As alterations of the redox homeostasis lie at the root of many pathophysiological processes in human health, redox proteomics holds the promise to shed further light on fundamental biological processes. In this review, the mechanisms of reactive oxygen species (ROS) and reactive nitrogen species (RNS) production are reviewed, mainly addressing those chemical phenomena which have already been associated with pathological conditions (of the central nervous system, cardiovascular system, or simply related to aging and altered-cell cycle regulation). From Alzheimer’s to Parkinson’s and Huntington’s disease, from ageing to cancer, oxidative stress (OS) appears to represent a common trait in so many relevant biological aspects of human health, that further investments in the field of redox proteomics ought to be mandatory.

For the foreseeable future, redox proteomics will likely play a pivotal role in the quest for new therapeutical targets and their validation, in the process of determining OS-triggered cellular alteration upon drug treatments and thus in the very heart of the design and testing of new drugs and their metabolites against those pathologies relying on altered redox homeostasis.

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1. **Introduction**

Redox proteomics is an emerging branch of proteomics aimed at investigating oxidative-stress induced modifications of proteins. Oxidative injuries to proteins are produced by chemically reactive species. Modifications could address oxygen species and thus generate Reactive Oxygen Species (ROS), such as hydroxyl, peroxide and superoxide radicals, or produce mixed nitrogen-oxygen species (RNS), viz nitric oxide (NO) and peroxynitrite (ONOO−).

The ROS/RNS are inevitably generated in metabolic pathways in all cells, and some of them might play important roles in cell signalling [1,2]. However, excessive levels of ROS from either the environment or aberrations in electron transport can produce such high levels of oxidative stress (OS) that large amounts of proteins can be irreparably altered [3]. Under chronic OS, damaged proteins can even accumulate up to reach toxic levels, often causing cell death as in a plethora of OS-associated physiological disorders and pathological diseases.

Among biomolecules, proteins are major targets of ROS/RNS thus complicating the whole proteome through side-chain modifications and covalent changes which have repercussions on protein activity, unfolding, degradation, as well as in cell functioning [4,5].

Thus, protein-oriented investigations upon prolonged OS-exposure, either under physiological or pathological conditions, are gaining momentum.

2. **Redox proteomics: a brief look at the basics**

Redox proteomics aims at detecting and analyzing redox-based changes within the proteome both in redox signalling scenarios and in OS [5]. The interested reader is referred to Table 1 for a rapid glimpse at the contents of this section, which is mainly focused on the assessment of oxidations/nitrosylations in thiol groups through gel-based and shotgun proteomic approaches and nitration on tyrosines. The basics of carbonylation-targeting redox proteomics are also briefly described.

2.1. **Oxidation/nitrosylation of cysteine thiol groups**

2.1.1. Gel-based approaches

To date, most of the proteomic studies of the oxidative stress response have used 2DE as a protein separation and quantification tool, coupled with mass spectrometry (MS) as a protein identification tool. Despite its weaknesses in the separation of certain protein categories (i.e. more hydrophobic and high molecular weight proteins, such as membrane proteins) and limitation in dynamic range, 2DE is still the best separation tool when dealing with redox-based protein changes. ROS/RNS add different footprints in the cells in the form of covalent modifications to proteins, thus it is often possible to reveal these changes by applying specific labelling followed by detection. A common strategy is to perform western blot analysis of proteins separated by 2DE [6].

Amongst the many kinds of amino acid residues susceptible to oxidative stress, cysteine is by far one of the most sensitive. Oxidation of its −SH groups can have functional significance by regulating protein function and can be the target of oxidative insult as well. For this reason, several experimental approaches have been developed for the systematic and exhaustive characterization of the so-called thiol proteome. One major limit in such an analysis is the chemical labile nature of Cys redox modifications, thus basically two critical steps are needed in analyzing the thiol proteome, which consist in a temporary trapping of free thiols and their subsequent reduction. Different strategies exist for quenching the thiol groups, ranging from the simple TCA (trichloroacetic acid)-based acidification [7] to the use of cell-permeable Cys-specific reagents such as the alkylating agents iodoacetamide (IAA) or N-ethylmaleimide (NEM) [8]. In this approach the subsequent use of more or less selective reducing agents will allow detection of a specific form of oxidation. For instance, cysteine residues in the sulfenic acid form are difficult to identify because of their unstable chemical nature, however this has been achieved by exclusive reduction of the sulfenic acid by sodium arsenite [9], or by its reaction with specific chemicals such as dithioglycol [10]. S-nitrosothiols are rather selectively reduced by ascorbate [11], whereas stronger reductants such as DTT reduce both nitrosothiols and disulfides. However, detection of protein S-nitrosylation is not easily performed with traditional gel-based methods such as immunoprecipitation and western blot analysis where the S—NO bond is broken during the electrophoresis step. On the other hand, commercially available anti-S-nitrosocysteine antibodies have been applied with good results only in immunohistochemistry studies [12,13] and little in the context of proteomics investigations.
2.1.2. Quantitative gel-based redox proteomics

A general workflow in redox proteomics consists of qualitative identification of oxidized proteins and subsequent quantitative determination of the extent of the redox status. Quantifying the redox state of a protein is surely more informative and represents the true challenge. Several thiol-reactive reagents have been used to reveal the extent of Cys oxidation by 2DE gels. They include, for example, the IAM-derivatives 5-iodoacetamidofluorescein [14] and BOD-IPY FL C1-IA [15] or the Cys-specific fluorescent reagent monobromobimane [16]. An improvement of 2DE-based fluorescence analysis of the “redoxome” (redox-proteome) has been obtained by applying the differential in gel electrophoresis (DIGE) technique. This strategy uses a set of fluorophores of similar molecular weights and chemical structures that differ by their spectral features (absorption and emission wavelengths). Redox-DIGE has been performed using the NEM or IAM derivatives of cyanine (Cy3, Cy5) [17–19] and DY-dyes [20]. An important limitation of all the methods described above is that only the most abundant proteins are usually detected, often missing “fancy” proteins such as transcription factors and other regulatory proteins. One way to circumvent this problem is to perform an upstream enrichment.
step for the oxidized protein-thiol fraction of the proteome using the biotin-switch method originally developed by Jaffrey et al. [11]. Since then, several variants have been developed, also with chemical entities alternative to biotin as tag [e.g., 21–23]. Biotin-based strategies are largely used for detection of S-glutathionylation [24,25] and S-nitrosylation [21], two Cys modifications which occur extensively in diseases characterized by oxidative stress.

2.1.4. Shotgun proteomics
Due to the technical limitations of 2DE-based methods, in recent years emphasis has gradually shifted towards gel-free technologies, such as shotgun-proteomics strategies. 2DE is, in fact, time- and labour-consuming with the major drawback that the extent of oxidation always corresponds to an average contribution of the Cys residues present in a polypeptide. The shotgun-proteomics approach in its most general sense refers to the direct analysis by MS/MS of proteolyzed protein mixtures to rapidly generate a global profile of the protein complement within the mixture itself. This mixture, however, is generally highly complex. A solution to overcome this hurdle is represented by alternative sample preparation strategies, which could be suitable to perform a preliminary enrichment of peptides containing redox-modified cysteines. Although various extraction and enrichment strategies have been developed for cysteinyl-peptide enrichment prior to MS analysis [27], only few methods are directed toward isolating peptides containing oxidized cysteines [28]. One of the most recent approaches designed for the specific enrichment of sulfopeptides in tryptic digests is based on anionic affinity capture using poly-arginine-coated nanodiamonds as high-affinity probes [28]. The method was applied to selectively concentrate peptides containing Cys sulfonic acid from either a highly dilute solution or a complex peptide mixture in which the abundance of the sulfonated analyte was as low as 0.02%.

2.1.4. Quantitative redox proteomics
The most attractive redox proteomic techniques are those related to quantification of changes in a thiol redox proteome upon OS exposure. In this respect, Sethuraman et al. described an interesting shotgun proteomic approach, which exploits isotope-coded affinity tag (ICAT) reagents [29] to quantify oxidant-sensitive protein thiols. ICAT reagents have been extensively used in quantitative proteomics to evaluate the abundance of expressed proteins [30] and, more recently, successfully applied to redox proteomics. This technique uses a certain type of marker which consists of three different parts: (i) a thiol-reactive compound (an iodoacetamide analogue), (ii) a linker containing either heavy or light isotopes, and (iii) a biotin moiety of the ICAT reagent. After exposing equivalent protein samples to either control or oxidant conditions in a non-reducing environment, they are differentially labelled with the heavy or light form of the ICAT. The protein samples are mixed and, after tryptic digestion, the labelled peptides are separated by affinity chromatography. Finally, the captured peptides are analyzed by LC-MS/MS for identification of the oxidant-sensitive cysteine thiols. Because oxidized cysteines are not susceptible to labelling with ICAT reagent, the labelling intensity decreases from the control to the oxidized sample [31,32]. This approach is potentially more powerful than conventional methods, especially because the protein identification and the quantification of the extent of thiol oxidation are made in the same analysis [32]. A remarkable development of this methodology has been recently presented and the adaptation of ICAT to redox proteomics has been definitively ratified with the coinage of the term OxICAT [33]. In the first step of OxICAT, proteins are denatured to gain access to all reduced cysteines which are irreversibly labelled with light ICAT. All reversible oxidative thiol modifications within the same sample are then reduced using the strong thiol reductant Tris(2-carboxyethyl) phosphine (TCEP) and all newly accessible cysteines are modified with heavy ICAT. Importantly, this procedure generates chemically identical proteins, which only differ in the specific mass of their ICAT-label (9 Da additional mass per heavy ICAT) depending on their previous redox state. Peptides that contain originally only reduced cysteines are predicted to yield single mass peaks corresponding to the light ICAT-labelled form. Peptides containing originally oxidized cysteines will have masses that are exactly 9 Da higher (or multiples thereof) than the corresponding light ICAT peptides, depending on the number of oxidized cysteines present. After tryptic digestion and affinity purification, the ICAT-labelled peptides are identified by LC-MS/MS, which also establishes the ratio of oxidized to reduced (heavy to light) Cys residues according to the relative MS intensities. As the extent of oxidation is given as an Ox/Red ratio, absolute protein amounts are not considered, therefore allowing cell extracts comparisons [33]. Contrary to the approach of Sethuraman and coworkers, where relative changes in the availability of free protein thiols are determined in two separate samples, the OxICAT method provides the absolute ratio of reduced to oxidized protein within a single sample making this technique suitable to monitor oxidative thiol modifications in vivo. Interestingly, slight modifications of the basic scheme, i.e. substitution of the nonspecific thiol reductant TCEP with more specific reductants, such as glutaredoxin or ascorbate, may allow the use of OxICAT to specifically detect glutathionylations or nitrosylations, respectively.

2.2. Analysis of nitrated peptides
Protein tyrosine nitration to form 3-nitrotyrosine (3-NT) is an important post-translational modification which has widely considered as a valid biomarker of protein RNS insult [34] occurring in a variety of diseases including cancers, neurodegenerative and age-related disorders [35]. 3-NT is also a relatively stable modification, thus it can be suitably analyzed by different specific techniques. Methods for separation, detection, and quantification of 3-nitrotyrosine in biological samples include immunochemical techniques using anti-3-nitrotyrosine antibodies, liquid and gas chromatography in combination with various detection systems [36,37]. However, with the introduction of MALDI and ESI as soft ionization methods for mass spectrometry of biomolecules, nitration has been also investigated directly at the protein level with the ability to determine polypeptide modification sites [38–40]. Indeed, application of mass spectrometry, in combination
with 2DE separation and western blot analysis [41] attempted a higher throughput characterization of protein targets for tyrosine nitration in cells and several tissues [42–45]. In the case of MS analysis of tyrosine-nitrated peptides, a characteristic mass increase of 45 Da corresponding to NO₂-Tyr is observed by both ESI and MALDI methods [44,46]. However, during MALDI analysis of the nitrated peptides, a series of additional modified peaks were observed as a consequence of photodecomposition reactions determining the formation of 3-NO-Tyr, 3-NHOH-Tyr and 3-NH₂-Tyr adducts [47,48]. This means that MALDI measurements yield to significant underestimation of the modification extent, highlighting the unreliability of this methodology for the sensitive detection of nitrated products. On the contrary, this phenomenon of artificial NO₂-Tyr fragmentation was not observed in ESI-MS measurements, allowing a complete evaluation of the protein nitration level. Moreover, the use of precursor ion scanning for the specific immonium ion at m/z 181.06 has found a broader application in the identification of nitrated peptides during LC-ESI-MS/MS analysis of protein digests [47]. At any rate, protein tyrosine nitration is typically a low-yield process and though mass spectrometry represents a sensitive analytical method, proteome-wide analysis of nitrated proteins/peptides is still challenging due to the lack of effective enrichment methods prior to mass analysis. This is likely due to the poor chemical reactivity of nitric oxide groups [34]. However, the nitro groups can be converted to the more chemically reactive amine groups which can be hence used as a chemical handle to employ tagging groups in nitrated residues. The tagged peptides are then extracted and isolated to solid supports and subsequently analyzed by MS which gives highly enhanced signal corresponding to the analyte peptide. To provide some recent examples in this regard, Nikov et al. reported a method for the enrichment of nitro-Tyr-containing proteins based on a first immunoprecipitation with anti-nitrotyrosine antibodies followed by a reduction of nitro-Tyr to amino-Tyr with sodium dithionite and subsequent biotin tagging. Protein sample was then proteolyzed, thereby the resulting biotinylated tryptic peptides were purified on a streptavidin affinity column and identified by MS [49]. Zhang et al. introduced an improved strategy where nitrotyrosines were firstly derivatized into free sulphhydryl groups through N-succinimidyl S-acetyltioacetate (SATA) and sulphhydryl-containing peptides were subsequently enriched with thiopropyl sepharose beads [50]. Very recently, Lee and co-workers introduced a new efficient chemical approach for the enrichment of nitrated peptides based on incorporation of a metal chelating motif into the modified tyrosine residues [51]. This strategy comprised a series of chemical modifications to convert the nitro groups on the tyrosine side chains to the metal chelating groups (bispyridinylated tyrosines) followed by solid-phase extraction with Ni²⁺-nitrilotriacetic acid (NTA) magnetic agarose beads [51]. Interestingly, the same research team developed another efficient enrichment method of nitrated peptides one year later [52]. The feasibility of this new approach was tested with success on in vivo model systems, providing an alternative tool for nitroproteome profiling. The method is based on fluorine-fluorine interaction affinity purification following chemical conversions of the nitro groups on tyrosines residues to highly fluorinated moieties [52]. Alternatively, an innovative approach involving dansyl chloride labelling of nitration sites that rely on the enormous potential of MS³ analysis has been reported by Amoresano et al. [53]. Briefly, discrimination between nitro- and unmodified peptides is based on two instrumental selectivity criteria obtained by combining a precursor ion scan and an MS³ analysis. Following MS/MS fragmentation, in fact, dansyl-derivatized peptides produce a stable fragment ion at m/z 170 useful for a precursor ion scan; moreover these species undergo a specific and diagnostic transition from m/z 234 to 170 in MS³, effectively providing the second selectivity criterion [53]. Interestingly, taking advantage of the experience made with the above strategy, the Amoresano’s laboratory also developed an analysis method for the simultaneous localisation and quantification of 3-NT residues in proteins, by exploiting the potential of iTRAQ reagents [54].

### 2.3. Carbonylations

After the reactions involving the sulphur-containing amino acids, carbonylation is the most commonly occurring oxidative protein modification. Lysine, arginine, proline, and threonine side-chains can be oxidatively converted to reactive aldehyde or ketone groups (carbonylation) causing inactivation, crosslinking or breakdown of proteins [55,56]. 2-Amino-adipic semialdehyde (AAS) and gamma-glutamyl semialdehyde (GGS) are the most abundant carbonyls in aged cells [57]. Multiple methods have been described for selection and recognition of carbonylated proteins, all of which exploit the relatively unique property of carbonyl groups to form Schiff bases. Affinity chromatography can be used to select oxidized proteins upon derivatization of carbonyls through dinitrophenylhydrazine (DNPH) or biotin hydrazide (BH2). This protocol ends up greatly enriching oxidized proteins or their proteolytic fragments in the process.

After a convenient method was developed for detecting protein carbonyls on PVDF membrane [58], electrophoretic and proteomic analyses of carbonylated proteins in OS-related pathologies have been extensively carried out by many groups [59–66]. As a rule of thumb, proteomic analysis of carbonylated proteins through BH2 tagging have been achieved in three different ways: (i) performing tryptic digest of biotinylated samples followed by affinity selection and RP-LC-MS/MS identification; (ii) targeting native proteins with subsequent affinity selection, proteolysis and RP-LC-MS/MS identification; (iii) further fractionating affinity selected biotinylated proteins by LC before proteolysis and identification of peptide fragments. Interestingly, Mirzaei and Regnier [67] compared these three different strategies. They found that performing the affinity selection and chromatography at the protein level before proteolysis and MS protein identification is more informative, due to the possibility of detecting also cross-linked or truncated protein forms, thus expanding the level of structural discrimination on the separation side. This strategy is not restricted to liquid chromatography, as 2DE can also be used. Unfortunately, DNPH derivatization changes the isoelectric point of proteins and can lead to sample loss during fractionation. One way to deal with this problem is by starting the fractionation with isoelectric focusing, then derivatizing with DNPH, and finally going to molecular weight separations [68]. However, as previously mentioned above, limitations of 2DE are low sensitivity, poor reproducibility, and limited dynamic range.
ROS represent a fundamental asset to immune cells in immune system responses against pathogens [72] and also play a physiological key role in normal plant cell physiology by triggering specific cascades [73]. Nonetheless, in humans OS is also involved in many diseases (Table 2). Examples include atherosclerosis/vascular aging [74], heart failure and myocardial infarction [75], nervous system diseases, such as Parkinson’s and Alzheimer’s disease (AD) [76], muscular dystrophy [77], fragile X syndrome [78] and chronic fatigue syndrome [79], but short-term OS may also be important in prevention of aging [80] through the induction of a process named mitochondrial hormesis (or mitohormesis) [81]. In parallel, as treatments for the diseases of youth and middle age have helped raise life expectancy significantly, OS has been growingly tied to aging through the gradual decline of cognitive capacity [82]. As it has progressively emerged, the central nervous system appears to be an eligible target for irreversible damage provoked by ROS.

In recent years, a role for redox proteomics has progressively emerged in delving into alterations redox poise which target protein species in several diseases. However, only preliminary results are currently available, as it will be discussed as follows.

3.1. Nervous system and OS

Due to its high rate of oxygen utilization, high content of unsaturated lipids and relative lack of antioxidant enzymes, the brain is very vulnerable to free radical damage. Indeed, OS is associated with the onset and pathogenesis of several prominent central nervous system disorders [83]. Along with neurotrophic support, a series of dramatically widespread nervous system diseases, such as Alzheimer’s, Parkinson’s and Huntington’s disease are thought to be triggered by oxidative damage [84]. OS can cause down-regulation of neurotrophic factors. While normal functioning of the nervous systems involves a positive feedback loop between antioxidant processes and neurotrophic support, breakdown of this feedback loop ultimately leads to diseased states [84].

Amyotrophic lateral sclerosis rightfully belongs to the group of the most relevant neurodegenerative syndromes worldwide. Again, the relationship between protein aggregation and the molecular events leading to neurodegeneration has not yet been exhaustively clarified, although it seems to involve mitochondrial dysfunction and thus, oxidative damage resulting in mutated and/or misfolded proteins [85].

Accumulating data indicate that OS plays a major role in the pathogenesis of multiple sclerosis as well. ROS generated in excess primarily by macrophages, have been implicated as mediators of demyelination and axonal damage in both multiple sclerosis and experimental autoimmune encephalomyelitis [86]. These preliminary observations made it desirable to pursue antioxidant treatments against this disease, although their efficacy has yet to be fully demonstrated [87].

OS to the central nervous system also leads to a wide array of behavioural disorders, such as anxiety [88], depression [89], bipolar disorders and schizophrenia [90].

An increasing number of individuals regularly consume a diet high in fat, with high-fat diet consumption known to be sufficient to promote metabolic dysfunction due to an increased susceptibility to OS, although the links between high-fat diet consumption, aging and brain aging, in particular, are only now beginning to be elucidated. Analogous considerations could be made for a wide array of metabolic disorders (type II diabetis, obesity, etc.).

<table>
<thead>
<tr>
<th>Disease/pathological condition</th>
<th>Oxidative stress alterations</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Neurodegenerative diseases (Parkinson’s, Alzheimer’s, Huntington’s diseases, amyotrophic lateral sclerosis)</td>
<td>Altered protein nitrosylation, carbonylation ubiquitination/degradation, (amyloid beta peptide, glutamine synthase, etc.); nitric oxide toxicity; DNA and lipid oxidative damage; MDA; GSH oxidation; Complex I electron transport/alteration; oxidation of NADH; PRDX1, 2, 6 and glutathione peroxidases induction; mitochondrial Cu,Zn-Superoxide dismutase 1 mutations; GSH-px, SOD, CAT activities; MDA; altered peripheral oxidative stress (erythrocytes, platelets, plasma);</td>
<td>[24,25,27,28] (AD)</td>
</tr>
<tr>
<td>Behavioural and mood disorders (depression, schizophrenia, psychosis, anxiety)</td>
<td>Nitric oxide homeostasis; xanthine oxidase; lipoxygenase; NADPH oxidase; altered mitochondrial redox homeostasis; peroxynitrite-mediated nitration and inhibition of MnSOD; decline in GSH content; Nr2f2/ARE dysfunction; dysfunctional electron transport chain; p66HSC</td>
<td>[78–81]</td>
</tr>
<tr>
<td>Atherosclerosis, vascular reperfusion injury, ischemia, hypoxic stress</td>
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<tr>
<td>Cellular/tissue/organ aging; cancer</td>
<td>Free radical accumulation; aerobic glycolysis; impaired electron transport (mitochondria);</td>
<td>[126,129] (ageing) [86] (cancer)</td>
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</tbody>
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3.1.1. Alzheimer’s, Parkinson’s and Huntington’s disease

3.1.1.1. Alzheimer’s disease. While life expectancy has increased over the last century, cognitive decline and progressive memory loss due to gradual neurodegeneration has emerged as one of the greatest health threats of old age, with nearly 50% of adults over the age of 85 afflicted with AD. Developing therapeutic interventions for such conditions demands a greater understanding of the processes underlying normal and pathological brain ageing. In this respect, the emerging field of neuroproteomics promises to provide powerful strategies for further characterizing neuronal dysfunction and cell loss associated with neurodegenerative diseases [91]. Recent advancements in the biology of ageing in model organisms, together with molecular and systems-level studies of the brain, are beginning to shed light on these mechanisms and their potential roles in cognitive decline [82].

Oxidative damage can lead to several events targeting, lipids, carbohydrates, DNA, RNA and proteins. As for the latter, the loss in specific protein function, abnormal protein clearance, depletion of the cellular redox balance end up interfering with the cell cycle, and, ultimately, lead to neuronal death. Protein carbonyls, a marker of protein oxidation, are increased in AD brain [92]. Some preferential protein targets have been identified through preliminary immunochemistry investigations, such as creatine kinase B8 and beta-actin [51,52], while others (glutamine synthase, and ubiquitin carboxy-terminal hydrolase L-1) have recently been added to the list through widespread redox proteomics approaches (2DE, western blot through anti-glutamine synthase antibodies, MALDI-TOF) [92]. Along with these markers, amyloid β-peptide (1-42) has been shown to induce OS in vivo [93]. Indeed, AD pathophysiology is characterized by the presence of extracellular senile plaques, intracellular neurofibrillary tangles, and synapse loss, the former being composed of an amyloid beta peptide (Aβ) core surrounded by dystrophic neurites. Aβ peptide is a 39-43 amino acid peptide that is derived from the proteolytic processing of amyloid precursor protein (APP), a ubiquitously expressed transmembrane glycoprotein. Aβ is produced by proteolytic cleavage of APP at the amino terminus by β-secretase and at the carboxy terminus within the lipid bilayer by γ-secretase. Cleavage by γ-secretase can occur at different positions within the carboxy terminus of the Aβ resulting in the production of Aβ peptides of varying length. Aβ1-40 and Aβ1-42 constitute the majority of the Aβ peptide found in the human brain [93]. As a result of oxidative damage, also anomalous protein nitrosylation (3-nitrotyrosines) at cysteine residues, carboxylations and altered composition of ubiquinated proteins (mainly are glial fibrillary acidic and tau proteins in AD-affected patients) have been widely observed in AD [94,95]. Tau proteins are also hyperphosphorylated in AD patients [95]. These recently gained insights might be important in providing potential targets for drug therapy in AD.

3.1.1.2. Parkinson’s disease. Parkinson’s disease (PD)-affected patients suffer from progressive dopaminergic neurodegeneration in basal ganglia and the substantia nigra, accumulation of Lewy bodies, as well as more widespread neuronal changes that cause complex and variable motor and non-motor symptoms. OS plays a dramatic role in PD, in that it is involved in dopamine cell degeneration and intimately linked to other components of the degenerative process, such as mitochondrial dysfunction, excitotoxicity, nitric oxide toxicity, S-nitrosylation and inflammation. It is therefore difficult to determine whether OS leads to, or is a consequence of these events [96]. Oxidative damage to lipids, proteins, and DNA occurs in PD, and toxic products of oxidative damage, such as 4-hydroxynonenal (HNE), can react with proteins to impair cell viability. There is a convincing evidence for the involvement of nitric oxide that reacts with superoxide to produce peroxynitrite and ultimately hydroxyl radicals production. The main concept involves the metabolism of dopamine, which might be responsible for the high basal levels of OS in substantia nigra. The degradation of dopamine by monoamine oxidase to produce hydrogen peroxide (H2O2) further emphasized how OS might arise [97]. Enzymatic oxidation of dopamine to H2O2 caused increased formation of oxidized glutathione (GSH), suggesting the occurrence of OS and impairment of a major antioxidant system.

Another mechanism which has recently been implicated as key to dopaminergic cell death in PD involves altered ubiquitination and degradation of proteins [98]. OS can impair these processes directly and products of oxidative damage, such as HNE, can damage the 26S proteasome. The ubiquitin-proteasome system and proteolytic stress underlie nigral pathology in both familial and sporadic forms of the illness [99]. Accumulating evidence suggests that mutations in alpha-synuclein, that cause the protein to misfold and resist proteasomal degradation, are at the root of familial PD. Similarly, mutations in two enzymes involved in the normal function of the ubiquitin-proteasome system, parkin and ubiquitin C-terminal hydrolase L1, are also associated with hereditary PD. Furthermore, structural and functional defects in 26S proteasomes with accumulation and aggregation of potentially cytotoxic abnormal proteins have been identified in the substantia nigra pars compacta of patients with sporadic PD [98].

In PD, especially in sporadic PD, an increase in OS damage to mitochondrial components is often observed. This is linked to systemic deficiency of the electron transport chain NADH-quinone oxidoreductase (Complex I) activity, and thus results in a reduced bioenergetic capacity, making it clear that mitochondrial dysfunction lies at the root of PD [100]. In molecular terms, oxidation of the catechol ring of dopamine results in the formation of ROS and the electron-deficient dopamine quinone. Exposure of mitochondria to oxidized dopamine results in uncoupling of mitochondrial respiration and mitochondrial swelling. Electron-deficient dopamine quinones will readily bind to thiol groups on proteins, often resulting in inactivation of the protein function [97]. In parallel, Bisaglia et al. have observed that the generation of dopamine quinones in isolated respiring mitochondria triggers the opening of the permeability transition pore, most probably by inducing oxidation of NADH [101], further impairing the bioenergetic activity of mitochondria.

Concordingly, products of several PD-associated genes, including SNCA, Parkin, PINK1, DJ-1, LRRK2 and HTR2A, show a degree of localization to the mitochondria under certain conditions [78]. Genetic therapies aimed at restoring Complex I activity have recently been proposed. One of these approaches exploits the yeast alternative NADH dehydrogenase, the Ndi1 protein, to reinstate the mitochondrial respiratory chain. The
benefits which stem from compensation for disabled complex I through Ndi1 seem to include retardation of PD [102].

The role of proteomics in research on PD has recently expanded, especially on animal models of PD [82]. The shift towards a protein-oriented scenario mainly lies on the accumulation of a plethora of data pinpointing an abnormal processing of the neuronal protein α-synuclein as a pivotal mechanism leading to aggregation, inclusions formation and degeneration [103].

3.1.2.1. Amyotrophic lateral sclerosis. Along with Parkinson’s, Alzheimer’s and Huntington’s diseases, amyotrophic lateral sclerosis (ALS, also referred to as Lou Gehrig’s disease) is a neurodegenerative disorder that results in loss of motor neurons, leading to a rapidly progressive form of muscle paralysis that is fatal [108]. There is no available cure and current therapies only provide minimal benefit at best. The real problem is the lack of in-depth knowledge of the molecular causes of ALS, as mutations in the Cu,Zn-Superoxide dismutase 1 (SOD1) gene account for only 20% of familial ALS. Nonetheless, recent evidence suggests that OS plays a central role in ALS as well. In a recent study, Baillet et al. [109] examined OS markers of 31 patients suffering from ALS against 30 matched controls, either aiming at determining oxidation levels for lipids (malondialdehyde — MDA) and proteins (plasma glutathione, carbonyls and thiols), and the activity of antioxidant enzymes i.e. erythrocyte Cu,Zn-SOD1, Glutathione peroxidase (GSH-Px) and catalase. MDA and thiols were significantly higher in ALS patients versus control population. In parallel, a trend for an increase in oxidized glutathione was noted in ALS patients. Moreover, univariate analysis showed that SOD activity was significantly decreased in ALS.

Analogously, Bonnefont-Rousselot et al. compared blood samples from 167 ALS patients against control subjects in order to assess peripheral oxidation levels related to this disease [110]. Significantly higher values of thiobarbituric acid-reactive substances and a significant enhancement of the erythrocyte SOD activity were observed in the ALS patients. Anomalously mutated SOD1 proteins have been related to ALS onset and mitochondrial dysfunction [111], which might thus represent a converging point of multiple pathways underlying ALS pathogenesis and progression.

Taken together, these observations emphasize the role of OS in ALS. So far, proteomics application to ALS research has been only limited to animal models [112]. Meanwhile, recent reviews highlighted an increased incidence of protein nitration in a series of neurodegenerative diseases, including ALS, which mainly target MnSOD and neurofilament-L [113]. In the light of these early results, experimentation on human samples will represent a significant area of research for the next few years.

3.1.2.2. Multiple sclerosis. Multiple sclerosis (MS, also known as disseminated sclerosis or encephalomyelitis disseminata) is an inflammatory disease in which the fatty myelin sheaths around the axons of the brain and spinal cord are damaged, leading to demyelination and scarring as well as a broad spectrum of signs and symptoms, viz. hypoesthesia and paraesthesia, ataxia, dysarthria, dysphagia and many others. OS plays a major role in the pathogenesis of MS. ROS, leading to OS, generated in excess primarily by macrophages, have been implicated as mediators of demyelination and axonal damage in both MS and experimental autoimmune encephalomyelitis (EAE), its animal model. ROS cause damage to cardinal cellular components such as lipids, proteins and nucleic acids (e.g., RNA, DNA), resulting in cell death by necrosis or apoptosis. In addition, weakened cellular antioxidant defense systems in the central nervous system (CNS) in MS, and its vulnerability to ROS effects may increase damage. Thus, treatment with antioxidants might theoretically prevent propagation of tissue damage and improve both survival and neurological outcome. Indeed, several experimental
studies have been performed to see whether dietary intake of several antioxidants prevents or reduces the progression of EAE. Although a few antioxidants showed some efficacy in these studies, little information is available on the effect of treatments with such compounds in patients with MS. Well-designed clinical studies using antioxidant intake, as well as investigations based on larger cohorts studied over a longer periods of time, are needed in order to assess whether antioxidant intake together with other conventional treatments, might be beneficial in treating MS [114].

3.1.3. Depression and behavioural disorders
If on the one hand OS has been increasingly related to neurodegenerative diseases, accumulating evidence suggests for a role of ROS and RNS in anxiety, depression, bipolar disorders and schizophrenia as well.

Anxiety is a normal emotional response to a threat or potential threat, which becomes pathological when the fear is disproportionate to the nature of the threat. Upon assessment of GSH-Px SOD, CAT activities and MDA levels in 20 patients affected by psychiatric disorders against matched controls, Kuloglu et al. [115] established a link between OS and certain anxiety disorders (obsessive–compulsive disorder and panic disorder), demonstrating that other systems, such as oxidative metabolism, can affect the regulation of anxiety. These observations were even more evident in patients affected by social phobia, while no relation between OS and post-traumatic stress disorders has been documented [116].

Conversely, there is mounting evidence of altered antioxidant enzyme activities and increased levels of lipid peroxidation in schizophrenia [117,118]. Zhang et al. reported that the activities of SOD and GSH-Px were decreased but levels of MDA were elevated in patients with a chronic form of schizophrenia as compared with normal controls [117]. SOD and GSH-Px activities were found to be significantly lower in paranoid and residual subtypes compared to both disorganized subtype and the control group. MDA levels were significantly higher in all subtypes (paranoid, disorganized and residual groups) compared to the control group. Concomitantly, a decreased GSH: GSSG ratio was also found in the schizophrenic group, along with a negative correlation to age of both GSSG and glutathione reductase levels in schizophrenic patients, when compared against matched controls [119].

Notably, a peripheral component to OS in psychiatric patients has been growingly reported, starting from observations of protein and lipid oxidation in erythrocytes [120], plasma [121] and platelets [122] of this category of patients. These observations are suggestive of a likely systemic oxidative unbalance which ultimately exerts its main negative effect, again, at the brain level.

Analogously, converging evidence suggests that systemic OS plays a critical role in the pathophysiology of depressive disorder and associated medical co-morbidities. Indeed, nearly 40 studies have identified biomarkers of oxidative damage in the venous peripheral blood of patients [123]. Nonetheless, Teysier et al. concluded that the pathogenic role of the OS in the cerebral mechanism of depression cannot be inferred from the alteration of peripheral parameters, as no significant differences were observed in the levels of SOD1, SOD2, CAT, glutathione peroxidase 1 (GPx1), 8-oxoguanine DNA glycosylase, nei-like 1, methionine sulfoxide reductase A, telomere repeat-binding factor 2 and C-FOS, when comparing prefrontal cortex of patients with depressive disorder against matched controls [123].

3.2. Vascular aging
Whereas the central nervous system represents the most susceptible target to OS, accumulation of OS in peripheral blood has deleterious effects on the cardiovascular system, proportionally to aging or pathophysiological conditions. The large and medium-sized arteries in elderly people show varying degrees of intimal and medial change. The medial change is known as age-related medial degeneration and sclerosis. The smooth muscle cells in the inner half of the aortic media of elderly people degenerate and undergo apoptosis. This causes degradation of elastin fibers and the accumulation of collagen fibers in the media, but the inflammatory infiltrates are scarce [74]. Age-related decrease of elastin and its crosslinks, and an increase of collagen and its crosslink have been positively related to an increased likelihood of glycation (Maillard reaction) and glyco-oxidative reaction [74].

ROS accumulation under pathophysiological conditions increases the incidence of cardiovascular diseases. ROS are released from different sources, such as xanthine oxidase, lipooxygenase, NADPH oxidase, the uncoupling of nitric oxide synthase and, in particular, mitochondria [124]. Endothelial dysfunction, characterized by a loss of nitric oxide (NO) bioactivity, occurs early on in the development of atherosclerosis, and determines future vascular complications.

Other factors triggering cardiovascular aging and atherosclerosis seem to involve anomalous protein nitration, especially of apolipoproteins (ApoB-100, APOE) or complement/ inflammation components (C3, complement factor H; CD5, prostacyclin synthase), even if most of these preliminary redox proteomics investigations have been performed on animal models [113].

Although the molecular mechanisms responsible for mitochondria-mediated disease processes are not clear, OS seems to play an important role. In general, ROS are essential to cell function, but adequate levels of antioxidant defenses are required in order to avoid the harmful effects of excessive ROS production. Mitochondrial OS damage and dysfunction contribute to a number of cell pathologies that manifest themselves through a range of conditions [125]. The molecular mechanisms responsible for age-related mitochondrial OS in the vasculature are multifaceted and likely involve cell-autonomous effects, including dysregulation of antioxidant defenses such as peroxynitrite-mediated nitration and inhibition of MnSOD, decline in GSH content, Nrf2/ARE dysfunction, and a dysfunctional electron transport chain, as reviewed by Ungvari et al. [125].

Mitochondrial redox status and induction of apoptotic cascades appear to be regulated by p66Shc, the “longevity gene”, since it has been related to an augmented lifespan in mammals [126,127].

3.3. Aging and cancer
OS produced by ROS activity might be responsible for apparently antithetic biological processes, such as cellular aging and
cancer, the former leading to cellular senescence, while the latter resulting in cellular immortalization.

Biological aging is a physiological process which represents a major risk factor in the development of neurodegenerative and cardiovascular diseases, as well as cancer, in vertebrates [128].

During the last decades, the common view of ageing as a stochastic process has been gradually replaced by the notion that a tight regulatory system modulates the maximum lifespan of an organism, through a series of mechanisms which involve ROS-triggered oxidative damage, and their correlation to life-span and incidence of age-related diseases [129,130].

While it was previously thought that a major role was merely played by telomere attrition [131], unbalanced regulation of mitochondrial ROS production appears to turn on cellular senescence programs through multifaceted mechanisms either involving p53 oncosuppressor or Ras oncogene, as elegantly schematized by Colavitti and Finkel [130]. Nonetheless, both pathways are realistically intertwined, as it recently emerged that telomerases might also play a telomere-independent survival function. Telomerase is a ribonucleoprotein that counteracts telomere shortening and can immortalise human cells, while it has recently been observed that TERT, the catalytic subunit of human telomerase, protects human fibroblasts against OS [132]. Impaired metabolic regulation leading to mitochondria-triggered ROS production seemingly lies at the root of neurodegenerative and cardiovascular diseases and aging, but also cancer and metabolic disorders.

Emerging evidence indicates that impaired cellular energy metabolism is the defining characteristic of nearly all cancers, regardless of cellular or tissue origin [133]. Nearly all cancers express aerobic glycolysis (the so-called “Warburg effect” [134]), regardless of their tissue or cellular origin. Aerobic glycolysis in cancer cells involves elevated glucose uptake with lactic acid production in the presence of oxygen, due to impaired oxidative phosphorylation. In parallel, numerous studies show that, in tumour cells, mitochondria are structurally and functionally abnormal and incapable of generating normal levels of energy [135,136]. Lipid abnormalities (altered levels of cardiolipin) and impaired electron transport capacity ultimately disrupt the regular flux of oxidative phosphorylation and provoke accumulation of ROS, which gradually but inevitably leads to genomic instability and cancer progression, as Seyfried and Shelton [133] recently reviewed.

4. Drugs and OS

Due to accumulating evidence relating OS to a large number of diseases (see Section 3), there is a growing need for simple, convenient, and reliable markers for the assessment both in vitro and in vivo of the metabolic/oxidative distress and of its modulation, if any, induced by the administration of pharmaceutical products [137]. In a pharmacological perspective, drugs may be designed to target OS-specific biomarkers or to prevent their generation by aiming at blocking ROS sources; conversely, certain classes of drugs might be created to target specific tissues (in cancer, for example) and thereby exert a pro-oxidant effect through local generation of ROS (Fig. 1). In this context, redox proteomics might result pivotal in highlighting which are the main targets of protein oxidations and which biological pathways are involved or compromised by these phenomena. While this application of proteomics to drug designing and development is but at its earliest phases, preliminary redox proteomics results have helped paving the way for further research in this field.

In the following sections a series of examples will be provided, while referring to examples of human pathophysiological conditions involving OS (Section 3 and Table 3).

4.1. CNS: neurodegenerative diseases and mood/psychiatric disorders

4.1.1. Treatment of neurodegenerative diseases through OS-related strategies

As it emerged from the previous section, although neurodegenerative diseases such as AD, PD and HD each have distinct clinical symptoms and pathologies, they all share common mechanisms such as protein aggregation, oxidative injury, inflammation, apoptosis, and mitochondrial injury that contribute to neuronal loss [138]. Therefore, in the treatment of neurodegenerative diseases, neuroprotective agents which target ROS sources and aim at preventing their generation represent one class of drug therapeutics of great interest in the pharmaceutical endeavour. Among them, the inhibitors of type B monoamine oxidase, such as selegiline and rasagiline, are the most promising neuroprotective agents to date, in that they prevent ROS generation through the mechanism reported in Section 3.1.3 [139]. These inhibitors protect neuronal cells against cell death induced in cellular and animal models. The neuroprotective functions are ascribed to the stabilization of mitochondria, the prevention of death signalling process and the induction of pro-survival anti-apoptotic Bcl-2 protein family and neurotrophic factors, thus counteracting mitochondria-mediated apoptotic pathways. In cellular models, selegiline and rasagiline increased the amounts of different neurotrophic factors classes, neurotrophins (nerve growth factor, brain-derive neurotrophic factor, neurophin 3) and ligands of glial cell line-derived neurotrophic factor. Studies in non-human primates and patients with PD confirmed further the induction of these specified neurotrophic factors.

Alternatively, targeting specific receptors in order to induce the cells to give an anti-oxidant/anti-apoptotic responses might represent an alternative (not yet definitive) clue to neurodegenerative diseases. PPARgamma agonists, for example, have the potential to modulate various signalling molecules/pathways, including matrix metalloproteinase-9, mitogen-activated protein kinases, signal transducer and activator of transcription, mitochondrial uncoupling protein 2, mitoNEET expression, amyloid precursor protein degradation, beta-site amyloid precursor protein cleaving enzyme 1 and Wnt signalling. In so doing, PPARgamma agonists prevent mitochondria-triggered oxidation and apoptosis [140]. Cigitazone, a PPARgamma inducer, has been shown to up-regulate apolipoprotein E (ApoE)mRNA expression [141]. ApoE, the most prevalent cholesterol transport protein in the central nervous system, has also been shown to co-localize with amyloid deposits and neurofibrillary tangles in AD. This is relevant in that ApoE takes
part in cholesterol catabolism (lipid oxidation and amyloid deposition) and acetylcholine dysfunction, two neuropathologic landmarks in AD [142].

While a wide array of clinical trials are currently in progress, no effective therapy exists at the moment. Alternative strategies have been suggested which aim at tackling OS by reducing iron levels while not affecting basal iron metabolism, as iron plays vital roles in sustaining cellular function, other than being involved in Fenton reactions from which most dangerous ROS are generated (HO). It has therefore been proposed that nutrition (low iron intake, diet restriction) and physical activity might contribute to fighting neurodegenerative diseases, by perhaps promoting neurogenesis (BDNF, NGF) or taking part in antioxidant/inflammation-related pathways (NF-kappa B pathways) [143]. In particular, nutrition might become relevant in AD patients, who could potentially benefit from the antioxidant potential of polyphenolic compounds obtained from dietary sources, such as anthocyanins from berries, catechins and theaflavins from tea, curcumin from turmeric, resveratrol from grapes and peanuts, the dihydrochalcones aspalatin and nothofagin from rooibos and the xanthone mangiferin from honeybush [144]. Analogously, vitamin E supplementation has been reported to be positively correlated with improved survival in a cohort of AD patients [145], although further trials are mandatory.

Conversely, early clinical trials have hitherto failed to demonstrate a potentially positive correlation between omega-3 fatty acid supplementation and the management of early AD [146].

Pharmaceutical intervention involving drugs which directly exert anti-oxidant activities includes coenzyme Q10 administration. Coenzyme Q(10) (CoQ(10)) is an essential cofactor in the mitochondrial electron transport pathway, and is also a lipid-soluble antioxidant. CoQ(10) deficiency has been implicated in several clinical disorders, including, but not confined to, heart failure, hypertension, Parkinson’s disease and malignancy. It is endogenously synthesised via the mevalonate pathway, and some is obtained from the diet. The efficacy of CoQ(10) supplements in the treatment of neurodegenerative diseases is currently under clinical evaluation [147].

Future strategies might involve cell-penetrating peptides (CPP), also called protein transduction domains, membrane-permeable peptides, or Trojan horse peptides, which have been used to promote intra-cellular delivery of heat shock proteins (HSPs) for re-folding of unfolded, misfolded or denatured proteins in models for apoptosis, necrosis, OS,
<table>
<thead>
<tr>
<th>Strategy</th>
<th>CNS</th>
<th>CVD</th>
<th>Ageing/cancer</th>
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<tr>
<td>Anti-oxidant Direct</td>
<td>– Vitamin E [99]; – Coenzyme Q10 [101]; – N-acetyl-cisteine [110]; – Lithium [113]</td>
<td>– Beta blockers (carvediol, atenolol, labetalol, metoprolol, pindolol, propranolol, sotalol, and timolol display antioxidant activity by testing them against superoxide radical, hydrogen peroxide, hydroxyl radical, hypochlorous acid, peroxyl radical, nitric oxide, and peroxynitrate) [120]; – Calcium antagonists (dihydropyridines eg, nifedipine, nisoldipine) [122]; – EUK8, EUK134 [131]; – Zn(II)-glycine, and CoQ10 [141]</td>
<td></td>
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<tr>
<td>Indirect</td>
<td>– Type B monoamine oxidase inhibitors (selegiline and rasagiline) [93]; – PPARgamma agonists (cigitazone) [94]; – Trojan horse peptides engineered to deliver HSPs in intracellular compartments [102]; – Fluoxetine and acetyl salicylic [105] or common antidepressants sertraline, paroxetine, and escitalopram) [107]; – Phenothiazines (trifluoperazine) and inhibition of mitochondrial permeability [109]</td>
<td>– Angiotensin-converting enzyme (ACE) inhibitors, captopril and enalapril [123]; – Sartans (losartan [128]); – Statins (fluvastatin, atorvastatin) [127]</td>
<td>– Metformin (insulin-pathways) [131]; – Vitamin E, vitamin C, coenzyme Q, carotenoids, vitamin A, flavonoids, polyphenol, resveratrol, antioxidant from virgin olive oil and selenium against doxorubicin (adriamicin) toxicity [146]; – Bcl-2 anti-sense protein expression modulators (G3139), Bcl-2 antagonist Bax delivery through viral vectors, BH3 mimetic peptides [147]; – Honokiol and betulinic acid [148]; – Oxidative homeostasis (cisplatin, ethidium bistercalinium, topoisomerase inhibitors) [148]; – Forcing mitochondrial respiration (isoniazide, oxamate, dichloracetate) [148]; – Glycolysis inhibition [149]</td>
</tr>
<tr>
<td>Pro-oxidant Direct</td>
<td>– Metal ion chelators bound to amyloid beta peptide [103]</td>
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<td>Indirect</td>
<td></td>
<td>– Flavonoids and polyphenols [128]; – Resveratrol (wine, berries) [129]</td>
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<td>Nutraceuticals</td>
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(*CNS = Central Nervous System; CVD = Cardiovascular disease. (Antioxidant: Direct = ROS-scavenging; metal chelators; Indirect: mitochondria stabilization; co-factor in anti-oxidant radical scavenging enzymatic activity; targeting receptors upstream of anti-oxidant defense pathways); (Pro-oxidant: Direct=stimulation of ROS generation through metal-based reactions, mitochondria stimulation, pro-apoptotic signalling; Indirect: stimulation of pro-oxidant signals, such as inhibition of mitochondrial apoptotic pathway blockade).
neurodegenerative diseases, stroke, cystic fibrosis, smooth muscle relaxation, myocardial injury, scar formation, and others [148].

A completely opposite approach could be based on promoting targeted OS in the very regions which lie at the root of the neurodegenerative disorder, thus blocking its spread and limiting the gravity and progression of the disease in the patients. In AD for example, metal ion chelators bound to amyloid beta peptide can promote aggregation and OS, and can thus have a local detrimental effect, while providing suitable targets towards amyloid beta peptide aggregates [149].

4.1.2. Mood and psychiatric disorders and OS-targeting approaches

Mood disorders such as bipolar disorder, major depressive disorder and psychiatric disorders (schizophrenia) are commonly treated with drugs targeting the proteins glycogen synthase kinase-3 (GSK-3) and protein kinase C (PKC), the purinergic system, histone deacetylases (HDACs), the melanergic/serotonergic system, the tachykinin neuropeptides system, the glutamatergic system, but also OS and bioenergetically-related molecules [150].

Most of the drugs which are routinely adopted for the treatment of psychiatric disorders have not been at first designed to tackle OS. Fluoxetine is one of these drugs. In the study by Galecki et al., anti-oxidant enzyme activities (CAT, SOD, etc.) and malondialdehyde levels were significantly higher in erythrocytes from 50 patients suffering from major depressive disorders against their matched controls, although these parameters were not significantly altered upon three months of successful fluoxetine treatment with remission of depression [151]. On the other hand, a combined treatment with fluoxetine and acetylsalicylic acid resulted in a reduction of enzymatic activity and total antioxidant status, suggestive of a reduction in OS in treated patients [152]. These observations are in agreement with Cumurcu et al. [153], who reported that treatment with common antidepressants (sertraline, paroxetine, and escitalopram) reduced total oxidative status [152]. These observations are in agreement with Cumurcu et al. [153], who reported that treatment with common antidepressants (sertraline, paroxetine, and escitalopram) reduced total oxidative status [152].

A wide series of drugs primarily aimed at counteracting OS have been proposed over the last decades. Some drugs which are routinely used in the treatment for schizophrenia, psychosis and anxiety, such as phenothiazines (PTZ), are known to act through stimulation of mitochondrial permeability, thus enhancing OS in target cells, although at the expenses of increased hepatotoxicity [154]. Conversely, trifluoperazine, a piperazinic PTZ derivative, has been reported to show antioxidant activity at relatively low concentrations, which has been associated with its inhibition of mitochondrial permeability [155].

Antioxidants, such as N-acetyl-cysteine, compounds that mimic GPX activity, and zinc exhibit antidepressive effects [156]. In a recent study, Kumar and Garg [157] demonstrated the efficacy of trazodone and imipramine in the treatment of sleep deprivation-induced anxiety-like behavior and oxidative damage in mice, through the restoration of reduced glutathione levels, catalase activity and attenuation of raised lipid peroxidation and nitrile concentrations as compared to untreated sleep-deprived animals.

Other drugs for the treatment of anxiety, such as venlafaxine, might be involved in NO modulation, although the mechanisms are not yet fully elucidated [158].

Another strategy involves metal ions, such as lithium, which is a mainstay in the acute and prophylactic treatment of bipolar affective disorder through its role in the homeostasis of the glutathione system [159].

Major depression is characterized by significantly lower plasma concentrations of a number of key antioxidants, such as vitamin E, zinc ions and coenzyme Q10, and a lowered total antioxidant status [157]. Therefore, a complementary approach to drug treatments involves dietary supplementation with antioxidant or pro-antioxidant biomolecules. CoQ10 supplementation might represent a winning strategy not only for the treatment of neurodegenerative diseases, as suggested in Section 3 [147], but also in chronic fatigue, depression and cardiovascular disorders [160].

An intestinal metabolite of ginseng, (S)-propanaxadiol (code name S111), demonstrated antidepressant-like activity as potent as fluoxetine, while S111, but not fluoxetine, significantly reduced brain OS and down-regulated serum corticosterone concentration in an animal model [161]. However, dietary supplementation alone might not represent an actual clue, since diet analyses of patients suffering from major depressive disorders showed that the dietary intake of vitamin E was not related to plasma alpha-tocopherol levels, while 89% of the subjects met or exceeded the recommended intake for vitamin E [162]. Nevertheless, administration of ascorbic acid to patients suffering from major depressive disorders caused a synergistic antidepressant-like effect with conventional antidepressants (fluoxetine, imipramine and bupropion), through a mechanism which might involve monoaminergic neurotransmission [163]. Finally, recent observations hint at a long-term association of vitamin B-6, folate, and vitamin B-12 with depressive symptoms among older adults over time, and a high dietary intake of these vitamins might play a protective role against depressive symptoms [164].

4.2. Cardiovascular aging

A key role of OS is evident in the pathologic mechanisms of endothelial dysfunction and associated cardiovascular diseases. Vascular enzymes such as NADPH oxidases, xanthine oxidase, and uncoupled endothelial nitric oxide synthase are involved in the production of ROS. The question remains whether pharmacologic approaches can effectively combat the excessive ROS production in the vasculature. Interestingly, registered cardiovascular drugs can directly or indirectly act as antioxidants, thereby preventing the damaging effects of ROS, although they had not been purposely designed for that end [165].

Beta blockers (sometimes written as β blocker) is a class of drugs used for various indications, but particularly for the management of cardiac arrhythmias, cardioprotection after myocardial infarction (heart attack), and hypertension. As beta adrenergic receptor antagonists, they diminish the effects of epinephrine (adrenaline) and other stress hormones. Gomez et al. showed that carvedilol, atenolol, labetalol, metoprolol, pindolol, propranolol, sotalol, and timolol display antioxidant activity by testing them against superoxide...
radical, hydrogen peroxide, hydroxyl radical, hypochlorous acid, peroxyl radical, nitric oxide, and peroxy nitrate, demonstrating their direct antioxidant capacity using ascorbic acid, lipoic acid, melatonin, rutin, and ebensol as positive controls [166]. Carvedilol, in particular, seems to inhibit the oxygen-free-radical-initiated lipid peroxidation and α-tocopherol depletion in vitro in rat brain homogenate through a two orders of magnitude greater than that of all the other β-blockers [167]. Carvedilol contains β- and α1-adrenoceptor-blocking pharma- cophore as well as a specific carbazole moiety to enhance its antioxidant activity. Moreover, its metabolites are active antioxidants as well [165].

Ca⁡²⁺ antagonists, such as dihydropyridines (eg, nifedipine, nisoldipine) have been shown to directly reduce H₂O₂-induced decreases in contractile function of rat hearts and low-density lipoprotein oxidation [168].

Indirect antioxidant activity (pro-oxidant activity, since these drugs promote cellular anti-oxidant defenses) have been observed for two angiotensin-converting enzyme (ACE) inhibitors, captopril and enalapril, which feature sulfhydryl groups [169]. The putative mechanism involves ACE inhibition, which in turn is known to promote NADPH oxidase, thus preventing O₂⁻ radical generation [165]. A similar indirect antioxidant effect may be expected from the Ang II receptor-blockers, the “sartans”, although recent proteomics investigations on platelets from moderately hypertensive patients upon treatment with olmesartan medoxomil showed that the treatment did not modify the expression of some inflammation and oxidation-related protein markers (HSPs, antioxidant enzymes) [170]. On the other hand, losartan reduced the insulin resistance score and the intensity of nitrotyrosine staining in a mouse model of diabetes [171].

Similarly, some of the mechanisms at the basis of the pleiotropic effects exerted by statins could be explained by an indirect antioxidant activity, through the inhibition of the coupling of the AT1 receptor to the NADPH oxidase and thus prevention of radical production [172]. In this respect, fluvastatin and that of atorvastatin have been shown to play a strong anti-peroxyl radical activity [173].

Finally, food-derived compounds appear to be effective inhibitors of OS and preserve vascular function. For example, flavonoids, highly abundant polyphenols of fruits and vegetables, appear to play beneficial cardiovascular effects, as an elevated intake of fruits has been correlated to decrease blood pressure in humans [174]. Flavonoids effect on ET-1 synthesis or arginase inhibition increases the availability of L-arginine, which forms the rate-limiting factor for cellular NO* production. It has further been proposed that the prevailing mode of action of flavonoids is inhibition of NADPH oxidase, thus lowering the O₂⁻ generation that leads to elevation of NO* levels in the cell and thus vasodilatory effects [175].

Analogously, resveratrol from wine, peanuts and berries, existing in both cis- and trans-isomeric forms, has been shown to act moderately both as a direct and indirect anti- oxidant, through regeneration of alpha-tocopherol [175]. For these reasons, resveratrol is believed to decrease circulating low-density lipoprotein cholesterol levels and reduce the cardiovascular disease risk, although convincing evidence is still lacking.

4.3. Aging and metabolism

Early attempts at antioxidant intervention as a means to delay aging were initiated soon after the free radical theory of aging was proposed. These attempts stemmed from the postulation of the free radical theory of ageing which posits that accumulation of oxidative damage underlies the increased cellular, tissue and organ dysfunction and failure associated with advanced age. However, these anti-oxidant interventions have so far failed to extend life span in most cases [176,177].

A series of encouraging, albeit preliminary results have been reported in C. elegans and Drosophila through the use of enzymatic synthetic drugs miming SOD and CAT activities, such as EUK-8 and EUK-134 [177]. However, while increasing anti-oxidant defenses in these organisms, the drugs did not produce any significant increase of the lifespan.

The direct impact of antioxidant enzyme treatment on life span is even more controversial in mammalian models. For instance, transgenic mice that constitutively over-express human CuZn-SOD did not live longer than control animals [178], while heterozygous mice with reduced MnSOD activity have a life expectancy that is similar to wild-type mice, although these animals have increased oxidative damage to DNA [179]. It is gradually becoming clear that antioxidant enzyme activities are not broadly correlated with longevity, as Page et al. concluded in their investigation on 14 mammalian and avian endotherm species with maximum life spans ranging from 3 years to over 100 years [180]. Analogously, maintenance of protein homeostasis through up-regulation of protein repairing and recycling machinery (glutaredoxin, thioredoxin reductase, etc.) is not associated with extended life spans in 15 vertebrate species [181]. Therefore, if free radicals are actually correlated to aging, a winning strategy should be targeted at preventing their production rather than increasing defenses and repairing mechanisms against ROS-induced damages. The Mitochondrial Free Radical Theory of Aging (MFRTA) proposes that mitochon- drial free radicals, produced as by-products during normal metabolism, are the major responsible of oxidative damage. According to MFRTA, the accumulation of these oxidative phenomena is the main driving force in the aging process. Although widely accepted, this theory remains unproven, because the evidence supporting it is largely correlative. In this view, resting metabolism should represent an advantage on the road to life span improvement. Nonetheless, moderate exercise produces changes in free radical production and oxidative damage without altering maximum life expectancy [182].

Ristow and Zarse recently reviewed accumulating evidence reporting that caloric restriction and specifically reduced glucose metabolism induces mitochondrial metabolism to extend life span in various model organisms, including Saccharomyces cerevisiae, Drosophila melanogaster, Caenor- habditis elegans and possibly mice [71]. In conflict with Harman’s free radical theory of aging, these effects may be due to increased formation of ROS within the mitochondria causing an adaptive response that results in increased stress resistance assumed to ultimately cause a long-term reduction of OS. Concordantly, it has been observed that hyperactive mice live longer than controls [183]. This type of retrograde response has been named mitochondrial hormesis or
mitohormesis, and may in addition be applicable to the health-promoting effects of physical exercise in humans and, hypothetically, impaired insulin/IGF-1-signalling in model organisms [71].

Recent findings shed further light on the strong linkage between aging and metabolism, opening new scenarios in the field of drug discovery [184]. Metformin, a biguanide drug commonly used to treat type-2 diabetes, has been noted to extend lifespan of nondiabetic mice and *C. elegans* [185]. Insulin-like signalling in *C. elegans* activates the transcription factor SNK-1, which is known to defend against oxidative stress by mobilizing the conserved phase 2 detoxification response and it is thus referred to as the longevity-promoting factor [186].

While ageing per se still remains a controversial issue, good results have been obtained in the field of cosmesis, as far as skin aging is concerned. Recent advances in anti-oxidant drug development against skin aging have been extensively reviewed [187]. A role has been proposed for ascorbic acid, alphatocopherol, carotenoids, polyphenols and other substances, such as ergothionine, Zn(II)-glycine, and CoQ10 in the treatment of skin aging [187]. In particular, topical application of CoQ10 and antioxidants like alpha-glucosylrutin diminished resistance in keratinocytes of old donors against UV irradiation (photoaging) either in vitro or in vivo study [188].

### 4.4. Cancer

Over the last decades, most of the efforts of the pharmaceutical establishment have focused on designing and testing effective drugs against cancer progression. As many noted [189], cancer shares some fundamental aspects with ageing. Cancer cells often display altered metabolism (aerobic glycolysis) and are rather prone to suffer from OS-induced injuries, often intertwined with the likelihood of accumulation of genetic mutations. These considerations have paved the way for the development of specific drugs aimed at preventing OS (in order to reduce the accumulation of genetic mutations), fighting it (to counteract ROS production induced by metabolism of chemotherapeutics or chelating radicals through metal-based drugs) or promoting it (thus exploiting ROS against OS-sensitive cancer cell populations).

Vitamin E (gamma-tocopherol in particular) belongs to the first group of biomolecules, as it represents a promising asset in prevention treatments for several cancers, including colon, prostate, mammary and lung tumorigenesis in animal models [190]. Analogously, curcumin, a hydrophobic polyphenol derived from rhizome (turmeric) of the herb Curcuma longa commonly called diferuloyl methane, may be used as chemopreventive or chemoprotectant, due to its antioxidant properties. Besides, curcumin may potentiate the anti-tumour effect of gemcitabine [191].

Anthracynes (doxorubicin, daunorubicin, epirubicin, and idarubicin) are currently the most effective group of anti-neoplastic drugs used in clinical practice. Of these, doxorubicin (also called adriamycin) is a key chemotherapeutic agent in cancer treatment, although its use is limited as a consequence of the chronic and acute toxicity associated with this drug. The molecular mechanisms of doxorubicin account for both the anti-cancer and the toxic side effects. Many antioxidants have been assayed, with positive or negative results, to prevent the toxicity of doxorubicin. In particular vitamin E, vitamin C, coenzyme Q, carotenoids, vitamin A, flavonoids, polyphenol, resveratrol, antioxidant from virgin olive oil and selenium have been assayed as to see whether (and to which extent) they might be used as antioxidant co-adiuvant in chemotherapeutic treatments [192].

An opposite strategy might involve drugs which aim at producing ROS with specificity to the cancer tissue. Targeting cancer cell mitochondria, through Bcl-2 family members which allow increasing mitochondrial membrane permeability in order to have them generate suicidal signals, might reveal a winning strategy [193]. These strategies include Bcl-2 antisense protein expression modulators (G3139), Bcl-2 antagonist Bax delivery through viral vectors, BH3 mimetic peptides (reviewed in [194]), which have been thought to trigger pro-apoptotic cascades mediated by cytochrome c release and ROS-induced damage in target cells. Permeabilization of mitochondrial membranes could be also achieved through honokiol and betulinic acid [195], while other strategies target mitochondrial DNA in order to prevent expression of specific respiratory proteins and thus alter the oxidative homeostasis (cisplatinum; ethidium ditercalinium, topoisomerase inhibitors) [196]. An emerging field of research takes into account the quasi-exclusiveness of the Warburg effect metabolism in tumour cells and thus aims at manipulating metabolic pathways for therapeutic purposes. Relevant studies include approaches of glycolysis inhibition or forcing mitochondrial respiration (lonidamine, oxamate, dichloroacetate) [194,195].

Alternatively, mitochondria could be targeted as to prevent angiogenesis which is necessary to sustain tumour growth and progression through nutritional and oxygen support [196].

Various metal complexes are currently used as therapeutic agents (e.g., Pt, Au, and Ru), due to their capacity for generating radical species (Fenton chemistry) in the treatment of malignant diseases, including several types of cancers [197,198]. Lowering the metal concentrations might reduce the OS potential by limiting metal-triggered ROS production and thus counteract malignant transformation and tumour growth and progression. In this view, the potential role of proteomics in target discovery and validation, metal-based drug design and development has been reviewed by Wang and Chiu [197]. Brewer’s group tested a drug known as tetramethylolbdate, which binds dietary copper before it can be absorbed by the body and thus reduces copper-triggered DNA and lipid peroxidation, in patients with different types of metastatic cancer. The drug was observed to act through the inhibition of cytokines, NFκB-pathways and angiogenesis [199].

The non-metal element selenium has been observed to play a protective role against several types of cancers, mainly prostate cancer, through a likely pro-oxidative rather than anti-oxidant activity [200], although proper prospective studies are currently under development.

Analogous considerations could be made for some nutraceuticals, that is to say a food or food product that provides health and medical benefits, including the prevention and treatment of disease. Resveratrol from red wines [201], polyphenols epicatechin and epigallocatechin-3-gallate from green tea [202] or olive oil phenolic extracts [203] might hold relevant clinical implications due to their likely pro-oxidant role in sensitizing cancer cells specifically. Nonetheless, most of the nutraceuticals exert their activity mainly by counteracting OS and free radicals, such...
as in the case of many compounds from vegetables (broccoli, red pepper and beans) [204], which might be helpful in the prevention of OS-induced damages caused by chemotherapeutic treatments (such as Aphanizomenon flos-aquae extracts attenuating cisplatinum-induced renal dysfunction [205]).

5. Conclusions

Redox homeostasis maintenance and modulation is a fundamental physiological process, which is compromised in several pathophysiological events of importance in human health. In this context, redox proteomics holds the promise to shed further light on these pivotal biological processes. While preliminary investigations have already contributed precious data, we are still far from looking at the whole picture in its full complexity. An increasing number of techniques are available which aim at investigating, either qualitatively or quantitatively, modifications to specific amino acids (cysteins, tyrosines, etc.) or specific groups (carbonylations, nitrosylations, etc.). In this review, the mechanisms of ROS and RNS production have been reviewed, mainly addressing those chemical phenomena which have already been associated with pathological conditions (of the central nervous system, cardiovascular system, or simply related to aging and altered-cell cycle regulation). In these fields, redox proteomics has yet to exhaustively contribute, although the increasing number of laboratories committed in these research endeavours argues for big strides in the upcoming years. In particular, redox proteomics might represent a valid tool to explore protein-targeting oxidative modifications prior to or upon drug administration, which could greatly contribute either to accumulate further basic knowledge or to produce the translational know-how which is necessary to speed the whole process of drug designing and testing up. Nonetheless, only preliminary research has been so far performed, mainly limited to animal models.

For the foreseeable future, redox (and quantitative [206]) proteomics will likely play a pivotal role in the quest for new therapeutical targets and their validation, in the process of determining OS-triggered cellular alteration upon drug treatments and thus in the very heart of the design and testing of new drugs and their metabolites against those pathologies relying on altered redox homeostasis. Redox-proteomics currently represents a breakthrough into the virgin forest of drug discovery and development, which is, nonetheless, yet to be fully explored. So far, therapeutic interventions have been thought to counteract oxidative stress (both directly and indirectly) or rather to promote it, strictly targeting in situ the area of pathological/diseased tissues. New redox-based drugs, like golden coins, have been sparsely found along the way. However the actual treasure trove has yet to be opened.

Abbreviations

HSP heat shock protein
IAA iodoacetamide
ICAT isotope-coded affinity tag
MDA malondialdehyde
NEM N-ethylmaleimide
NO nitric oxide
OS oxidative stress
PD Parkinson’s disease
PTZ phenothiazines
RNS reactive nitrogen species
ROS reactive oxygen species
SOD1 Superoxide dismutase 1
TCA trichloroacetic acid

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