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Protein oxidation - Formation mechanisms, detection and relevance as biomarkers in human diseases

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ABSTRACT

Generation of reactive oxygen species and related oxidants is an inevitable consequence of life. Proteins are major targets for oxidation reactions, because of their rapid reaction rates with oxidants and their high abundance in cells, extracellular tissues, and body fluids. Additionally, oxidative stress is able to degrade lipids and carbohydrates to highly reactive intermediates, which eventually attack proteins at various functional sites. Consequently, a wide variety of distinct posttranslational protein modifications is formed by protein oxidation, glycoxidation, and lipoxidation. Reversible modifications are relevant in physiological processes and constitute signaling mechanisms ("redox signaling"), while non-reversible modifications may contribute to pathological situations and several diseases. A rising number of publications provide evidence for their involvement in the onset and progression of diseases as well as aging processes. Certain protein oxidation products are chemically stable and formed in large quantity, which makes them promising candidates to become biomarkers of oxidative damage. Moreover, progress in the development of detection and quantification methods facilitates analysis time and effort and contributes to their future applicability in clinical routine. The present review outlines the most important classes and selected examples of oxidative protein modifications, elucidates the chemistry beyond their formation and discusses available methods for detection and analysis. Furthermore, the relevance and potential of protein modifications as biomarkers in the context of disease and aging is summarized.

1. Introduction

Biological macromolecules are constantly exposed to oxidants and oxidative damage to cellular components has been increasingly recognized as a significant pathophysiological event leading to disease and aging processes. Free radicals and other oxidizing species are derived from both endogenous sources such as mitochondria, peroxisomes, and phagocytic cells, but also exogenous sources such as tobacco smoke, pollution, alcohol, and certain drugs.

The most important oxidants originate from oxygen (reactive oxygen species, ROS) and nitrogen (reactive nitrogen species, RNS). The oxygen molecule itself is a biradical with two unpaired electrons. Additionally, important primary oxygen-containing compounds with reactive properties are superoxide ($O_2^{\bullet-}$) and the most reactive hydroxyl radical ($^{\bullet}OH$), formed from $O_2^{\bullet-}$ and hydrogen peroxide (H_2O_2) in the presence of metal ions (Fenton reaction) with a very short half-life of approximately 10^{-9} s [1]. Less powerful ROS are the alkoxyl radical ($RO^{\bullet}$) as well as peroxyl radical ($ROO^{\bullet}$), both key intermediates in lipid peroxidation chain reactions. Nitric oxide ($^{\bullet}NO$) is a slowly reacting molecule, with a high relevance as signaling molecule. However, nitric oxide reacts with superoxide at a high rate constant to give peroxynitrite ($ONOO^-$), able to decompose spontaneously to yield nitrogen dioxide ($^{\bullet}NO_2$) and hydroxyl radicals [2].

Under normal circumstances, the formation and subsequent degradation of reactive species is regulated by cellular defense systems, including scavenging enzymes able to remove oxidants or their precursors such as superoxide dismutases, catalase, thioredoxin/thioredoxin reductase, and glutathione peroxidase. Non-enzymatic antioxidants such as tocopherols and ascorbic acid, but also metal binding proteins delay oxidation reactions or prevent the development of reactive species. Repair and removal systems such as methionine sulfoxide reductases, disulfide reductases/isomerases, and the ubiquitin-proteasome-system complete the damage defense. However, even if

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Abbreviations

3-NT	3-Nitrotyrosine
4-HNE	4-Hydroxy-2-nonenal
AD	Alzheimer's disease
AGE	Advanced glycation endproduct
ALC	Alcoholic liver cirrhosis
ALD	Alcoholic liver disease
ALE	Advanced lipoxidation endproduct
ALS	Amyotrophic lateral sclerosis
AOPP	Advanced oxidation protein product
CAD	Coronary artery disease
CHD	Chronic heart disease
CKD	Chronic kidney disease
CML	Carboxymethyl lysine
COPD	Chronic obstructive pulmonary disease
CSF	Cerebrospinal fluid
CVD	Cardiovascular disease
DHP	Dihydropyridine
DNPH	2,4-Dinitrophenylhydrazine
DOPA	Dihydroxyphenylalanine
DTT	Dithiothreitol
ELISA	Enzyme linked immunosorbent assay
EPR	Electron paramagnetic resonance
ESI	Electrospray ionization
FLD	Fluorescence detection
HBV	Hepatitis B virus
HIV	Human immunodeficiency virus
IHC	Immunohistochemistry
HPLC	High pressure liquid chromatography
MALDI	Matrix assisted laser desorption ionization
MCI	Mild cognitive impairment
MDA	Malondialdehyde
MetS	Metabolic syndrome
MRM	Multiple reaction monitoring
MS	Mass spectrometry
NAFLD	Non-alcoholic fatty liver disease
NOS	Nitric oxide synthase
PBMC	Peripheral blood mononuclear cells
PD	Parkinson's disease
RA	Rheumatoid arthritis
RCS	Reactive carbonyl species
RNS	Reactive nitrogen species
ROS	Reactive oxygen species
Skin AF	Skin autofluorescence
T2D	Type 2 diabetes
TBARS	Thiobarbituric acid reactive substances
WB	Western Blot

these preventive and repair systems are interconnected and work efficiently, they cannot fully prevent oxidative damage to cellular components. Furthermore, a higher level of damage may arise from increased oxidant generation and/or a decrease or failure of defense systems under several conditions such as diseases and aging [3,4]. This imbalance between the excessive production of reactive species and the ability to detoxify them or repair the resulting damage is termed "oxidative stress" (the full concept on oxidative stress is reviewed in Ref. [5]).

Generally, reactive species can generate damage to all cellular components, including proteins, carbohydrates, lipids, and DNA. It has been estimated that proteins can scavenge a majority (50%–75%) of generated reactive species [6]. Highly reactive species damage numerous sites at side-chains and backbones of proteins, while less reactive species have higher selectivity regarding targeted residues [7]. The variety of reaction sites generates a wide range of posttranslational protein modifications consequently changing composition and folding, the net charge, as well as the hydrophobicity/hydrophilicity of proteins. This affects their functions as receptors, enzymes, carrier or structural proteins [8]. It has been established that reversible modifications are thought to be relevant in physiological processes and constitute signaling mechanisms under appropriate conditions ("redox signaling"), while non-reversible modifications may contribute to pathological situations and several diseases [9].

Since free radicals are very reactive and short-lived, their detection is challenging and techniques with fast response times such as electron paramagnetic resonance (EPR) are needed. However, detection of more stable products, resulting from reactions with free radicals, such as oxidative protein modifications yield more convincing data and often result in higher-quality quantitative data. Over the last years considerable advances have been made in the development of techniques to detect, identify, and quantify protein modifications.

Studies reveal an age-related increase in the level of oxidatively modified proteins [10,11] and many diseases have an oxidative etiology [9,12–14]. It can be therefore assumed that proteins also accumulate evidence of oxidative damage in relation to disease, although some of these are only associations and oxidation is not always causal, but a contributing factor. To function as suitable biomarkers, it is critical that protein oxidation products are stable, accumulate in detectable concentrations, and correlate with disease severity. Sample availability is another important factor limiting the reliability of a biomarker. Protein oxidation products can be determined in blood and urine samples, but also in specific tissue or cell samples.

In this review, we focus on the chemistry of reversible and irreversible protein modifications by oxidants and available methods for detection and analysis. Discussion whether protein oxidation provides suitable biomarkers in the context of disease and aging is another major aim of this review.

2. Mechanisms of oxidative protein modifications

2.1. Oxidation of sulfur-containing amino acids

Sulfur-containing moieties such as the sulfhydryl group of cysteine and the thioether group of methionine are targeted by oxidative stress and a plethora of posttranslational protein modifications is formed (Fig. 1) [15]. Formation of cysteine thiyl radical and sulfenic acid is a reversible process and both intermediates are highly unstable. Both serve as precursors for several oxidized cysteine modifications (Fig. 1A) [16].

One example with huge biological relevance is the formation of disulfide bonds between cysteine and thiols under oxidative conditions. Reaction of cysteine with another cysteine under non-enzymatic as well as enzyme mediated conditions leads to cystine. Cystine is a vitally important modification, because it stabilizes protein structures via intra- and intermolecular disulfide bridges. Formation and cleavage of disulfide bonds is a reversible process, which is controlled by several enzymes *in vivo* [17]. Cysteine residues can be protected against "overoxidation" by *S*-glutathionylation, which is the reversible addition of glutathione via disulfide linkage. Overoxidation describes the further oxidation of cysteine sulfenic acid to cysteine sulfinic and finally sulfonic acid [18]. Cysteine sulfenic and sulfinic acid modifications can be reversed by glutaredoxin mediated reduction of sulfinic acid, conjugation of sulfenic acid via *S*-glutathionylation, and deglutathionylation by glutaredoxin or sulfiredoxin. The overoxidation product cysteine sulfonic acid is irreparably damaged and the affected protein has to be degraded by the proteasome [19].

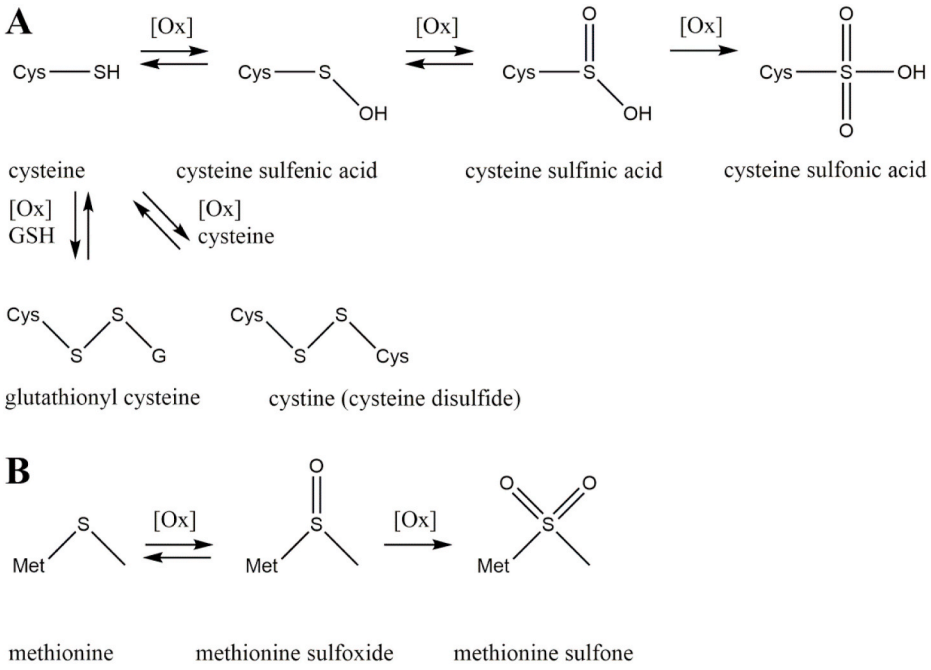

Fig. 1. Oxidation of sulfur-containing amino acids. Cysteine is oxidized in a multi-step reaction to the respective sulfenic, sulfinic, and sulfonic acid modifications or oxidatively conjugated with glutathione (GSH) or another cysteine residue (A). Methionine is reversibly oxidized to methionine sulfoxide and irreversibly oxidized to methionine sulfone (B).

Another sulfur containing amino acid targeted by oxidative modification is methionine (Fig. 1B). In contrast to the non-enzymatic oxidation of methionine to methionine sulfoxide, the reduction is catalyzed by methionine sulfoxide reductases [20]. Further oxidation results in methionine sulfone formation, which is not targeted by methionine sulfoxide reductases and has to be considered as a stable modification [21].

2.2. Oxidation of aromatic moieties

Aromatic functionalities of amino acids are excellent targets of protein oxidation (Fig. 2). In particular, tyrosine is a redox active structure. The phenolic side-chain is easily oxidized, because the intermediary tyrosyl radical is stabilized by mesomeric delocalization of the unpaired electron (Fig. 2A) [22]. The tyrosyl radical can react with another tyrosyl radical. After enolization, the main product of this reaction is the fluorescent protein crosslink *ortho,ortho*-dityrosine [23].

Reaction of protein bound tyrosyl radicals with hydroxyl radicals leads to 3-hydroxytyrosine, which is an analogue of the neurotransmitter 3,4-dihydroxyphenylalanine (DOPA) [24]. Beside proteinogenic *para*-tyrosine, the abnormal isomers *ortho*- and *meta*-tyrosine can be formed by oxidation of phenylalanine residues via hydroxyl radicals (Fig. 2B) [25]. Tryptophan is oxidized by hydroxyl radicals to hydroxytryptophan, which is cleaved by oxygen to yield *N*-formyl kynurenine (Fig. 2C) [26]. Oxidation of histidine by metal catalyzed reaction with hydroxyl radicals leads to 2-oxohistidine (Fig. 2D) [27,28].

2.3. Glycoxidation

The basic amino acids lysine and arginine are readily modified by glycation. The reaction results in the formation of intermediate Amadori products and subsequently advanced glycation endproducts (AGEs). The protein modifications are formed by the nucleophilic reaction of amino acid residues with reductive sugars or their reactive degradation products (α-dicarbonyl compounds). It is important to note that the formation of AGEs does not generally require oxidative conditions and only selected AGEs are generated by oxidation. This special subset of AGEs is called glycoxidation products, because they are formed by a combination of glycation and oxidation (Fig. 3) [29]. This review focuses on glycoxidation products as feasible markers of combined oxidative and carbonyl stress. A more general overview of AGE structures and underlying mechanisms of the Maillard reaction *in vivo* can be found elsewhere [30,31].

The most prominent and one of the most abundant AGEs *in vivo* is carboxymethyl lysine (CML), which was extensively reviewed before [32–34]. CML was the first discovered glycoxidation product and is formed by oxidative degradation of fructoselysine (Amadori product) [35]. An alternative pathway is the reaction between the α-dicarbonyl compound glyoxal and lysine leading to CML formation via an isomerization mechanism [36]. Although the latter mechanism is non-oxidative, the reactive precursor glyoxal is mainly formed by oxidative degradation of carbohydrates, lipids, nucleotides, and serine [37]. The oxidation of the central enaminol intermediate of this isomerization cascade results in the formation of the α-oxoamide AGE glyoxylyl lysine. Compared to CML this structure is an even more sensitive marker of oxidative stress, because the precursor glyoxal and in addition the formation of glyoxylyl lysine itself rely on oxidative processes [38].

Another well-known glycoxidation product is pentosidine, a crosslink between lysine and arginine residues. Despite the rather low abundance of pentosidine, it was one of the first discovered AGEs due to its specific fluorescence [39]. Pentoses were at first postulated as the main source of the C5 backbone in pentosidine [40]. Later studies proved the oxidative cleavage of C6 structures such as glucose to yield pentosidine as well [41]. Beside oxidatively formed glycoxidation products some AGEs are mainly formed by precursors which are generated by oxidation. An excellent example is the oxidation of glucose or Amadori product thereof to glucosone [42]. A major product of lysine mediated cleavage of glucosone is formyl lysine [43]. Recently, formyl lysine was discovered in very high quantities in various murine tissues and histones [44,45]. Alternative pathways of formyl lysine formation such as formyl phosphate [46] and formaldehyde metabolism [47] were reported in the literature. All postulated mechanisms require an oxidation step and first experiments in cell culture give rising evidence of

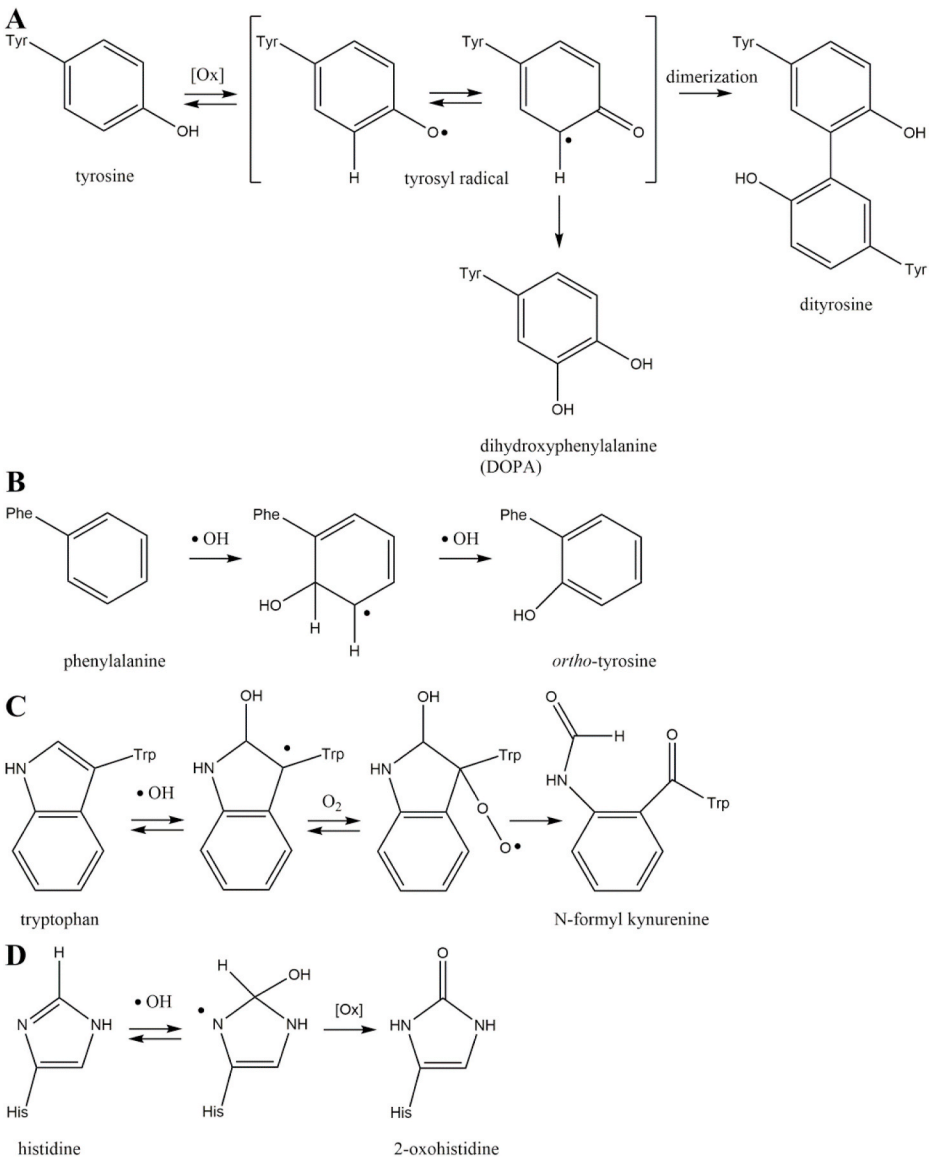

Fig. 2. Oxidation of aromatic amino acids. Oxidation of tyrosine is favored by the intermediary tyrosyl radical and leads to the stable modifications dihydroxyphenylalanine and dityrosine (A). Unusual tyrosine isomers like *ortho*-tyrosine are generated by oxidation of phenylalanine residues (B). Oxidation of tryptophan is the initial step of the *N*-formyl kynurenine reaction cascade (C). The stable modification 2-oxohistidine is formed by histidine oxidation (D).

correlation between formyl lysine concentration and oxidative stress [46].

2.4. Lipoxidation

As mentioned above the CML precursor glyoxal can be formed by carbohydrate degradation and lipid oxidation. Hence, CML is not only considered as an AGE, but also as an advanced lipoxidation endproduct (ALE) [48]. ALEs are protein modifications formed by the nucleophilic reaction of proteins with reactive carbonyl species (RCS) originating from oxidative lipid degradation [49]. A complete overview of ALE structures with their respective precursors and mechanisms of formation was previously published [50].

During lipid oxidation, a plethora of distinct RCS is formed, which can be categorized as α/β unsaturated, keto aldehyde, and dialdehyde RCS. One of the most abundant dialdehyde RCS resulting from lipid peroxidation is malondialdehyde (MDA). MDA is less reactive compared to other RCS, because at physiological pH it enolizes to β-hydroxyacrolein and the enolate is formed by loss of a proton [51]. Only the protonated β-hydroxyacrolein is attacked by lysine yielding the key intermediate *N*-propenal lysine (Fig. 4A) [52]. An enamine crosslink is formed by reaction of *N*-propenal lysine with a second lysine residue [53]. Alternatively, reaction with a second MDA molecule and acetaldehyde, which is a MDA degradation product, results in formation of the fluorescent dihydropyridine (DHP) product lysine 4-methyl-1,4-dihydro-3,5-dicarbaldehyde [54]. This DHP derivative can react with another lysine residue and MDA to form the fluorescent 3,5-diformyl-1,4-dihydropyridin-4-yl-pyridinium crosslink [55]. Reaction between *N*-propenal lysine and arginine is the main source of the lysine-arginine crosslink 2-ornithinyl-4-methyl(1ε-lysyl)-1,3-imidazole [56].

Another important example for α/β unsaturated RCS is 4-hydroxy-2-nonenal (4-HNE). The carbonyl function pulls away electrons from the alkene group resulting in electron deficiency at the β-carbon of 4-HNE. This positive partial charge is attacked by nucleophiles such as cysteine and histidine and stable Michael adducts are formed (Fig. 4B) [50]. Nucleophilic attack of lysine forms unstable Michael adducts, which require reduction, e.g., by sodium borohydride prior to analysis [57]. The open forms of Michael adducts of 4-HNE and nucleophiles are

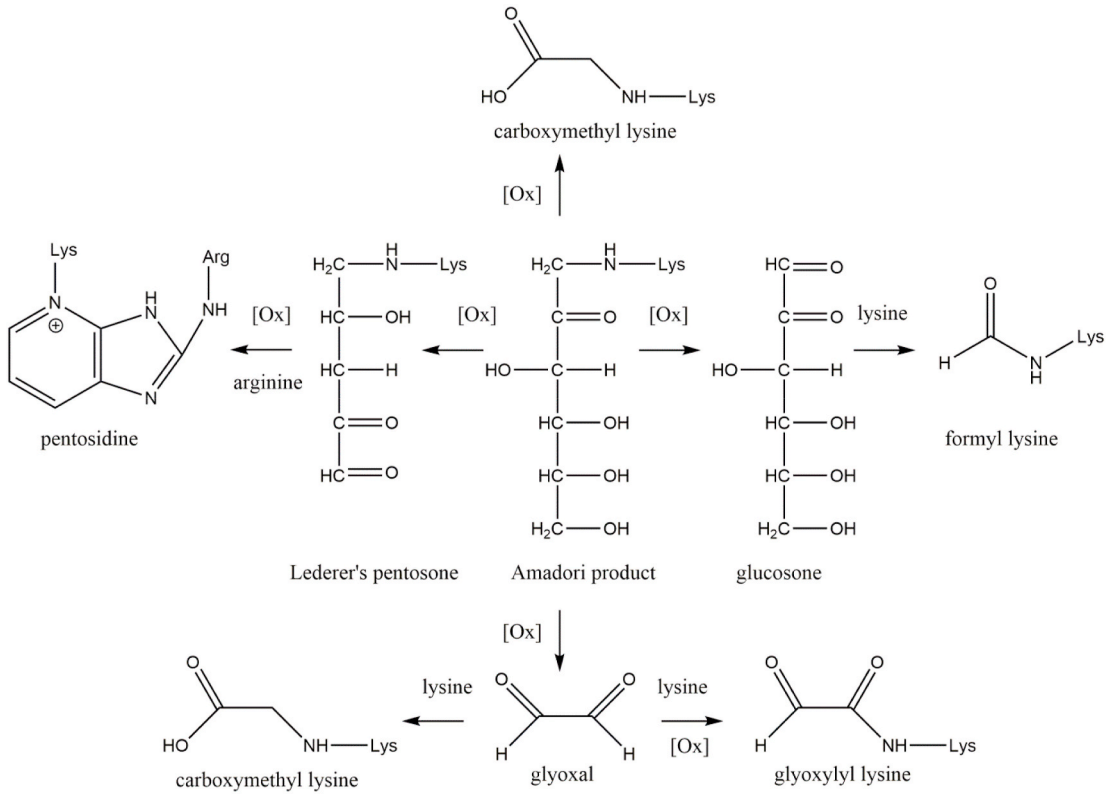

Fig. 3. Formation of glycoxidation products. A special subset of advanced glycation endproducts is exclusively formed under oxidative conditions including the prominent modifications carboxymethyl lysine and pentosidine. The novel modifications glyoxylyl and formyl lysine are potential biomarkers of oxidative stress based on their formation pathways.

in an equilibrium with their favored cyclic hemiacetal form [58]. Beside nucleophilic attack at the alkene group, reversible formation of Schiff bases between the carbonyl function and lysine residues is a common reaction. In analogy to the Michael adduct the Schiff Base is stabilized by cyclization as a pyrrole [59]. Alternatively, attack of nucleophiles results in a mixed Michael adduct and Schiff base intermediate [60], which is stabilized as a cyclic hemiaminal [61]. Oxidation of the Schiff base and attack of a second lysine residue yields a fluorescent pyrrolinium crosslink [62].

2.5. Carbonylation

Protein carbonylation is a stable modification induced by ROS via three pathways: direct oxidation of protein-bound amino acids, oxidative cleavage of the protein backbone, and incorporation of carbonyls from glycoxidation or lipoxidation [63]. The latter option has been described in the above chapters. Aminoadipic and glutamic semialdehyde are examples for direct oxidation of amino acids and responsible for about 60% of total protein carbonylation in liver [64]. Formation of aminoadipic semialdehyde requires hydroxyl radical mediated abstraction of the hydrogen located next to the N^{ε}-amino function of lysine, metal-catalyzed oxidation of the carbon-centered radical, and hydrolysis of the resulting imine (Fig. 5A). A similar reaction scheme leads to glutamic semialdehyde, in which the proton next to the guanidino function of arginine or the proton next to the nitrogen of the proline pyrrole ring is abstracted, the carbon radical is oxidized, and the intermediate is hydrolyzed (Fig. 5B) [65].

Generally, oxidative cleavage of the peptide backbone is initiated by superoxide mediated alkoxyl radical formation at the α-carbon next to a peptide bond. The alkoxyl radical fragments either by the homolytic cleavage of the carbon-carbon bond (diamide pathway) or the carbon-nitrogen bond (α-amidation pathway) (Fig. 5C). The diamide pathway results in a diamide and isocyanate, while a N^{α}-ketoacyl derivative and an amide are the endproducts of the α-amidation pathway [66]. Additionally, oxidative cleavage of the peptide backbone by specific mechanism can be facilitated by prolyl, glutamyl, and aspartyl residues [67]. As an example, protein bound proline is oxidized to the 2-pyrrolidone peptide under carbon-carbon cleavage (Fig. 5D) [68].

2.6. Nitration/nitrosylation

Nitric oxide ($^{\bullet}$NO) is an important signaling molecule involved in vasodilation and neurotransmission via activation of soluble guanylate cyclase and generation of the second messenger cGMP [69]. Formation of $^{\bullet}$NO is catalyzed by three isoforms of nitric oxide synthase (NOS) (Fig. 6): endothelial NOS, neuronal NOS or inducible NOS. All NOS isoforms use arginine and oxygen to produce citrulline and $^{\bullet}$NO [70]. Apart from its role in cell signaling $^{\bullet}$NO readily reacts with superoxide anions forming RNS such as peroxynitrite and nitrogen dioxide [71]. These RNS are potent oxidizing agents *in vivo* [72]. Additionally, $^{\bullet}$NO or higher nitrogen oxides including dinitrogen trioxide (N_2O_3) can directly and reversibly modify cysteine residues by *S*-nitrosation [73]. The irreversible modification 3-nitrotyrosine (3-NT) is formed by nitration of tyrosine via attack of the nitrogen dioxide radical at the *ortho*-position of the aromatic ring [74].

3. Analysis of oxidative protein modifications

The high chemical diversity and plethora of oxidative protein modifications is reflected in various techniques which are currently used to detect modified proteins. This can be accomplished by analyzing the whole proteome, specific proteins, and even solely certain modifications. The following section focuses on general techniques to analyze protein oxidation which are summarized in Fig. 7.

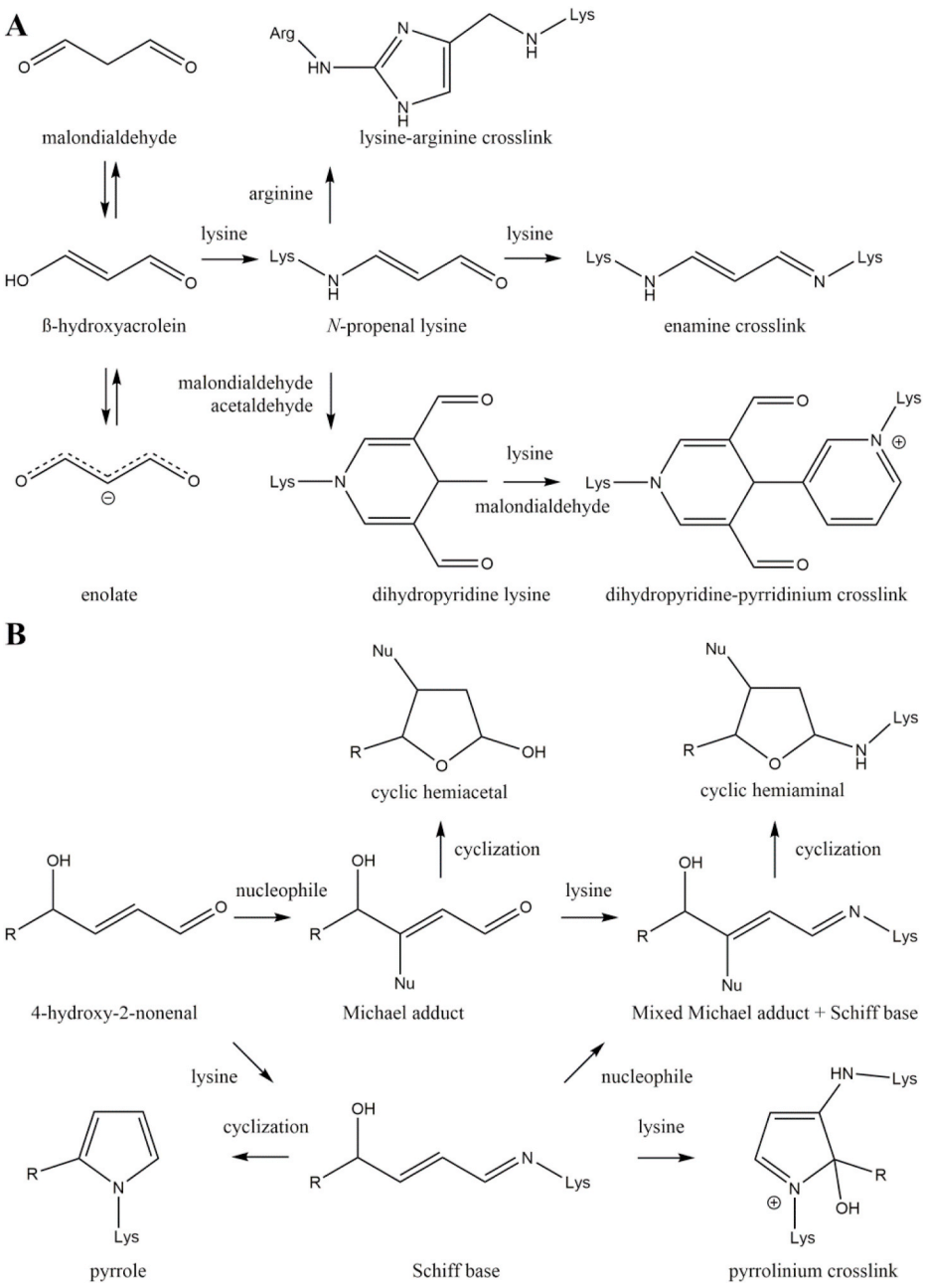

Fig. 4. Formation of lipoxidation products. Complex reaction cascades of malondialdehyde (A) and 4-hydroxynonenal (B) are briefly summarized and important protein modifications presented.

3.1. Sample preparation

Great attention should be paid on sample preparation to ensure reliable and reproducible analysis results. Preservation of biologically oxidized modifications and minimization of artifactual events at the same time are the biggest challenges and of utmost importance. Rogowska-Wrzesinska et al. [75] point out that proteins are not the only target of oxidation processes in biological samples. Oxidized molecules, such as nucleic acids and lipids, can cause high background signals, interfere with analysis procedures or can further promote oxidation reactions. An example for the latter is the formation of AGEs and ALEs from reaction of oxidized carbohydrates or lipids with proteins. Extraction steps might be applied to reduce the number of interfering compounds. Simultaneously, this leads to enrichment of proteins-of-interest which might be of importance for low abundant proteins [76]. Chemicals used for protein extraction should be chosen with caution since some of them can interfere with the protein oxidation analysis. For example, mild reducing agents such as dithiothreitol (DTT) and mercaptoethanol may reduce some protein oxidation products such as disulfides and carbonyl groups, respectively, making them unavailable for detection [77,78]. In addition, atmospheric oxygen and free metal ions can accelerate oxidation. To avoid overestimation of modified proteins due to sample preparation, it might be helpful to use derivatization reagents such as 2,4-dinitrophenylhydrazine (DNPH) or *p*-aminobenzaldehyde [75]. With derivatization, biological modifications are captured and stabilized and new modifications, resulting from further sample handling, are not contributing to the measured values. Chemical labeling of modifications offers the possibility of enrichment by isolating a specific modification from complex samples by affinity chromatography. Enrichment can be performed based on chemical reactivity, resin capture agents or antibodies for the modification or chemical tag [79]. Depending on the subsequent analysis technique,

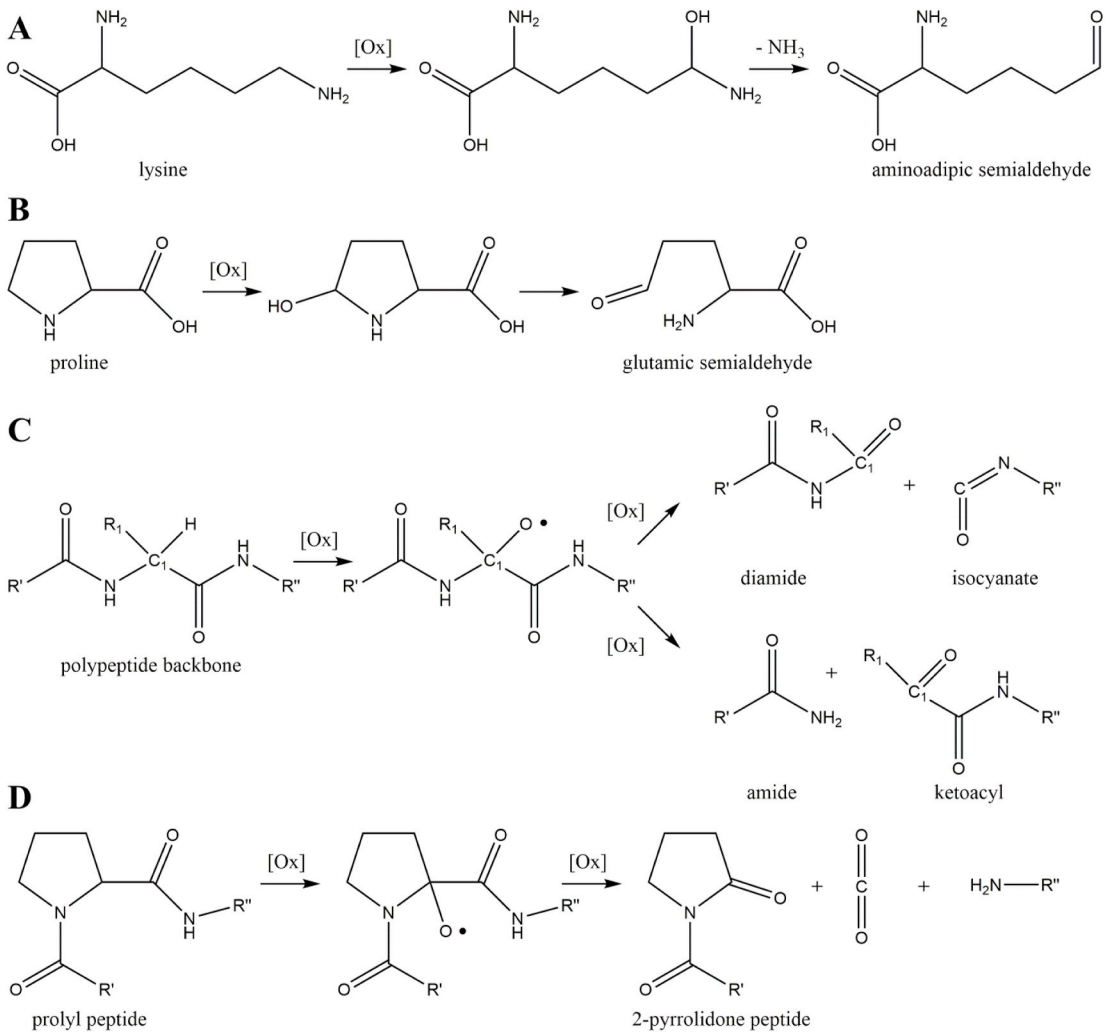

Fig. 5. Pathways of protein carbonylation. Lysine and proline residues are vitally important precursors of protein carbonylation by oxidative formation of aminoadipic semialdehyde (A) and glutamic semialdehyde (B), respectively. Oxidative cleavage of peptide backbone results in protein carbonylation via diamide and α-amidation pathways (C). Formation of 2-pyrrolidone by oxidation of prolyl containing peptide (D).

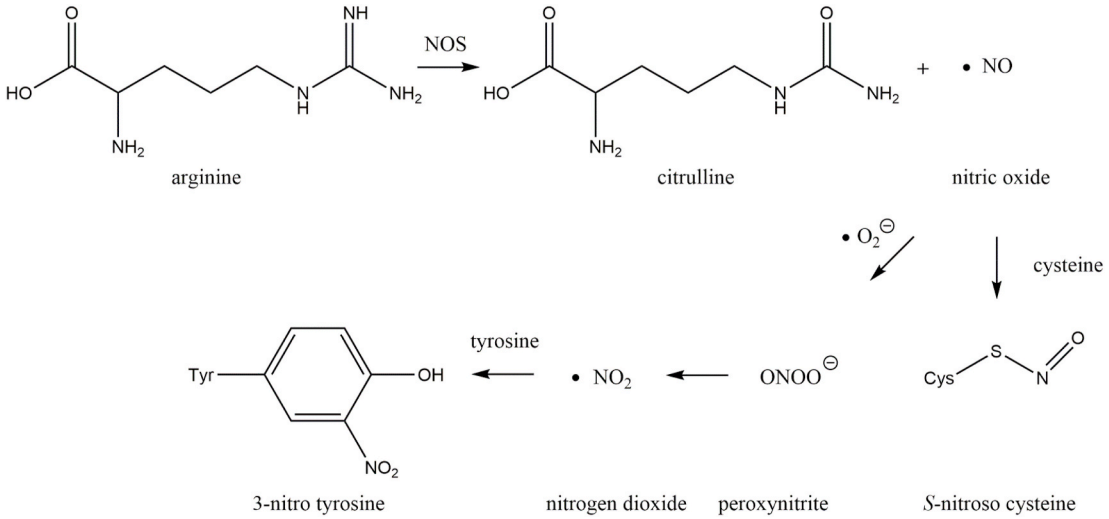

Fig. 6. Generation of reactive nitrogen species (RNS). Nitric oxide synthase (NOS) catalyzes the generation of nitric oxide, which is the precursor of *S*-nitrosylation and nitration.

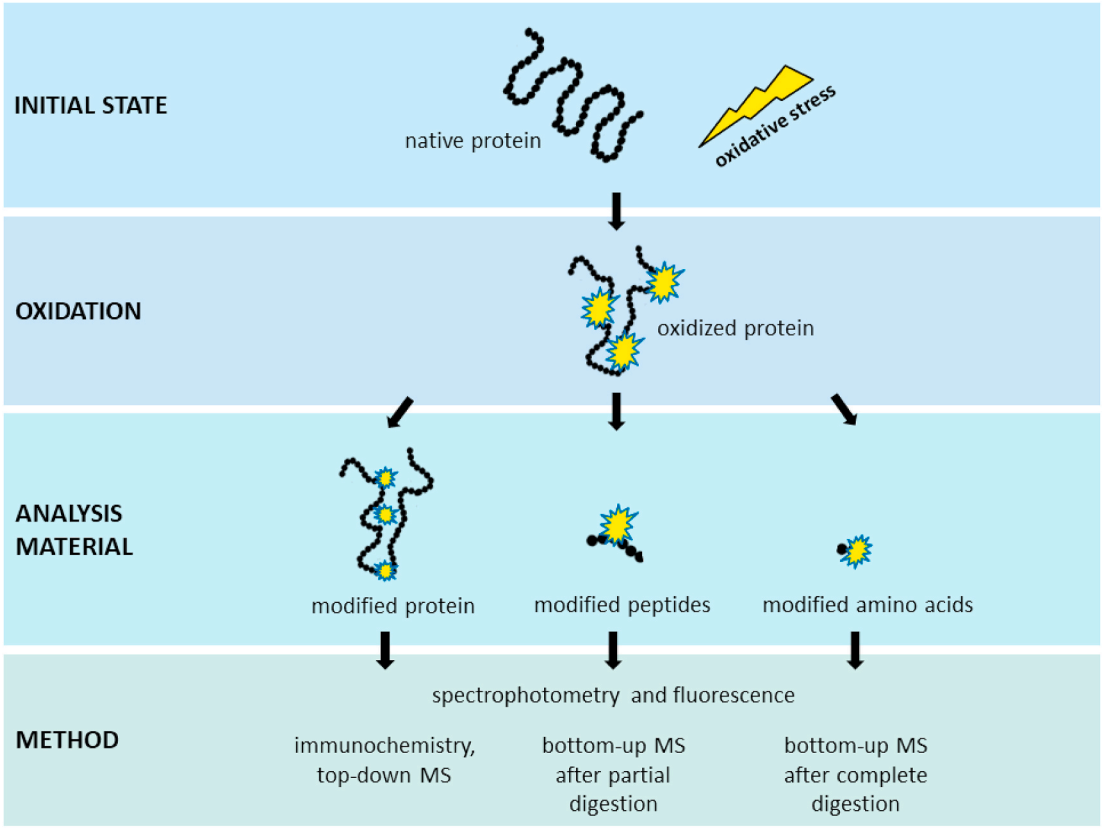

Fig. 7. Analysis of oxidative protein modifications. Proteins are oxidized by ROS. Subsequent molecular changes can be analyzed for individual proteins, peptides or modified amino acids. Depending on the choice of analytes, different analysis techniques are available. Among the presented methods, mass spectrometry is the most accurate technique and its application shall be further increased for biological samples to advance the knowledge regarding protein oxidation.

chemical tags might be removed or can even facilitate downstream analysis (e.g. DNPH as a matrix for matrix assisted laser desorption ionization mass spectrometry (MALDI-MS) of carbonylated proteins) or chemical tags function as reporters during MS analysis [80]. It can be crucial to perform sample preparation as quickly as possible after sampling to prevent changes from the biological oxidation pattern or formation of artifacts. Moreover, standardization of methods and reference material can help to obtain consistent results. There are commercially available oxidized proteins which can be used to regularly check analytical results. However, data on exact degree of modification provided by the supplier is limited. In most cases, the specific types and sites of modifications are not identified.

3.2. Analysis of specific oxidation products

The analysis of specific oxidation products is crucial to reveal the chemistry behind oxidation and give information about the protein involved in oxidation. Furthermore, depending on the method, targeted analysis provides quantitative or at least semi-quantitative data.

3.2.1. Spectrophotometric methods

Protein oxidation can lead to the introduction of carbonyl groups into amino acid residues of the protein (see Fig. 5). The derivatization of this functional group with carbonyl-specific reagents provides a method to detect and quantify protein carbonylation [81]. The most common method to analyze protein carbonylation is the reaction with 2,4- dinitrophenylhydrazine (DNPH) (Fig. 8).

The resulting hydrazones are quantified spectrophotometrically or by immunological methods. Sample preparation and derivatization conditions for the determination of protein carbonyls were extensively reviewed elsewhere [82]. In brief, oxidized proteins are incubated with an excess of DNPH for complete derivatization of carbonyl groups. Unbound DNPH needs to be removed from the sample, because it absorbs at the same wavelength as hydrazones (λ = 370 nm). Several washing steps

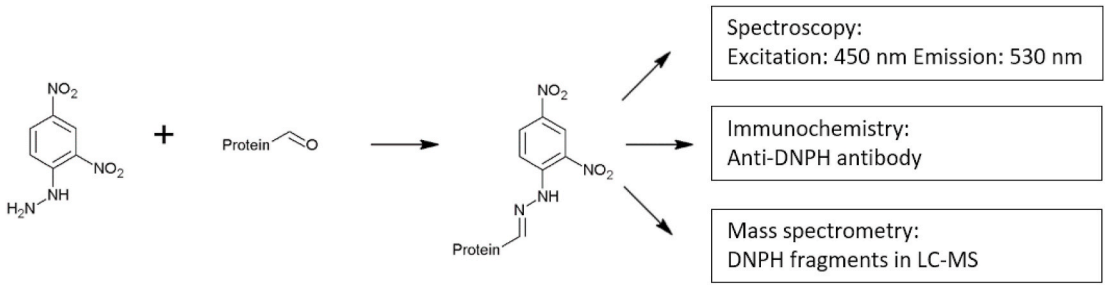

Fig. 8. Quantitation of protein carbonylation. Carbonyl groups are stabilized by derivatization with 2,4-dinitrophenylhydrazine (DNPH). The resulting hydrazones are quantified using a spectrophotometer, specific antibodies, or are analyzed using LC-MS.

of the protein are required to remove unbound DNPH leading to an inevitable loss of protein of up to 10–15% [75]. An alternative assay to overcome the limitations of the classical DNPH assay was developed by Mesquita et al. [83]. DNPH-derivatized protein solution is neutralized with sodium hydroxide prior to spectrophotometric measurement. Neutralization shifts the absorbance of protein-conjugated hydrazones from 370 nm to 450 nm, thereby eliminating the interference with unbound DNPH. According to the authors, no further washing steps are required. It is important to notice that carbonyl compounds can also be formed during lipid peroxidation and the Maillard reaction, leading to an overestimation of protein oxidation. The accessibility and reactivity of carbonyl groups depends on the tertiary structure of proteins. Protein crosslinking or pronounced tertiary structures can hide DNPH-active carbonyl structures inside of proteins making them unavailable for detection. An important drawback of the DNPH method is its unspecific nature towards other oxidation products besides carbonyl groups. It was mentioned by Hellwig [84] that sulfenic acids, which are not of carbonyl form, still react in the assay.

In 2019, Ma and colleagues described a novel method for the detection of carbonylated proteins by frequency-shift based surface-enhanced Raman spectroscopy immunoassay, eliminating potential interferences by introducing a capture antibody [85]. Besides this, LC-MS/MS has been also used for proteomic analysis to identify carbonylated proteins, providing a more sensitive approach, but reveal limitations for quantitative assessments [86].

3.2.2. Fluorescence

Fluorescence spectroscopy can serve as a sum parameter for overall protein oxidation. It needs to be considered that several oxidative and also glycoxidative modifications of amino acid residues can contribute to fluorescence signals. The limited selectivity is a major drawback of fluorescence spectroscopy for studying protein oxidation products. However, because of its sensitivity, robustness and the minimal instrumental requirements, fluorescence spectroscopy became a popular approach in protein modification analysis. A selection of fluorescent oxidation products is shown in Table 1. Dityrosine and *N*-formyl kynurenine are formed by oxidation, whereas other compounds, such as pentosidine and the "aging pigment" lipofuscin, are formed by glycation and/or oxidation reactions. Since several fluorophores, each with a different specific fluorescence, contribute to the total fluorescence of biological samples, no quantitative calibration can be achieved. Since fluorescence is not limited to oxidation products, the method without prior chromatographic separation is only of minor importance for biomarker analysis of protein oxidation.

3.2.3. Immunochemical methods

Immunological techniques are widely used to identify modified protein residues. Reduced antibody binding to native epitopes indicates modification of binding sites and can be a marker of oxidative changes. Depending on the degree of modification, oxidative damage can also lead to increased antibody binding. This was shown in several studies examining the effect of hypochlorous acid on extracellular matrix proteins [87,88]. The rather high sensitivity of immunological methods and easy-to-carry-out protocols boosted the number of biological studies working with immunoblotting or enzyme linked immunosorbent assay (ELISA). Various commercially available monoclonal and polyclonal antibodies promise to detect specific protein oxidation products. Due to minimal instrumental requirements and straightforward experimental implementation, immunoassays became very popular for the analysis of protein modifications, especially in clinical applications. Immunological detection can be performed in complex mixtures or after separation, e.g., gel electrophoresis. Full chemical and structural characterization of antibody binding is crucial for the evaluation of its selectivity and sensitivity [89,90]. However, most commercial antibodies lack this characterization and their target epitopes remain unknown. Most antibodies detect different oxidation products which can be explained by poor antigen preparation and characterization. An alternative to the rather unspecific binding of antibodies to oxidation products might be the use of more selective antibodies raised against derivatization reagents. A prominent example is the anti-DNP antibody, which detects hydrazones resulting from the reaction of DNPH and carbonyl groups [91]. Besides structural uncertainty, absolute quantification is problematic with immunochemical methods. Most commercial suppliers of immunoassays provide standards in their kits. Since there is no market wide standardization of reference material, concentration values may vary depending on the used kit. Protein carbonyls, 3-NT and methionine sulfoxide are biomarkers of protein oxidation which are commonly analyzed via immunochemical methods [75,82,92].

Table 1
Fluorophores derived from oxidation or glycation reactions [84,163].

Fluorescent structure	$\ddot{e}_{ex/em}$ [nm]	Reference
Dityrosine	283/409	[201]
N-Formyl kynurenine	325/434	[202]
Kynurenine	365/480	[202]
Lipofuscin	366/570-605	[203]
"AGE fluorescence"	330-350/420-440	[204,205]
Imidazolone	320/398	[206]
Argpyrimidine	320/385	[207]
Pentosidine	335/385	[40]

3.2.4. Mass spectrometric methods

Mass spectrometry (MS) has been used for many years to identify and characterize proteins. Consequently, MS-based methods were developed to analyze protein oxidation products. Compared to other methods available, MS is currently the gold standard and arguably the most informative technique for protein analysis. It can be divided in "top-down" or "bottom-up" approaches which basically describes the protein treatment prior to analysis. "Top-down" means that intact proteins are introduced in the MS and their fragmentation patterns are assessed, whereas "bottom-up" requires enzymatic digestion of proteins to peptide fragments prior to MS analysis [79]. The latter is more common in proteome research and helped to sequence and identify proteins in biological samples. A special case of the "bottom-up" approach is complete digestion of the protein down to individual (modified) amino acids followed by MS analysis. It was used to investigate a wide range of oxidized amino acids, thus, identifying new oxidation products and allowing total quantification of a particular product. A drawback of complete digestion is the lack of information on the specific protein that has been modified, or the exact site of modification.

3.2.4.1. Top-down. The mass of intact proteins can be analyzed via MALDI (matrix-assisted laser desorption/ionization) or ESI (electrospray ionization) ionization techniques connected to high resolution mass analyzers, such as quadrupole-time-of-flight or orbitrap instruments. ESI results in various m/z ratios of the protein of interest, since more than one charge during ionization is usually acquired. Regarding ionization, ESI is the more common and often sensitive technique since the compilation of peaks can be used to calculate protein mass more accurately. Analysis of intact proteins requires no extensive handling of protein samples prior to MS analysis. However, since experimental spectra are compared to theoretical spectra to determine the modification status of proteins, mostly individual proteins are processed. Purification of complex protein mixture is especially challenging in biological samples. Top-down approaches can be helpful to evaluate the total modification status on an individual protein [93–96]. Small modifications with low abundancies require more sophisticated, high-resolution instruments such as Q-TOFs, Orbitraps or FT-ICR MS. Another option to explore minor modifications occurring on a small

percentage of proteins is fragmentation of intact proteins. A selected protein ion ("precursor ion") can be dissociated resulting in smaller charged fragments ("product ions"). Dissociation methods such as electron capture dissociation (ECD) and electron transfer dissociation (ETD) are particularly good at preserving labile posttranslational protein modifications. Besides recent advances in top-down MS approaches, a number of disadvantages require the additional performance of bottom-up measurements to receive valid data. For example, sequence coverage in biological samples remains quite low using top-down MS due to overlapping and deconvolution effects [79]. Moreover, complex proteins often need to be dissolved in buffer systems or detergents to ensure complete solubility, but ESI mass spectrometers are not compatible with many buffers and especially not with detergents. Thus, buffer salts need to be removed before analysis and if a protein is insoluble in detergent-free solutions, it cannot be analyzed by top-down MS. With regard to biological samples the main drawback of top-down MS is its limitation to analyze mostly individual proteins and its focus on targeted analysis of known proteins. Complex mixtures need to be purified extensively by liquid chromatography before top-down analysis. Finally, top-down analysis requires high amounts of purified proteins to acquire highly resolved peaks of precursor and product ions with sufficient intensity [97]. Taken together, top-down MS remains an attractive technique to evaluate the overall modification status of proteins, but due to its various drawbacks, complementary techniques, such as bottom-up proteomics, need to be employed to acquire a comprehensive understanding of the protein structure.

3.2.4.2. Bottom-up. Digestion of proteins to the peptide level prior to analysis is characteristic for bottom-up MS approaches. After digestion, specific labels (also called "tags") can be introduced into the molecule, whereby a specific modification reacts with a chemical label which can then be analyzed with reporter ions. This technique facilitates the analysis of specific modifications in a peptide and is also able to determine the modification status of peptides. A prominent example for a labeling approach is the tagging of cysteine to monitor the reversible oxidation status [98,99]. However, more common in standard proteomics are unlabeled methods which have the advantage of less sample manipulation.

Bottom-up approaches can be divided in untargeted ("discovery" or "shot-gun") and targeted approaches. For the detection of oxidative protein modifications, untargeted methods are only of limited help. The selection of precursor ions for subsequent fragmentation is usually performed automatically and depends on the abundancy of the corresponding ion in the MS scan. However, modified peptides often occur only at low abundancies making it unlikely that they are covered by untargeted approaches. New techniques use upstream scanning methods, which identify unique ions of oxidized residues that reveal the presence of oxidative modifications and subsequent fragmentation of the according peptides. In general, untargeted approaches are mostly used to scan for certain proteins in biological samples and check for proteome changes in samples of healthy versus diseased individuals and not to analyze specific protein modifications. The specificity for the identification of oxidative modifications is improved with semi-targeted and targeted methods. In the latter, precursor and product ion are known, whereas in semi-targeted approaches only the product ion can be used as a diagnostic tool. The presence of a specific fragment ion serves as a reporter for a certain modification. Especially on low resolution instruments, data evaluation should be treated with caution since isobaric ions from non-modified peptides can yield false-positive results [100]. At best, it is recommended to confirm modification sites with synthetic modified peptides. Fully targeted analysis of oxidative protein modifications is the strategy with the highest sensitivity and most accurate quantification. This includes single reaction monitoring (SRM) and multiple reaction monitoring (MRM), where both the precursor ion and product ion masses are known for the corresponding analyte. For quantification, the abundance of the analyte ion intensities after appropriate normalization can directly be compared. The modification of a peptide may alter its ionization properties greatly, especially when ionizable groups are introduced or removed. This needs to be considered when comparing ion intensities between a certain peptide and its modified form. The most accurate method is certainly quantification with standard material of the oxidative modification of interest. However, since standard material is often not available, especially not in peptide form, this approach requires synthesis efforts or cannot always be implemented. To gain as much information as possible about the modification status of a protein of interest, it is useful to combine top down and bottom up mass spectrometric approaches and check whether a specific modification analyzed by targeted approaches can be confirmed by the loss of parent structures.

3.3. Analysis of total protein modification

A major challenge in analyzing protein modifications in biological samples is that unmodified reference material is commonly not available. However, the comparison of different sample groups (such as healthy and diseased) can provide important information without examining the exact modification site. One plausible approach is the analysis of protein structure that can provide information concerning the degree of protein modification without analyzing specific oxidation products. For example, aggregate formation can be monitored by gel electrophoresis or biophysical methods, such as light scattering or X-ray crystallography. However, these methods do not give any information about the type or site of modification and are therefore only of limited use for biomarker studies. A method which reveals modification site, but not the type of modification is amino acid analysis. By analyzing hydrolysates of native and modified protein samples, the amino acid composition can provide information about the loss of parent proteins, but also the formation of products [101]. Various protocols for the analysis of amino acids exist using derivatization with dansyl chloride or *ortho*-phthalaldehyde and subsequent HPLC-FLD [102,103] or HPLC-UV [104,105] or preparation of butyl esters followed by ion-pair LC-MS/MS [106]. For analysis of protein-bound amino acids, hydrolytic cleavage needs to be performed. Acid hydrolysis conditions lead to a loss of cysteine, cystine, and some tryptophan species, whereas alkaline conditions preserve tryptophan species, but breaks down arginine, serine, threonine and cysteine [107]. A mild method for proteolysis is enzymatic cleavage with nonspecific proteases. Depending on the parent protein, it might be useful to apply multistep enzymatic approaches [108]. Quantitative data can easily be achieved by using external calibration and, in case of MS analysis, internal isotope-labeled standards. Thus, amino acid analysis provides valuable information about the modification state of proteins. For detailed information on modification products and the types of proteins, MS analysis of peptides, as described above, is inevitable.

4. The relevance of biomarkers of protein oxidation in the context of human disease and aging

Research over the past decades displayed a substantial impact of protein oxidation in various human diseases, among others diabetes, cardiovascular disease (CVD), cancer, atherosclerosis, arthritis and neurodegenerative disease. It has been shown that most of these pathologic conditions increase as a function of age, indicating that oxidation of proteins occurs simultaneously to aging and age-related diseases [109–111]. Therefore, the identification of valid biomarkers of protein oxidation has an indispensable relevance to improve the understanding of human disease pathogenesis and provide an important tool to capture disease state as well as develop potential treatment strategies. In general, biomarkers for clinical application, typically measured in samples from body fluids and diverse tissues, have to fulfill certain requirements ranging from high sensitivity, specificity, reproducibility to reliability.

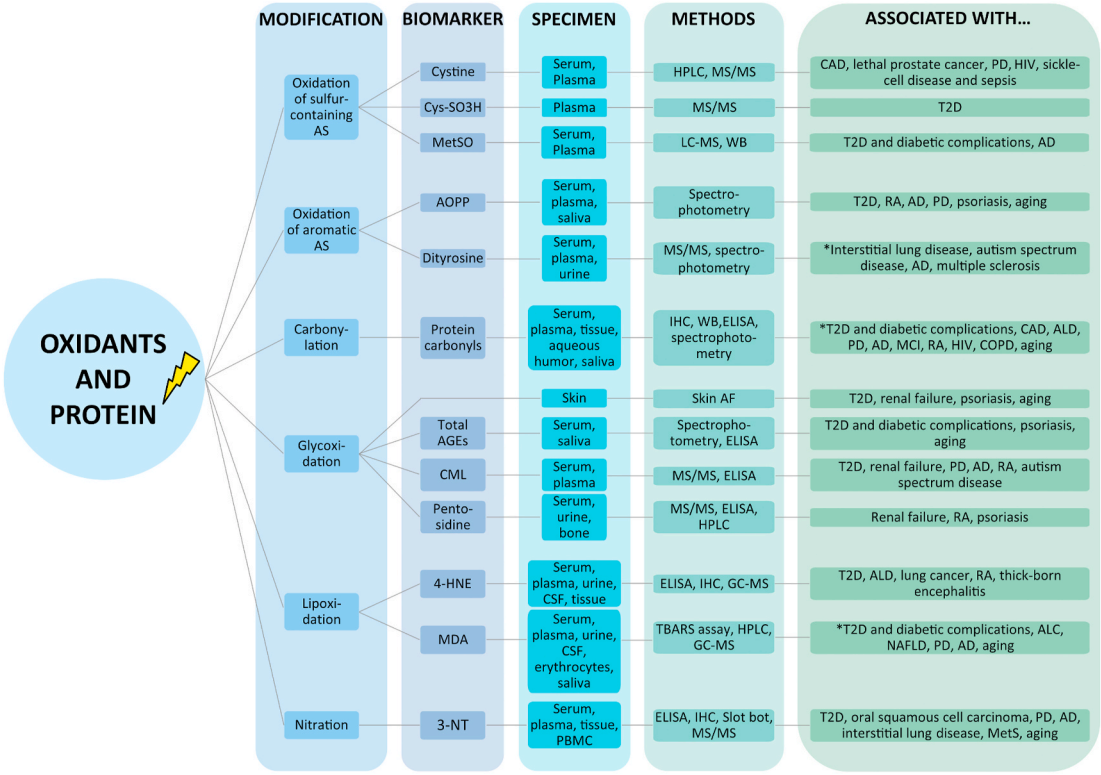

Fig. 9. Schematic overview of frequently used biomarkers of protein oxidation. Their strengths, limitations, and relevance as biomarkers in human diseases and aging are discussed in sections 4.1–4.6, based on the current state of research. For further details see also Tables 2–7 3-NT – 3-Nitrotyrosine, 4-HNE – 4-Hydroxy-2-nonenal, AD – Alzheimer's disease, AGE – Advanced glycation endproduct, ALD – Alcoholic liver disease, AOPP – Advanced oxidation protein product, AS – Amino acids, CAD – Coronary artery disease, CML – Carboxymethyl lysine, COPD – Chronic obstructive pulmonary disease, CSF – Cerebrospinal fluid, Cys-SO3H – Cysteine sulfonic acid, ELISA – Enzyme linked immunosorbent assay, HIV – Human immunodeficiency virus, HPLC – High pressure liquid chromatography, IHC – Immunohistochemistry, NAFLD – Non-alcoholic fatty liver disease, MCI – Mild cognitive impairment, MDA – Malondialdehyde, MetS – Metabolic syndrome, MetSO – Methionine sulfoxide, MS – Mass spectrometry, PBMC – Peripheral blood mononuclear cells, PD – Parkinson's disease, RA – Rheumatoid arthritis, Skin AF – Skin autofluorescence, T2D – Type 2 diabetes, TBARS – Thiobarbituric acid reactive substances, WB – Western Blot. *among others.

Moreover, due to high sample throughput in clinical studies, methods should be affordable, convenient and robust as well as rapid and easy to perform [112–115]. Here, we aim to review the most chemically stable and frequently used biomarkers of protein oxidation in routine clinical diagnostics (summarized in Fig. 9), and discuss their strengths and limitations, based on the current state of research. Since several outstanding reviews of single or multiple biomarkers and their clinical and biological relevance have been published previously [110,116,117], we selected and focused on recent clinical studies between 2015 and 2020. The search strategy on PubMed database in November 2020 was composed of search terms of the individual biomarkers plus "human disease", "human age-related disease", or "human aging".

4.1. *Sulfur-containing amino acids in human diseases and aging*

Sulfur-containing amino acids, such as cysteine and methionine are highly susceptible to diverse forms of oxidation processes [118,119]. In particular, cysteine residues are major targets of ROS undergoing a large number of posttranslational modifications, leading to functional and structural alterations of proteins. Thus, cysteine and its oxidized forms, such as cystine and further cysteine disulfide species, are considered as potential biomarkers of protein oxidation in human disease [120,121]. However, a strong limitation for the analysis of cysteine species is their instability due to the dynamic nature, since oxidation products of protein cysteine residues can be easily reduced by other thiols [122,123]. Cysteine residues have been detected in serum and plasma samples via HPLC or different combinations of chromatographic techniques with MS or MS/MS. In particular, the latter approaches have been used in recent human studies, providing high sensitivity, specificity and reproducibility, but are limited for routine clinical applications, because of high costs for laboratory equipment and low sample throughput. By using these methods, researchers found higher cystine levels in patients with Parkinson's disease (PD) and human immunodeficiency virus (HIV) compared to healthy individuals as well as elevated serum cystine levels associated with reduced risk for lethal prostate cancer (Table 2). Another promising method has been described in 2019 by Fu and colleagues. They used a UPLC-MS/MS approach for simultaneous quantification of thiols as well as disulfides and found higher levels of cysteine disulfides in plasma of patients with sickle-cell disease and sepsis compared to healthy donors [124]. All of these studies are related to the oxidation of free amino acids or glutathione and can therefore not be considered as studies investigating direct protein oxidation. Quantitative detection of cysteine trioxidation, a stepwise and irreversible cysteine modification generating sulfonic acid motifs, has been recently developed by Paramasivan and colleagues. By using a LC-MS/MS approach, they found elevated cysteine sulfonic acid in human serum albumin of type 2 diabetes (T2D) patients compared to control subjects, indicating the potential of trioxidized peptides as prognostic biomarker of protein oxidation in human disease [125]. The use of cysteine modifications as biomarkers of aging has not been investigated in recent years, but a review by Oliveira and Laurindo in 2018 highlights the association of changes in the $thiol_{red}/thiol_{ox}$ redox couple in aging and age-related disease, including cancer, CVD, and neurodegenerative disease [121].

Besides cysteine, methionine is another sulfur-containing amino acid that reacts to methionine sulfoxide under oxidative stress conditions. In

Table 2
Selected studies using modifications of sulfur-containing amino acids as biomarkers of protein oxidation in clinical settings.

Disease/aging	Specimen	Study population	Method	Main outcome	Reference
Cystine and cysteine sulfonic acid					
Coronary artery disease (CAD)	Plasma	N = 247 patients (of n = 1411) experienced primary outcome of death	HPLC	Higher cystine levels associated with increased risk for all-cause mortality in CAD patients	[208]
Lethal prostate cancer	Serum	Control subjects vs. lethal prostate cancer patients (n = 523 per group)	LC-MS/MS	Higher serum cystine levels are associated with reduced risk for lethal prostate cancer	[209]
PD	Serum	Control subjects (n = 30) vs. PD patients (n = 20)	UPLC-MS	Higher cystine levels in PD patients	[210]
HIV	Serum	HIV-negative (n = 21) vs. HIV-positive (n = 113) patients	GC-MS	Increased levels of cystine in HIV-positive patients	[211]
Sickle-cell disease and sepsis	Plasma	Healthy donors (n = 18) vs. sickle-cell disease (n = 9) and sepsis patients (n = 6)	UPLC-MS/MS	Elevated levels of cysteine disulfides (protein-bound cysteine) in disease patients	[124]
T2D	Plasma	Healthy subjects vs. T2D patients (n = 8 per group)	LC-MS/MS	Higher cysteine sulfonic acid levels in T2D patients	[125]
Methionine sulfoxide					
T2D and diabetic complications	Serum (serum albumin)	Healthy subjects (n = 18) vs. T2D patients with (n = 23) and w/o (n = 12) renal failure	LC-MS	Higher levels of methionine sulfoxide in T2D patients with and w/o renal failure compared to controls	[212]
AD	Plasma	Healthy subjects (n = 23) vs. patients with mild-cognitive impairment (MCI) (n = 13) or AD (n = 25)	WB	Elevated levels of methionine sulfoxide in plasma of AD patients compared to other groups	[130]

contrast to other thiol oxidation products, methionine sulfoxide shows higher stability and is easily detectable via a conventional amino acid analyzer [116,126,127]. Only a few publications are available, determining levels of methionine sulfoxide in clinical settings. However, higher methionine sulfoxide levels have been found in plasma and serum samples of T2D patients with and without renal failure and Alzheimer's disease (AD) in comparison to control subjects, measured via immunoblotting or LC-MS (Table 2). To our knowledge, studies investigating methionine sulfoxide levels as a function of age have not been published, recently. The fact that methionine sulfoxide levels increase age-dependently in human skin collagen, as shown earlier [128,129], and are elevated in AD patients, suggests an impact in the aging process [130].

4.2. Aromatic amino acids in human diseases and aging

Aromatic amino acid residues are major targets for several forms of ROS and RNS, leading to the formation of dityrosine-containing crosslinks, classified as AOPPs [131,132]. In clinical settings, AOPPs have been primarily detected via spectrophotometric assays, based on the

Table 3
Selected clinical studies using AOPP and dityrosine-containing crosslinks as biomarkers of protein oxidation.

Disease/aging	Specimen	Study population	Method	Main outcome	Reference
AOPP					
T2D or rheumatoid arthritis (RA)	Serum	Control subjects (n = 17) vs. T2D or RA patients (n = 20 per group)	Spectrophotometry	Higher AOPP levels in RA and T2D patients	[213]
T2D and aging	Plasma	Non-diabetic subjects (n = 38) vs. T2D patients (n = 50)	Spectrophotometry	Age-dependent increase of AOPP levels No differences between non-diabetic subjects and T2D patients	[135]
AD	Plasma	Control subjects (n = 34) vs. AD patients (n = 38)	Spectrophotometry	Higher levels of AOPPs in AD patients	[214]
PD	Plasma	Control subjects (n = 46) vs. PD patients (n = 40)	Spectrophotometry	Elevated AOPP levels in PD patients	[185]
Mild to moderate psoriasis	Serum	Healthy individuals (n = 40) vs. patients with mild to moderate psoriasis (n = 80)	Spectrophotometry	Increased levels of AOPP in psoriasis patients	[136]
Mild psoriasis	Plasma	Healthy subjects vs. patients with mild psoriasis (n = 84 per group)	Spectrophotometry	No difference in AOPP levels	[138]
Mild and moderate to severe psoriasis	Plasma	Healthy subjects (n = 14) vs. psoriasis patients (n = 29; mild n = 14, moderate to severe n = 15)	Spectrophotometry	Higher levels of AOPP in mild and moderate to severe psoriasis patients compared to controls	[137]
Aging	Saliva and plasma	Children (2–14 years), adults (25–45 years), elderly (65–85 years, n = 30 per group)	Spectrophotometry	Higher AOPP levels in saliva and plasma of elderly people compared to other groups	[141]
Dityrosine					
Interstitial lung disease	Plasma	Healthy individuals vs. patients with interstitial lung disease (n = 42 per group)	LC-MS/MS	Elevated levels of dityrosine in patients with interstitial lung disease	[142]
Autism spectrum disorders	Plasma, urine	Healthy children (n = 31) vs. children with autism spectrum disorders (n = 38)	LC-MS/MS	Elevated plasma and urine dityrosine levels in children with autism spectrum disorders	[143]
AD	Serum	Control subjects (n = 32) vs. AD patients (n = 52)	Spectrofluorometry	Higher dityrosine levels in AD patients	[215]
Chronic liver diseases	Plasma	Healthy subjects vs. patients with chronic liver disease (n = 30 per group)	Spectrophotometry	Elevated dityrosine levels in patients with chronic liver diseases	[216]
Multiple sclerosis	Plasma	Healthy subjects (n = 11) vs. patients with multiple sclerosis (n = 14)	Spectrophotometry	Estimated dityrosine levels were higher in patients with multiple sclerosis	[217]

Table 4
Selected studies using protein carbonyls as a biomarker of protein oxidation in clinical settings.

Disease/aging	Specimen	Study population	Method	Main outcome	Reference
T2D with vascular complications	Serum	Healthy individuals (n = 94) vs. T2D patients with vascular complications (n = 94)	ELISA	Higher protein carbonyl levels in T2D patients with vascular complications	[149]
T2D with NASH	Serum	Control subjects (n = 50) vs. T2D patients with NASH (n = 60) or T2D w/o NAFLD (n = 50)	ELISA	Elevated protein carbonyl levels in T2D patients with NASH compared to other groups	[218]
T2D with and w/o CKD	Serum	Healthy subjects vs. T2D patients with and w/o CKD (n = 50 per group)	Spectrophotometry/ HPLC	Higher protein carbonyl levels in T2D patients with and w/o CKD compared to control subjects	[219]
Diabetic nephropathy	Serum	Control subjects (n = 142) vs. T2D patients with diabetic nephropathy (n = 153)	ELISA	Elevated levels of protein carbonylation in patients with diabetic nephropathy	[150]
CAD	Plasma	Control subjects vs. premature CAD or normal CAD (n = 30 per group)	ELISA	Higher plasma protein carbonyl levels in premature and normal CAD patients compared to control groups	[220]
Acute coronary syndrome or chronic periodontitis	Saliva	Control subjects vs. patients with acute coronary syndrome, chronic periodontitis or both (n = 24 per group)	Spectrophotometry	Higher protein carbonyl levels in all disease groups compared to controls	[221]
Alcoholic liver cirrhosis (ALC)	Serum	Healthy control subjects (n = 130) vs. ALC patients (n = 57)	Spectrophotometry	No difference between control and ALC groups	[222]
Normal and end stage ALD	Hepatic tissue	Normal ALD (n = 8) vs. end stage ALD (n = 9)	IHC, WB	Increased protein carbonylation in liver sample of end stage ALD patients	[223]
PD and AD	Plasma	Control subjects (n = 34) vs. AD (n = 40) and PD patients (n = 70)	ELISA	Higher protein carbonyl levels in male AD patients compared to PD patients and controls	[151]
MCI, AD	Plasma	Healthy control (n = 24) vs. MCI (n = 24) and AD patients (n = 18 mild AD, n = 15 moderate AD, n = 14 severe AD)	WB	Higher levels of protein carbonyls in all AD groups compared to control and MCI groups	[224]
RA	Serum	Healthy control subjects (n = 41) vs. RA patients (n = 29)	Spectrophotometry	Higher levels of protein carbonyls in RA patients	[225]
RA	Plasma	Control subjects (n = 53) vs. RA patients (n = 120)	Spectrophotometry	Higher protein carbonyl levels in RA patients	[226]
Acute rheumatic fever in children	Serum	Healthy control (n = 31) vs. patients with acute rheumatic fever (n = 31)	Spectrophotometry/ HPLC	Higher serum protein carbonyl levels in patients with acute rheumatic fever	[227]
HIV	Plasma	Control group (n = 300) vs. HIV infected Antiviral Therapy (ART) naive (n = 100), on first line ART (n = 100), on second line ART (n = 100)	ELISA	Higher protein carbonyl content in HIV-infected patients on first- and second-line ART	[228]
Chronic obstructive pulmonary disease (COPD)	Plasma	Control patients (n = 15) vs. COPD patients (n = 42)	ELISA	Elevated levels of protein carbonyls in COPD patients	[229]
MetS	Serum	Healthy women (n = 77) vs. women with MetS (n = 106)	Spectrophotometry	Higher levels or protein carbonyls in women with MetS	[230]
Leptospirosis	Serum	Control subjects (n = 30) vs. mild (n = 50), severe (n = 60) Leptospirosis or Dengue Leptospirosis (n = 30)	ELISA, Spectrophotometry	Higher serum protein carbonyl levels in severe Leptospirosis patients compared to all other groups	[231]
Glaucoma	Serum, aqueous humor	Control subjects (n = 64) vs. glaucoma patients (n = 96)	ELISA	Higher levels of protein carbonyls in plasma and aqueous humor of glaucoma patients	[232]
Aging	Saliva, plasma	Young (n = 104) vs. middle-aged (n = 90) and elderly subjects (n = 79)	ELISA	Age-dependent increase in saliva and plasma protein carbonyl levels	[148]
	Serum	Adolescents (n = 30, mean age 17.1 years) vs. adults (n = 34, mean age 33.3 years) with bipolar disorder	ELISA	Higher protein carbonyl content in adults	[233]
	Plasma	Healthy individuals divided into 4 groups: group 30 (28–34 years), group 40 (35–45 years), group 50 (47–54 years) and group above 60 (57–79 years)	ELISA	Higher protein carbonyl levels in groups 40, 50 and 60 compared to group 30	[234]
	Plasma	3 study groups from MARK-AGE project: RASIG (n = 794), GO (n = 493), SGO (n = 272) Participants divided in 4 groups: 55-59, 60–64, 65–69 and 70–75 years	ELISA	Positive correlation between protein carbonyl levels and age in RASIG group No correlation between protein carbonyl levels and age in GO and SGO groups	[194]

oxidation reaction of iodide to iodate, as firstly described by Witko-Sarsat and colleagues in 1996 [133]. This technique has been modulated and optimized by different groups in recent years [134,135]. For instance, Taylor and colleagues described a modification by determining total iodide ion oxidizing capacity of plasma and eliminating the effect of sample precipitation that improves accuracy and reproducibility of the assay [135]. Due to the assay's simplicity, allowing for high sample throughput, most researchers used this technique in recent years. However, spectrophotometric assays for the determination of AOPPs are highly unspecific, detecting a wide range of optically absorbing species or total oxidative capacity of plasma and the exact contribution of AOPPs to this reaction remains unknown. Therefore, studies performed with this assay and subsequent conclusions regarding AOPPs should be treated with caution. Nevertheless, groups using this method indicated higher AOPP levels in patients with several diseases, including T2D, rheumatoid arthritis (RA), neurodegenerative disease as well as aging, but conflicting results have been found for psoriasis, a common immune-mediated skin disorder (Table 3). While Haberka et al. and Yazici et al. showed higher concentrations of AOPPs in psoriasis patients compared to healthy subjects, no differences have been found in the study by Skoie and colleagues [136–138]. This might be explained by the inclusion of patients with only mild severity of the disease, generally associated with lower levels of oxidative stress [138]. In line with previous findings [139,140], Maciejczyk et al. and Taylor et al. found elevated AOPP levels in plasma and saliva of humans at advanced age, suggesting their relevance in the aging process [135,141].

Besides this, crosslinks such as dityrosine are implicated in a number of pathologies and have not been only detected via spectrophotometric or spectrofluorimetric assays, but also by chromatographic separation followed by MS. Although MS approaches are expensive, require special laboratory equipment and are therefore often not applicable in routine clinical diagnostics, MS is the most specific and sensitive technique for the detection of dityrosine. With this, elevated concentrations of dityrosine have been found in plasma and urine samples of patients with interstitial lung disease and autism spectrum disorders [142,143]. Dityrosine levels have not been determined as a function of age in recent years, but in 2011 Campos et al. found a positive correlation between age and levels of dityrosine in urine samples of healthy smoker and non-smoker controls using unspecific spectrofluorometric detection [144]. Moreover, dityrosine crosslinks have been previously observed in amyloid plaques and cerebrospinal fluid (CSF) of AD patients via LC-ESI-MS/MS, indicating a potential role in aging and age-related diseases [145].

4.3. Carbonylation in human diseases and aging

Protein carbonylation is an irreversible protein modification, associated with crucial alterations in their functional and structural integrity, contributing to cellular dysfunction and tissue damage [146,147]. Due to relatively early formation during oxidative stress, higher stability in comparison to other oxidation products and simple analysis methods, protein carbonyls are one of the most commonly analyzed biomarkers in human metabolic and age-related diseases [63,111]. Traditionally, protein carbonyls are determined in plasma, serum, and tissue samples, but also in aqueous humor and saliva (Table 4). The latter provides advantages for patients due to convenient, non-invasive, pain- and infection-free sample collection, but might be affected by oral hygiene [148]. Protein carbonyls can be determined by ELISA, immunoblot techniques, immunohisto- and cytochemistry via specific anti-DNP antibodies, and direct spectrophotometric or HPLC measurements. These methods are based on the derivatization of carbonyl groups with DNPH, forming a stable hydrazone adduct, as described in section 3.2. In 2015, two independent articles compared the available standard methods for the detection of protein carbonylation and highlighted advantages and disadvantages of the individual techniques in detail. Since immunoblotting and LC-MS/MS approaches rather provides insights into molecular mass and the nature of carbonyl modifications, the use of ELISAs has been declared as the method of choice for quantification of protein carbonyls [82,86]. As shown in Table 4, this technique has been primarily used to determine protein carbonyls in clinical studies, because of its suitability for high-throughput analysis, small sample volumes and minimal laboratory requirements. Although Goycheva et al. and Almogbel and colleagues included T2D patients with different diabetic complications (vascular complications vs. diabetic nephropathy), protein carbonyls levels measured by ELISA were comparable in control as well as disease groups [149,150]. Independent of the used methods and specimens, almost all selected studies consistently revealed elevated levels of protein carbonyls in multiple diseases, including T2D and diabetic complications, lung, liver, infectious, or rheumatological disease (Table 4). The study by Sharma and colleagues, however, found lower levels of protein carbonyls in female AD patients compared to healthy control subjects, whereas male AD patients revealed higher concentrations. These sex-specific differences were also observed for other markers of protein oxidation, including AGEs, MDA, or 3-NT, and should be considered in future clinical assessments [151]. Although Weber et al. found no correlation between protein carbonyls and aging in two of three cohorts from the European MARK-AGE study, presumably related to genetic and lifestyle factors, all other recent studies revealed higher concentrations of protein carbonyls in adults and elderly compared to younger individuals (Table 4). In addition, elevated protein carbonyl levels have been found in neurodegenerative disease, such as AD, together indicating the relevance of protein carbonyls in aging and age-related diseases [63,151].

4.4. Glycoxidation in human diseases and aging

The formation and accumulation of glycoxidation products occurs during normal, but especially under certain physiological conditions, such as hyperglycemia, hyperlipidemia, and oxidative stress. This indicates their relevance in the progression of metabolic diseases, including neurodegenerative disease, chronic kidney disease (CDK), CVD, and cancer, but in particular, T2D and related complications as reviewed elsewhere [152]. Moreover, a growing body of evidence reveals that levels of glycoxidation products increase as a function of age in several tissues, dependent on the dietary intake and the presence of hyperglycemic conditions [152,153]. One major difficulty in detection of glycoxidation products for standardized clinical research is their structural complexity and heterogeneity, deriving from various formation mechanisms [154,155]. Among glycoxidation structures, CML and pentosidine are most frequently measured in clinical studies [156,157]. Several methods for their detection in blood, urine, and tissue samples have been established in recent years, such as spectrophotometry, immunohistochemistry (IHC), immunoblot, and ELISA techniques, whereas the latter has been most commonly used. Although ELISAs are easy to perform and ensure high sample throughput, their specificity and reproducibility have been criticized, especially by the limited description of applied antibodies, associated with different outcomes [158]. This becomes obvious by the comparison of two studies. Guerin-Duborg and colleagues used an AGE-ELISA Kit preferentially quantifying CML and revealed no differences between non-diabetic subjects and T2D patients as well as T2D patients with and without vascular diseases. In contrast, Farhan et al. found elevated AGE levels comparing the latter groups by using a competitive ELISA Kit without specification of detected glycoxidation products [159,160]. AGEs can be also detected via non-invasive skin autofluorescence (skin AF) readers [161]. The measurement correlates well with the total amount of AGEs in skin biopsies and has been applied in clinical settings, indicating elevated AGE levels in several diseases and aging, as shown in Table 5. However, this technique is highly unspecific to capture glycoxidation products since several other fluorescent structures, such as non-oxidatively formed AGEs or nicotinamide adenine dinucleotide are detected simultaneously. The method has to be standardized in terms of using the same

Table 5
Selected studies using glycoxidation products as biomarkers of protein oxidation in clinical settings.

Disease	Specimen	Study population	Method	Main outcome	Reference
T2D with or w/o vascular disease	Plasma	Non-diabetic controls (n = 31) vs. T2D patients (n = 75) with (n = 44) or w/o (n = 31) vascular disease	Spectrophotometry, ELISA	No difference in fluorescent-AGEs or AGE levels between non-diabetic subjects and T2D patients No differences in AGE levels between T2D patients with or w/o vascular disease Higher fluorescent-AGE levels in T2D patients with vascular disease compared to T2D patients w/o vascular disease	[160]
T2D with or w/o vascular complications	Serum	Healthy subjects (n = 20) vs. T2D patients with or w/o vascular complications (n = 25 per group)	ELISA	Higher AGE levels in T2D patients with and w/o vascular complications Highest AGE levels in T2D patients with vascular complications	[159]
Renal failure or T2D	Serum	Control individuals vs. patients with renal failure (n = 30 per group) Control subjects (n = 49) vs. T2D patients (n = 95)	Spectrophotometry, skin AF, LC-MS/MS	Higher total AGE, pentosidine and free CML levels in patients with renal failure Elevated free CML but not pentosidine and total AGE levels in T2D patients Higher skin AF in patients with renal failure and T2D compared to controls	[165]
Prediabetes	Plasma	Women with normal fasting glucose vs. women with impaired fasting glucose (n = 30 per group)	LC-MS/MS	No difference between groups in free CML levels	[169]
CKD	Plasma	Patients with CKD (n = 150)	LC-MS/MS, ELISA	No association between pentosidine levels and CKD	[235]
PD and AD	Plasma	Control subjects (n = 34) vs. AD (n = 40) and PD patients (n = 70)	UPLC-MS/MS	Higher protein-bound CML levels in AD and PD patients compared to controls	[151]
Schizophrenia	Red blood cell lysates	Control subjects vs. schizophrenia patients (n = 23 per group)	WB	No differences in pentosidine levels	[236]
Autism spectrum disorders	Plasma	Healthy children (n = 31) vs. children with autism spectrum disorders (n = 38)	LC-MS/MS	Increased plasma CML levels in children with autism spectrum disorders	[143]
RA	Serum	Control subjects (n = 30) vs. RA patients (n = 80)	ELISA	Higher CML and pentosidine levels in RA patients	[237, 238]
Psoriasis	Skin, serum	Healthy individuals and psoriasis patients (n = 40 per group)	Skin AF, ELISA	Higher skin AF, total AGEs and pentosidine levels in patients with psoriasis	[239]
Multiple sclerosis	CSF, plasma, brain tissue	Non-demented control subjects (n = 10) vs. multiple sclerosis patients (n = 15)	UPLC-MS/MS, IHC	No difference in plasma free and protein-bound CML levels	[168]
>Aging (and Diabetes)	Skin	Age groups: group 20–30 years, n = 18 group 30–40 years, n = 10 group 40–50 years, n = 14 group 50–60 years, n = 12 group >60 years, n = 11 diabetes group, n = 13	Skin AF	Skin AF increases with age Higher skin AF in diabetes patients compared to groups aged <50 years	[171]
	Skin, urine, serum, bone	Patients with spine disease	Skin AF, ELISA, HPLC	Age-related increase in serum, urine, bone and skin pentosidine levels	[170]
	Saliva, plasma	Children (2–14 years), adults (25–45 years), elderly (65–85 years, n = 30 per group)	Spectrophotometry	Higher AGE levels in saliva and plasma of elderly people compared to children and adults	[141]

Table 6
Selected clinical studies using 4-HNE and MDA as biomarkers in different diseases.

Disease	Specimen	Study population	Method	Main outcome	Reference
4-HNE					
T2D	Serum	Control subjects (n = 9) vs. T2D patients (n = 11)	ELISA	Higher levels of 4-HNE in patients with T2D	[240]
Alcoholic liver disease (ALD)	Human hepatic tissue	Normal ALD (n = 8) vs. end stage ALD (n = 9)	IHC	Elevated 4-HNE in liver sample of ALD patients	[223]
Lung cancer	Serum	Control subjects (n = 40) vs. lung cancer patients (n = 92)	ELISA	Higher levels of 4-HNE in male and female lung cancer patients	[183]
RA	Plasma, urine	Healthy subjects vs. RA patients (n = 73 per group)	GC-MS, ELISA	Elevated levels of free and protein-bound 4-HNE in RA patients	[177]
Thick-born encephalitis	CSF, plasma, urine	Healthy subjects (n = 56) vs. patients with thick-born encephalitis (n = 60)	GC-MS, ELISA	Elevated free and protein-bound 4-HNE levels in plasma of thick-born encephalitis patients	[178]
MDA					
T2D and NAFLD	Serum	T2D patients with (n = 73) and w/o NAFLD (n = 51)	TBARS assay	Higher MDA levels in T2D patients with NAFLD	[241]
T2D and CKD	Plasma	Healthy subjects vs. T2D patients with or w/o CKD (n = 50 per group)	TBARS assay	Higher MDA levels in T2D patients with and w/o CKD compared to control subjects	[219]
T2D with vascular complications	Plasma	Healthy individuals (n = 94) vs. T2D patients with vascular complications (n = 93)	TBARS assay	Higher levels of MDA in T2D patients with vascular complications	[149]
ALC	Serum	Healthy subjects (n = 130) vs. ALC patients (n = 57)	TBARS assay	Higher MDA levels in patients with ALC	[222]
NAFLD	Plasma	Healthy subjects (n = 40) vs. NAFLD patients (n = 67)	TBARS assay	Higher MDA levels in NAFLD patients	[85]
Lung cancer	Serum	Control subjects (n = 40) vs. lung cancer patients (n = 92)	TBARS assay	No differences in MDA levels between the groups	[183]
PD	Serum	Control subjects (n = 46) vs. PD patients (n = 40)	TBARS assay	Elevated MDA levels in PD patients	[185]
PD and AD	Plasma	Control subjects (n = 34) vs. AD (n = 40) and PD patients (n = 70)	RP-HPLC	Lower MDA levels in AD and PD patients compared to control subjects	[151]
RA	Plasma, urine	Healthy subjects vs. RA patients (n = 73 per group)	GC-MS	Elevated levels of MDA in plasma and urine of RA patients	[177]
Thick-born encephalitis	CSF, plasma, urine	Healthy subjects (n = 56) vs. patients with thick-born encephalitis (n = 60)	GC-MS	Elevated MDA levels in all specimens of thick-born encephalitis patients	[178]
	Plasma	Healthy individuals divided into 4 groups: group 30 (28–34 years), group 40 (35–45 years), group 50 (47–54 years) and group above 60 (57–79 years)	HPLC	No differences between the groups	[234]
	Serum	Adults (Age 20–50 years) vs. elderly (Age $\leq$ 60 years, n = 30 per group)	TBARS assay	Higher MDA levels in elderly	[242]
	Erythrocytes	Healthy subjects aged $\leq$65 years (n = 27) and $\geq$65 years (n = 30)	TBARS assay	No differences between the groups	[243]
Aging	Saliva, plasma	Children (2–14 years), adults (25–45 years), elderly (65–85 years, n = 30 per group)	TBARS assay	Higher MDA levels in saliva and plasma of elderly people compared to children and adults	[141]
	Plasma	3 study groups from MARK-AGE project: RASIG (n = 794), GO (n = 493), SGO (n = 272) Participants divided in 4 groups: 55-59, 60–64, 65–69 and 70–75 years	RP-HPLC	No correlation in all groups between MDA levels and age	[194]

Table 7
Selected studies using 3-NT as biomarker of protein oxidation in clinical studies.

Disease	Specimen	Study population	Method	Main outcome	Reference
T2D	Serum	Non-diabetic subjects (n = 14) vs. T2D patients (n = 72) T2D patients with (n = 14) or w/o (n = 46) cardiovascular events	ELISA	Elevated levels of 3-NT in T2D patients Higher 3-NT levels in T2D patients with cardiovascular events	[189]
T2D	Serum	Non-diabetic subjects (n = 35) vs. T2D patients (n = 60)	ELISA	Higher levels of protein-bound 3-NT in T2D patients	[244]
T2D and CAD	Plasma	T2D patients with (n = 36) and w/o (n = 32) CAD	ELISA	No differences between the groups	[190]
Mortality in CHD patients	Serum	Patients with acute coronary syndrome (n = 342)	ELISA	No relationship between 3-NT and mortality during 4-years of follow-up	[245]
Oral squamous cell carcinoma	Tissue samples (oral mucosa)	Normal oral mucosa (n = 16) vs. oral leukoplakia (n = 42) or oral squamous cell carcinoma with (n = 46) and w/o (n = 39) metastases	IHC	Higher 3-NT levels in oral squamous cell carcinoma samples with metastases compared to samples w/o metastases	[246]
PD and AD	Plasma	Control subjects (n = 34) vs. AD (n = 40) and PD patients (n = 70)	ELISA	Higher 3-NT levels in male AD patients compared to PD patients and controls	[151]
AD	PBMC	Non-demented subjects vs. AD patients	Slot blot	Increased total 3-NT levels in PBMC of AD patients	[191]
AD	Plasma	Healthy subjects (n = 37) vs. AD patients (n = 48)	ELISA	Higher plasma 3-NT concentrations in AD patients	[192]
Interstitial lung disease	Plasma	Healthy individuals vs. patients with interstitial lung disease (n = 42 per group)	MS/MS	Elevated levels of 3-NT in patients with interstitial lung disease	[142]
MetS	Plasma	Healthy vs. MetS subjects (n = 25 per group)	LC-ESI-MS/MS	Higher protein-bound 3-NT levels in MetS subjects	[247]
Aging	Plasma	3 study groups from MARK-AGE project: RASIG (n = 794), GO (n = 493), SGO (n = 272) Participants divided in 4 groups: 55-59, 60–64, 65–69 and 70–75 years	ELISA	Positive correlation between 3-NT levels and age in RASIG group No correlation in GO and SGO groups between 3-NT levels and age	[194]

(tattoo-free) body region as well as multiple determination and might be limited by the use of body creams, bronzer as well as solarium [162]. The most sensitive approaches for detection of glycoxidation products are methods based on chromatography, including HPLC und UPLC that are mainly combined with MS/MS. These methods represent the gold standard for the identification of glycoxidation structures and detection in free and protein-bound forms, but their use in routine clinical studies is limited, in particular, due to high costs [158,163,164]. For a detailed review of advantages and disadvantages of various immunochemical, bioanalytical, and biochemical methods for the measurement of AGEs, including glycoxidation products, see Ashraf et al. [158]. Studies from recent years, using UPLC- or LC-MS/MS approaches show very heterogenous outcomes in different diseases, depending on the detected structure. This emphasizes the importance of discrimination and selective analysis of the various structures formed by glycoxidation reactions and the limited usefulness of some methods in this field. For instance, O'Grady et al. found higher CML levels in plasma of T2D patients compared to healthy subjects but similar pentosidine concentrations [165]. The latter finding is in contrast to previous investigations, using ELISA or HPLC techniques, revealing higher serum pentosidine levels in diabetes patients compared to healthy controls [166,167]. Moreover, Wetzels and colleagues as well as Teichert et al. show similar plasma levels of various AGE structures, including free and protein-bound CML between non-demented subjects and patients with multiple sclerosis or non-diabetic and pre-diabetic women, respectively [168,169]. In line with earlier findings on glycoxidation levels in aging [153], studies from 2019 suggest an age-related increase in skin AF, pentosidine levels and total amount of AGEs [141,170,171]. In particular, Kida and colleagues performed their analysis in different specimens of spinal surgery patients and found strong correlations between serum, urine, bone, and skin pentosidine levels with age [170]. The fundamental role of AGEs, including glycoxidation products, in human aging, but also in aging of vertebrate and invertebrate model organism has been reviewed in 2018 by Chaudhuri and colleagues [153], together indicating the relevance of AGEs as biomarkers of aging and age-related diseases.

4.5. Lipoxidation in human diseases and aging

The biological process of lipid peroxidation has been implicated in a high number of human pathologic conditions [172,173]. Recent clinical studies focused mainly on reactive intermediates 4-HNE and MDA, which are prominent precursors of ALE formation [174]. Qualitative and semiquantitative immunological methods, such as ELISA and IHC, using commercially available antibodies, have been primarily performed to detect 4-HNE in biological samples of various human diseases, as shown in Table 6 and reviewed elsewhere [175]. These antibodies are raised against protein-bound 4-HNE, ensuring relatively high specificity. In general, detection by IHC is valuable for the evaluation of morphological alterations in tissue samples, but not advisable for parallel quantitative assessments due to several limitations, such as the use of different fixatives, potential background staining, quality of dyes or humidity [176]. A more sensitive approach has been used by Luczaj and colleagues via GC-MS, but also ELISA, measuring free 4-HNE and protein-bound 4-HNE, respectively, in two independent studies [177,178]. For instance, RA patients consistently revealed higher levels of free and protein-bound 4-HNE in urine and plasma compared to control subjects [177]. To our knowledge, levels of 4-HNE have not been determined as a function of age in recent years. However, 4-HNE is activating or inhibiting certain age-related signaling pathways, including NF-κB, Nrf2 or mTOR pathways, and might be therefore contributing to the aging process [179,180].

To our knowledge, MDA modified proteins have not been used as biomarkers in recent clinical investigations although antibodies are commercially available and highly selective MS methods have been established, previously [57]. Nevertheless, free MDA potentially leading to stable protein modifications, has been quantified in several studies via batch thiobarbituric acid reactive substances (TBARS) assay. This approach is the method of choice for the quantification of free MDA due to simplicity and low costs, but is often criticized as being unspecific and rather measuring total oxidative stress instead of MDA alone [181,182]. Except of Zablocka-Slowinska and colleagues, showing no differences between healthy subjects and patients with lung cancer [183], all clinical investigations using the TBARS assay consistently found elevated MDA levels in diverse human metabolic disease, as shown in Table 6. Beside this assay, further methods have been developed in order to improve the specificity of MDA measurement, such as HPLC combined with UV or fluorescence detection [182,184]. Reversed-phase HPLC coupled with fluorescence detection has been further used by Sharma and colleagues, revealing lower levels of MDA in AD and PD patients compared to controls [151]. This is in contrast to the findings by

Medeiros and colleagues, indicating higher MDA concentrations in PD patients that might be explained by the use of different methods, but also potential differences in the study populations [185]. The GC-MS approach in the study by Luczaj and colleagues as mentioned above, has been also used for the measurement of MDA, revealing elevated levels in different specimens of RA and thick-born encephalitis patients compared to controls [177,178]. Independent of the applied method, three of five studies found no correlation between MDA and advanced age although they partially included large cohorts, e.g., from the European MARK-AGE study (Table 6). In combination with lower MDA levels in elderly male and female AD and PD patients compared to healthy subjects as well as unchanged concentrations between lung cancer patients and control individuals at advanced age [151,183], this suggests that MDA is no suitable marker for human aging and age-related diseases.

4.6. Nitration in human diseases and aging

3-NT, the stable endproduct of peroxynitrite-mediated oxidation formed via nitration of free or protein-bound tyrosine, has been primarily used for the determination of protein nitration and nitrosative stress in clinical settings [186,187]. Already in 2008, Pacher et al. published an outstanding and detailed review about the role of peroxynitrite and 3-NT formation in human health and disease, indicating their relevance as diagnostic biomarker for several pathologic conditions [188]. Elevated levels of 3-NT have been also found in a variety of human diseases in recent research, including T2D, cancer, and neurodegenerative diseases, but the available data are partially conflicting (Table 7). For instance, while Ravassa et al. found higher 3-NT levels in T2D patients with additional cardiovascular events, no differences were observed by Segre and colleagues although baseline parameters of the study populations and the used methods were similar [189,190]. Moreover, recent research in AD patients revealed higher 3-NT levels compared to healthy volunteers, whereas no differences were found in a previous investigation by Ryberg and colleagues, but these studies are only partially comparable since different specimens and methodological approaches have been used [151,191–193]. To our knowledge, only Weber et al. determined 3-NT as a function of age in three different cohorts of the MARK-AGE study [194]. Two of three cohorts showed no differences between the age groups, but elevated 3-NT levels in age-related diseases, such as AD and PD, indicate the impact of 3-NT accumulation in human aging [191,192,195]. The measurement of 3-NT can be performed in plasma, serum as well as tissue samples by different methods, including IHC, ELISA, HPLC or the combination of LC with MS/MS, whereas MS approaches have been considered as gold standard. Nevertheless, commercially available ELISAs are preferentially used for 3-NT quantification in clinical studies due to standardization, easy sample preparation, the need of conventional equipment, and high sample throughput, as described for other biomarkers. In turn, several limitations have been highlighted in the literature, such as low sensitivity, minor specificity of applied antibodies, the susceptibility to errors, or missing detection of free 3-NT [196–199]. However, Weber et al. provided a simple and fast indirect ELISA method for the measurement of protein-bound 3-NT in human serum and plasma samples. High detection limits, small sample volumes, and the opportunity for high throughput-screening makes this method useful for clinical applications [196]. Moreover, Tramutola and colleagues used a slot blot approach to detect total 3-NT levels in PBMC (peripheral blood mononuclear cells) of AD patients, followed by a MS/MS-based proteomic analysis of 3-NT-modified proteins [191]. In 2016, Ng et al. demonstrated a novel detection scheme of 3-NT by using a nickel-doped graphene synthetic receptor combined with a localized surface plasmon resonance biosensor that might be of potential relevance for the diagnosis of 3-NT in clinical settings. As declared by the authors, this approach provides advantages compared to other techniques, such as immunoassays, due to label-free detection, higher specificity and capture of 3-NT by direct chemisorption [200]. The most accurate detection of 3-NT, however, requires the combination of LC with MS/MS providing the opportunity to distinguish between free, protein-bound, or total 3-NT, but the use of this method is not feasible for clinical research, because of high costs and low throughput [198].

5. Conclusion

Reactive oxygen and nitrogen species are ubiquitous side-products of metabolism. Impaired balance between their generation and degradation in aging and disease causes oxidative and nitrosative stress. As a result, an incredible number of distinct protein modifications is formed. These modifications are generally more stable compared to their reactive precursors and, thus, important biomarkers for monitoring medium- to long-term oxidative and nitrosative stress. Selection of the appropriate technique to study protein oxidation in biological samples is the responsibility of the user and depends on the experimental question and the available equipment. Many biological disciplines use immunoassays as routine methods. Immunological methods are often based on monoclonal antibodies or polyclonal antisera obtained after immunization with immunogens, lacking full chemical or structural characterization. In many cases, the epitopes recognized by the antibodies remain unknown. To advance the knowledge in protein oxidation and its structural mysteries, a further increase of MS analysis for biological samples would be desirable. Among the presented methods, MS is certainly the most accurate and informative technique, capable of resolving remaining questions regarding protein modifications.

Since many frequently used routine methods are less than optimal, the clinical relevance of most protein modifications as biomarkers is questionable, although protein oxidation seems to play a critical role in several pathologies and in the aging process. Reversible modifications, including cysteine disulfide and methionine sulfoxide have been associated with various human and age-related diseases, but in most studies only oxidized free amino acids have been determined, not reflecting the extent of protein oxidation. Also, the widely used spectrophotometric detection of AOPPs is highly unspecific and detects a wide range of oxidative species, therefore, these studies should be treated with caution. The determination of specific crosslinks such as dityrosine by chromatographic separation followed by MS analysis is more reliable. Moreover, drawing a final conclusion on glycoxidation products as relevant biomarkers of protein oxidation is hardly possible based on the included studies published in the last years. The selection of specific and reliable analytes and detection methods is of utmost importance and can influence the study outcome significantly. Thus, further longitudinal studies with standardized methods and conditions are needed to determine the relationship between these oxidation products and human diseases in more detail. Regarding advanced lipoxidation endproducts, recent studies mainly focused on the reactive intermediates 4-HNE and MDA, which presumably correlate with the amount of protein modifications formed by reaction with this carbonyl species. However, future studies should focus on measuring the corresponding stable protein modifications. Despite partially inconsistent results in studies of recent years, elevated 3-NT levels are associated with multiple diseases and aging, indicating its potential as biomarker for protein oxidation in clinical research. Also, elevated levels of protein carbonyls have been observed in several human diseases and are associated with the aging process. Therefore, protein carbonyl content can be seen as a biomarker of global oxidative protein damage, with the advantage of early formation and long circulation periods compared to other parameters of oxidative stress.

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