteins).

Oxidative stress has a major role in the pathogenesis of AD, manifested by protein oxidation, lipid peroxidation and ROS formation. Whether oxidative stress is a primary or secondary event, it is clearly an important neurodegenerative element, which might contribute to neuronal loss [10–12].

Oxidative damage is common in the aging brain but is more severe in AD. PCO content was shown to be increased by 42% in AD hippocampus and by 37% in AD inferior parietal lobule, relative to AD cerebellum, a brain region that shows little degenerative change in AD (whereas the other two regions are rich in β-amyloid plaques) [13]. PCO levels in control hippocampus and inferior parietal lobule were similar to control cerebral levels. Therefore, proteins in AD brain appear to be more carbonylated than in age-matched controls, and mainly in regions that contain severe histopathological alterations [13] (although PCOs increase in both the cell bodies of neurons without visible pathomorphological changes and the cell bodies of neurons with neurofibrillary abnormalities [14]). Furthermore, carbonyl levels correlate well with NFT [15].

The in vivo carbonylation of the neurofilament heavy (NFH) subunit suggests that carbonyl modification is associated with a generalized cytoskeletal abnormality that could be crucial in AD neurofibrillary pathology. However, this ‘pathological’ response (the modification of the NFH subunit by carbonyls) and the pathological lesions of the disease (senile plaques and NFT) could be a part of the cellular defence, playing a role in the protection of neurons from oxidation [16]. Intriguingly, although NFH has a long half-life, the extent of carbonyl modification does not change throughout the normal aging process, or along the length of the axon, and NFH might be uniquely adapted as a carbonyl scavenger, owing to its high lysine content. It is tempting to consider NFH carbonylation as an augmentation of the neuronal defences that are important in protecting the axon (the major site of neurofilaments) from reactive aldehydes, which are among the most toxic products of oxidation. The slow turnover rate of proteins in the axon (up to several years) might necessitate this protection.

Degradation of oxidized proteins is normally enhanced, mainly via the proteasomal system [17]. Furthermore, neuronal proteins can be synthesized locally in dendrites near synaptic sites as well as in the axon [18,19]. These observations might undermine the suggestion that carbonylation is necessary for protecting neuronal-process proteins, because it is possible that these proteins are synthesized locally, independent of cell-body protein synthesis. However, the proteasome might be at least partially inactivated by oxidants, particularly during aging [17], and the translation machinery is also affected by carbonylation [20]. Hence, a ‘protective’ role for neuronal protein carbonylation cannot be ruled out.

Identification of specific targets of protein oxidation will be crucial for establishing a relationship between oxidative modification and neuron death. Two-dimensional gel fingerprinting, coupled to immunochromatographic detection of PCOs, has led to the identification of creatine kinase BB and β-actin as brain proteins that are specifically carbonylated in AD [14,21]. Proteomic analysis has identified other specific targets of protein carbonylation, such as glutamine synthase, ubiquitin C-terminal hydrolase L-1, dihydropyrimidinase-related-protein-2 (which is involved in axonal growth and guidance) and α-enolase [8,22]. The identification of these carbonylation targets has suggested plausible mechanisms for neurodegeneration in AD brain: energy depletion (creatine kinase BB and β-actin as brain proteins that are specifically carbonylated in AD [14,21].

Chronic lung disease

Several different environmental and infectious stimuli have been implicated in the initiation of the lung injury that culminates in chronic lung disease (CLD), but the most important are mechanical ventilation and oxygen toxicity. Pre-term infants are often exposed to increased oxidative stress, owing to supra-physiological oxygen concentrations (hyperoxia), in combination with low surfactant concentrations, reduced antioxidant defences,
and decreased ability to induce antioxidant enzymes. Of infants weighing <1.5 kg, 15–25% develop bronchopulmonary dysplasia (BPD), a CLD that develops after prolonged acute lung injury and appears to evolve partly from early inflammatory responses in the lung. The molecular mechanisms of hyperoxia-induced lung injury (including that to vascular endothelium) and cell death are complex, and are regulated by the generation of high levels of ROS, cytokine-mediated inflammation, loss of antioxidant defence mechanisms, and modulation of the signal transduction pathways that regulate expression of stress-responsive and apoptotic-regulatory genes [23].

Oxidative damage to pulmonary-fluid proteins can be quantified in terms of carbonyl content in samples obtained during routine suctioning of neonates receiving ventilation. The amount of carbonylated proteins can provide a quantitative assessment of oxygen toxicity and of pulmonary antioxidant defences [24].

Oxidative stress in the lungs of pre-term babies undergoing ventilation, especially those who subsequently developed CLD, has been demonstrated in bronchoalveolar lavage (BAL) fluid [25]. Premature babies show high concentrations of PCOs, ascorbate and urate during the first 72 h of life, which then fall progressively over the next six days. A similar increase in PCOs observed in tracheal aspirates correlates strongly with myeloperoxidase activity, suggesting a possible contribution by neutrophil-derived ROS to the lung injury [26].

Increased carbonylation of Clara-cell secretory protein (CCSP), and decreased expression of CCSP, were observed more consistently in tracheal aspirate fluids from infants who later developed BPD than from infants who did not develop BPD [27]. This suggests that Clara-cell function and CCSP expression might be crucial for normal bronchoalveolar-fluid homeostasis, and that maintenance of CCSP concentration and function could be useful targeted therapies for inhibition of BPD development. Furthermore, it is possible that early alterations in CCSP oxidation or expression could be used to identify subsets of premature babies with greater sensitivities to lung oxidant stresses and to the development of adverse pulmonary outcomes.

**Chronic renal failure**

Oxidative stress has been implicated in the progression of renal diseases, and dialysis patients combine a massive generation of ROS during each dialysis session with a chronic deficiency in the major antioxidant systems.

Oxidative stress in patients with chronic renal failure (CRF) and in patients on chronic haemodialysis therapy is manifested in an increase in plasma protein oxidation, including PCO formation and thiol-group oxidation [28], with albumin being the major target [29]. Oxidation of albumin, which is an important plasma antioxidant, decreases plasma antioxidant defences, and hence this patient population is at considerable risk of oxidant-stress-induced tissue injury and cardiovascular disease. Furthermore, patients that have CRF with proteinuria show increased carbonylation of urinary protein compared with plasma protein, and urinary albumin is the major target, showing a 71% increase in carbonylation relative to plasma albumin [30].

Muscle weakness and reduced exercise capacity are frequent complaints of patients with chronic uraemia. Increased oxidative damage to proteins and lipids from skeletal muscle has been observed in uremic patients on haemodialysis, suggesting a role for oxidative damage in the pathogenesis of skeletal myopathy in haemodialysed patients [31]. Advanced glycation end-product (AGE) and advanced lipoxidation end-product (ALE) levels in plasma and matrix proteins are elevated to several times those of normal subjects. The accumulation of reactive dicarbonyl compounds and/or of glyoxidation and lipoxidation products is termed ‘carbonyl stress’ [32]. Hence, uraemia is associated with carbonyl overload (carbonyl stress), caused by excess oxidative formation of reactive carbonyl groups in proteins, lipids and carbohydrates, and the attendant irreversible protein modifications that might have a role in long-term complications associated with CRF and haemodialysis, such as dialysis-related amyloidosis and arteriosclerosis [33,34]. Carbonyl stress has also been observed in diabetes and arteriosclerosis, and has been implicated in the accelerated vascular damage observed in both of these conditions [34].

**Diabetes**

Diabetes mellitus is one of the most common chronic diseases and is characterized by elevated blood glucose and urinary glucose excretion. Sufferers are predisposed to markedly increased cardiovascular mortality and to the development of nephropathy, neuropathy and retinopathy. The prevalence of type-II (non-insulin-dependent) diabetes among adults varies from <5% to >40% for different populations. Type-I (juvenile-onset) diabetes is less common [1].

Elevated PCO levels have been detected in both type-I and type-II diabetes [35–38]: in arteriosclerotic tissues, in the thickened intima of arterial walls, and co-localized with glyoxidation and lipoxidation products. In addition, AGEs and ALEs accumulate in the characteristic diabetic glomerular lesions, such as the expanded mesangial matrix and nodular lesions. The rate of AGE and ALE accumulation is related to the severity of complications (neuropathy, nephropathy, retinopathy and lens disorders), and their concentration also rises linearly with age and correlates with the severity of microvascular disease [33]. AGEs exist in very young healthy children (<6 years) but diabetic individuals have much higher concentrations [34]. Carbonyl stress might result from hyperglycaemia (lipidaemia), oxidative stress and/or impaired detoxification of RCOs. Treatment of carbonyl stress in diabetes, as well as in uraemia and arteriosclerosis, offers new therapeutic approaches, including redox modulation, RCO detoxification and inhibition of carbonyl stress. Widely used hypotensive agents, such as angiotensin-converting-enzyme inhibitor and angiotensin-II-receptor antagonist, are potentially useful treatments, because they do not produce the side effects (vitamin B6 deficiency and neurotoxicity) typical of the first generation of carbonyl-stress inhibitors, such as aminoguanidine, which function as RCO-trapping agents.
The newer compounds act on the production of RCO precursors by scavenging a variety of radicals and altering oxidative stress.

An imbalance in the oxidant:antioxidant ratio (systemic oxidative stress) is already present upon early onset of type-I diabetes and increases into early adulthood. Plasma PCO levels are significantly higher in diabetic children and adolescents without complications compared with control subjects, indicating that oxidative protein damage occurs at the onset of disease and tends to increase in the later stages. Furthermore, decreased antioxidant defences might increase the susceptibility of diabetic patients to oxidative injury [35]. Protein carbonylation in young type-I-diabetic patients that are clinically free of complications was confirmed in other studies [37]. This work also showed that type-I-diabetic patients with complications have higher plasma PCO levels than patients without complications [37]. There are no significant differences between diabetic patients with and without complications in the levels of other markers of oxidative protein damage (nitrotyrosine and protein thiols) [36].

Patients with type-II diabetes without complications also show an increased concentration of plasma PCOs compared with controls, but no significant differences in plasma thiol concentration [38]. PCO increases are also well correlated with the ophthalmic complications observed in diabetes [39].

Sepsis
Sepsis is a complex syndrome caused primarily by overwhelming release of bacterial endotoxin from Gram-negative organisms. This occurs secondary to inflammatory responses, including production of tumour necrosis factor α, which induces the release of ROS, reinforcing the level of oxidative stress and protein damage. In infants and immunocompromised patients, septicaemia carries an almost 50% mortality rate, and effective therapies have remained elusive until recently.

Winterbourn and co-workers investigated whether oxidative injury occurs in patients who are critically ill with severe sepsis or major trauma [40]. They collected plasma and BAL fluid regularly during the first 10 days following trauma or the onset of sepsis, and both fluids were analysed for PCO concentrations (as a measure of protein oxidation) and for thiobarbituric-acid-reactive substances (TBARS) (as a measure of lipid peroxidation). In both patient groups, PCO concentrations were initially highly elevated compared with those of healthy adults, in both the plasma and BAL fluid, indicating that oxidation is not restricted to the lungs. PCOs fell significantly within the first few days but remained above control values. There was a strong correlation between carbonyl concentrations in lavage fluid and plasma, and between PCOs, TBARS and myeloperoxidase (an index of neutrophil activation) in the lungs, indicating that neutrophil oxidants could be responsible for the injury. These results provide evidence of early oxidation in severe sepsis and major-trauma patients, with PCOs being a sensitive index of this process. A recent study has confirmed that severe sepsis results in carbonylation of plasma proteins [41].

Conclusions and perspectives
The current rapid progress in the identification of carbonylated proteins should provide new diagnostic biomarkers for human diseases that are associated with oxidative stress. For example, increases in total oxidized protein levels were observed in blood from both AD subjects and AD relatives when compared with non-AD controls. A protein that is uniquely oxidized in the plasma of AD subjects was shown to be much more susceptible to oxidation than the corresponding control protein when plasma was subjected to oxidative stress in vitro [42]. This observation becomes even more interesting following recent studies of protein carbonylation in prokaryotic systems [43,44]. These studies showed that misfolded proteins are more susceptible to carbonylation than native ones, suggesting that carbonylation, being an irreversible protein modification, could signal that a protein is irreparable and, hence, act as a tagging system for the proteolytic pathway. It is thought that protein carbonylation targets the modified (and generally dysfunctional) protein to degradation by the proteasomal system in oxidatively stressed mammalian cells [17]. Studies using prokaryotes have raised the possibility that, in AD and other diseases, some proteins are more susceptible to carboxylation because they are misfolded (and, consequently, dysfunctional), rather than being dysfunctional because carbonylation has made them misfolded. The principal carbonylated plasma proteins are isoforms of the fibrinogen-γ-chain precursor protein and of α-1-antitrypsin, which exhibit a two- to six-fold greater specific-oxidation index in plasma from AD subjects than from age-matched controls. This suggests that these oxidized plasma proteins could be useful as diagnostic (pre-symptomatic) biomarkers for AD [45].

Protein carbonylation has been associated with important functional alterations in a variety of structural and enzymatic proteins. For example, actin carbonylation is a sign of severe functional impairment associated with filament disruption [46], and occurs at an extent of oxidative insult higher than that causing the oxidation of methionine residues crucial for actin function [47]. This suggests that methionine oxidation could be a damaging event that precedes the actin carbonylation observed in AD. Inflammatory bowel disease and rat myocardial ischaemia [14,48,49]. Interestingly, a significant decline in the activity of methionine-sulphoxide reductase (which regenerates methionine residues within oxidized proteins, thereby restoring function) has been observed in AD brain regions showing a significant PCO elevation [50].

The potential involvement of ROS in the pathogenesis of several diseases suggests that free-radical scavengers and antioxidants might have therapeutic uses. Although there is significant experimental evidence demonstrating the protective effects of antioxidants in in vitro models (for example of neurodegeneration) and in some animal models, the clinical evidence for such protection is relatively scarce and/or controversial.

Several free-radical scavengers, such as vitamin E, selegline (a monoamine-oxide inhibitor) and Ginkgo biloba extract Egb 761, have produced positive therapeutic results in AD, as have anti-inflammatory drugs,
oestrogens and desferrioxamine (an iron-chelating agent), all of which have an antioxidant effect [51]. With regard to carbonyl stress, possible therapeutic interventions to limit carbonyl toxicity (both genetically and pharmacologically) are currently being explored [52]. These strategies could be employed as interventions during disease progression or as preventive measures.

By contrast with AD, there is no clear evidence for a beneficial effect of α-tocopherol (a form of vitamin E) or selegiline in patients with amyotrophic lateral sclerosis, even though levels of 4-hydroxynonenal, nitrotyrosine and PCOs are increased, indicating that oxidative stress might be involved in its pathology [53–55].

One possible limitation of antioxidant therapy is that significant damage to macromolecules, and tissue injury, which ultimately lead to cell death, will already have occurred by the time overt symptomatology of disease is observed. Hence, antioxidant therapy can only, at best,
rescue undamaged macromolecules and surviving cells, an effect that might not be sufficient to attenuate the symptomatology. It is therefore important to begin therapy at an early stage of the disease (Fig. 3). Another serious limitation of antioxidant therapies is that clinicians cannot yet determine which individuals might be benefit from which antioxidant therapy. The optimum daily doses, even of common antioxidants such as α-tocopherol and vitamin C, are subject to debate, and no guidelines have ever been considered for less common, but not necessarily less significant, antioxidants such as γ-tocopherol. In addition, some subgroups of individuals might react negatively to particular antioxidants, as shown by the apparent exacerbation of lung cancer among patients taking β-carotene [2]. Therefore, clinicians will need to determine both the type of antioxidant supplementation appropriate for a specific individual, and the responsiveness of this patient to the prescribed treatment.

It should be emphasized that the detection of more than one marker for oxidative stress is key, because a single marker might give misleading results. Furthermore, it is important to examine whether the level of a specific oxidative marker reflects the severity of the oxidative stress exerted on the subject (as verified in some cases). It is also crucial to determine whether particular markers, alone or in combination with others, can serve as a true indicator of the health status of the subject, thereby allowing the success (or the failure) of a treatment to be monitored (Fig. 3). Although the level of PCOs is currently the best overall marker of oxidative-stress-mediated protein damage, the development of analytical procedures that can measure the levels of each kind of protein modification (like that showing that glutamic acid and aminoadipic semialdehydes are the main carbonyl products of metal-catalysed protein oxidation [56]) should be encouraged. Identification of each kind of protein modification is necessary for determining why individuals vary in their susceptibility to oxidative stress, as well as in their responsiveness to antioxidant therapy. This will also help to establish the relative importance of particular types of oxidative stress in the development of specific diseases.

The challenges for the future are to elucidate of the mechanisms that lead to disease-associated oxidative stress, to develop effective therapeutic antioxidants, and to demonstrate their benefit to patients. However, before antioxidant therapy becomes accepted, detailed longitudinal studies will need to be conducted, evaluating panels of oxidative biomarkers along with traditional clinical endpoints in patients undergoing treatment for diverse chronic illnesses. The completion of these studies will usher in a new era in diagnosis and therapy of human diseases associated with oxidative stress.

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References
27 Ramsay, P.L. et al. (2001) Clara cell secretory protein oxidation and
42 Ballesteros, M. et al. (2001) Bacterial senescence: protein oxidation in non-proliferating cells is dictated by the accuracy of the ribosomes. EMBO J. 20, 5280–5289
52 Ferrante, R.J. et al. (1997) Evidence of increased oxidative damage in both sporadic and familial amyotrophic lateral sclerosis. J. Neurochem. 69, 2064–2074