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Lipid peroxidation derived reactive carbonyl species in free and conjugated forms as an index of lipid peroxidation: limits and perspectives

Alessandra Altomare [a], Giovanna Baron [a], Erica Gianazza [b], Cristina Banfi [b], Marina Carini [a], Giancarlo Aldini [a,*]

[a] Department of Pharmaceutical Sciences (DISFARM), Università Degli Studi di Milano, Via Mangiagalli 25, 20133, Milan, Italy
[b] Centro Cardiologico Monzino, IRCCS, Via Parea 4, 20138, Milan, Italy

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ABSTRACT

Reactive carbonyl species (RCS) formed by lipidperoxidation as free forms or as enzymatic and non-enzymatic conjugates are widely used as an index of oxidative stress. Besides general measurements based on derivatizing reactions, more selective and sensitive MS based analyses have been proposed in the last decade. Untargeted and targeted methods for the measurement of free RCS and adducts have been described and their applications to in vitro and ex vivo samples have permitted the identification of many biological targets, reaction mechanisms and adducted moieties with a particular relevance to RCS protein adducts. The growing interest in protein carbonylation can be explained by considering that protein adducts are now recognized as being involved in the damaging action of oxidative stress so that their measurement is performed not only to obtain an index of lipid peroxidation but also to gain a deeper insight into the molecular mechanisms of oxidative stress. The aim of the review is to discuss the most novel analytical approaches and their application for profiling reactive carbonyl species and their enzymatic and non-enzymatic metabolites as an index of lipid-oxidation and oxidative stress. Limits and perspectives will be discussed.

1. Introduction

Oxidative stress can be defined as an imbalance between the production and elimination of oxidant species in favor of the first. The reactive species causing oxidant stress can firstly be classified, on the basis of their oxygen or nitrogen content, as reactive oxygen-(ROS) and nitrogen-(RNS) species. ROS and RNS can then be classified as free radicals (including hydroxyl radicals, $HO^{\bullet}$; peroxyl radicals, $ROO^{\bullet}$; alkoxyl radicals $RO^{\bullet}$; superoxide anions $O_2^{\bullet-}$ and nitric oxide $NO^{\bullet}$) and non-radical oxidizing species (H_2O_2, ROOH, ONOOH). ROS/RNS are able to react with and oxidize any biological molecules, such as proteins, lipids, and nucleic acids, causing structural and functional changes [1,2]. In physiological conditions, such oxidant events/reactions are prevented by the antioxidant defense mediated by enzymatic and non-enzymatic antioxidants which compete with the oxidizable substrates thus significantly delaying or inhibiting their oxidation. An additional defense system is provided by the enzymatic removal or repair of the damaged biomolecules before their accumulation which would result in alterations in the cellular metabolism [3]. The antioxidant defense system also permits ROS/RNS to mediate cell signaling and

Abbreviations: **ACR**, acrolein; **ADH**, alcohol dehydrogenase; **ALDH**, aldehyde dehydrogenases; **APL**, aminophospholipids; **AKR**, aldo-keto reductase; **dG**, deoxyguanosine; **DHN**, 1,4-dihydroxy-2-nonene; **DNPH**, dinitrophenylhydrazine; **dR**, deoxy-ribose; **eNOS**, endothelial nitric oxide synthase; **ESR**, Electron Spin Resonance; **ELISA**, enzyme-linked immunosorbent assay; **F2-IsoPs**, F2-isoprostanes; **GC-MS**, gas chromatography coupled to mass spectrometry; **GS-HNA**, 3-(s-glutathionyl)-4-hydroxynonanoic acid; **GSH-DHN**, 3-(s-glutathionyl)-1,4-dihydroxynonane; **GO**, glyoxal; **HPLC**, high-performance liquid chromatography; **isoLG**, isolevuglandins; **isoK**, isoketals; **HPLC-MS**, high-performance liquid chromatography coupled to mass spectrometry; **3-PMA**, 3-hydroxypropyl mercapturic acid; **HNA**, carboxyl 4-hydroxy-2-nonenoic acid; **HNE**, 4-hydroxy-nonenal; **HHE**, 4-hydroxy-hexenal; **LLOQ**, lower limit of quantification; **LPO**, lipid peroxidation; **MA**, mercapturic acid; **MRM**, multiple reaction monitoring; **MS**, mass spectrometry; **MDA**, malondialdehyde; **MGO**, methylglyoxal; **Nrf2**, nuclear factor erythroid 2–related factor 2; **ONE**, 4-oxo-nonenal; **PE**, phosphatidylethanolamine; **PS**, phosphatidylserine; **PUFA**, poly unsaturated fatty acids; **RCS**, reactive carbonyl species; **RCS-PA**, RCS-protein adducts; **ROS**, reactive oxygen species; **RNS**, reactive nitrogen species; **TBARS**, thiobarbituric acid-reactive substances; **ULOQ**, upper limit of quantification.

* Corresponding author.
E-mail address: giancarlo.aldini@unimi.it (G. Aldini).

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redox regulation that subsequently lead to an increased expression of the genes for various antioxidant proteins which modulate the appropriate induction of adaptation processes or alternatively, the activation of cell death mechanisms [4].

Hence, in those conditions characterized by elevated ROS/RNS levels and by impairment of the antioxidant defense and of the adaptive response, a redox imbalance occurs, resulting in uncontrolled oxidative stress. As above mentioned, in these conditions, ROS/RNS induce structural and functional alterations to cell components thus leading to oxidative damage which is involved in the onset and propagation of aging and of several diseases [5].

Oxidative stress is involved in numerous physio-pathological conditions and is recognized as an important target to be modulated in order to have preventive/curative effects [6]. Several molecular approaches based on dietary/synthetic compounds able to act as direct or indirect antioxidants (activators of the nuclear factor erythroid 2–related factor 2 [Nrf2] system) have been considered with the aim of inhibiting oxidative damage and the consequent cell and tissue damage. More recently other strategies based on scavengers of oxidation products such as lipid and glucose-oxidation products have been reported as efficient molecular strategies [7]. Much thought has also been devoted to how to measure oxidative stress with the aim not only of correlating the onset and propagation of oxidative stress with the clinical outcomes but also of measuring the efficacy of the antioxidant approaches.

2. Oxidative stress biomarkers

Measurement of oxidative stress in in vitro and ex vivo conditions is a research topic which still attracts great interest due to the fact that validated and easy to measure biomarkers of oxidative stress are still lacking. From an analytical point of view, the measurement of oxidative stress is quite complex due to the fact that the oxidant species causing oxidative stress are very reactive and unstable and so are short lived species [8,9]. A direct measurement of these species can be performed in the presence of radical spin trappers for the radical species (ESR experiments) [10] or using suitable fluorescence probes which form a fluorophore when undergoing oxidation [2]. These techniques are mainly performed in in vitro conditions because their measurement requires that the spin trapper/fluorescent probe is added concomitantly to the oxidant formation. In ex vivo conditions, oxidative stress is usually evaluated by measuring the reaction products between oxidant species and substrates, including proteins, lipids and nucleic acids. Even though the measurement of oxidative stress products is affordable, it is still complicated because the reaction products of oxidative stress are chemically and metabolically unstable, are heterogeneous, and present in a reduced amount in complex biological matrices. So far, the measurement of oxidative stress in ex vivo conditions is based on the analysis of the reaction products between ROS/RNS and their biological substrates: lipids, nucleic acid and proteins. Besides proteins [11], lipids are also a main target of oxidative stress due to their abundance and sensitivity to oxidation and different oxidative reaction pathways and oxidation products have been identified and characterized and selected as an index of oxidative stress. Of the lipid oxidation pathways, that targeting poly unsaturated fatty acids (PUFA) and involving a chain-reaction (lipid-peroxidation) has been the most studied and the resulting lipid peroxidation (LPO) end products, the corresponding metabolites and their reaction products, are widely accepted as an index of oxidative stress and are largely used in pre-clinical and human studies [12,13]. The aim of the review is to discuss the most novel analytical approaches and their applications in the field of LPO focusing on reactive carbonyl species (RCS) as break-down products and their enzymatic and non-enzymatic metabolites.

3. Lipid peroxidation in oxidative stress

The general process of LPO consists of three stages: initiation, propagation, and termination [2]. Hydroxyl and hydroperoxyl radicals are the most powerful initiators of LPO. Hydroxyl radicals are generated from hydrogen peroxide during cellular oxygen metabolism via the Fenton and Haber–Weiss reactions, in the presence of free iron or copper ions, and from peroxynitrite [5]. Superoxide and nitric oxide, which are generated by the activity of NADPH oxidase, xanthine oxidase, and nitric oxide synthase, are not able to initiate LPO, but they do react to give peroxynitrite, which may initiate chain reactions [3].

The membrane lipids, mainly phospholipids, containing PUFA are predominantly susceptible to the lipid chain autoxidation (Fig. 1). In PUFA, the –C═C– units are separated by methylene (–CH_2–) groups whose hydrogen atoms (bis-allylic hydrogens) are susceptible to attack by hydroxyl radical and peroxynitrite (initiation). In particular the abstraction of a hydrogen atom from the methylene group by $HO^{\bullet}$ generates a lipid radical ($L^{\bullet}$) which in turn reacts with molecular oxygen to form a lipid peroxyl radical ($LOO^{\bullet}$). The $LOO^{\bullet}$ can abstract hydrogen from an adjacent fatty acid to produce a lipid hydroperoxide (LOOH) and a second lipid radical thus propagating the reaction (LPO chain reaction, propagation phase). The LOOH thus formed can be cleaved by reduced metals, such as Fe^{2+}, producing lipid alkoxyl radicals ($LO^{\bullet}$) which further stimulate the chain reaction of LPO by abstracting additional hydrogen atoms or decomposes forming RCS [14]. Termination of the chain reaction occurs when two $LOO^{\bullet}$s encounter one another to yield nonradical products or when the $LOO^{\bullet}$ abstracts an H atom from radical trapping antioxidants such as α-tocopherol [2].

4. Lipid peroxidation markers: a general overview

The LPO cascade forms different intermediates including conjugated dienes, hydroperoxides, alkoxyl and peroxyl radicals, alcohols as well as a multitude of break-down products most of which are characterized by carbonyls (aldehyde and ketone groups) and an unsaturated moiety acting as soft and hard electrophiles (RCS) (Fig. 2). To date, several analytical methods have been reported and also applied to measure intermediate and breakdown LPO products [1]. Regarding intermediates, lipid hydroperoxides have been measured by using HPLC with post-column chemiluminescence, and electrochemical reduction [15] but nowadays their application is quite limited.

It is now widely accepted that F2-isoprostanes (F2-IsoPs), a series of free radical-catalyzed LPO products of arachidonic acid, are a reliable marker of LPO and in general of oxidative stress in in vivo condition. F2-IsoPs are relatively stable in urine [16] and two main analytical approaches are used for their analysis: those based on mass spectrometry (HPLC-MS or GC-MS) and immunological techniques. Studies, which compared both methods, found that 8-isoprostane concentrations determined with chromatography/mass spectrometry-based assays did not agree with those determined with immunoassays, suggesting that GC–MS and immunological techniques do not measure the same compounds. GC–MS is the gold standard and it is recommended for the analysis of F2-IsoPs [17].

Many analytical approaches have been performed on the break-down products characterized by a carbonyl group which is a quite characteristic moiety since it is absent in non-oxidized lipids, proteins and nucleic acids and can be easily detected and/or enriched by derivatization reactions.

The lipidperoxidation by-products characterized by a carbonyl moiety (RCS), are quite heterogenous and their formation depends on the attacking PUFA. The most studied are the short-chain RCS which can be put into three main classes: α,β-unsaturated-aldehydes (4-hydroxy-nonenal, HNE; 4-hydroxy-hexenal, HHE; nonenal; acrolein, ACR), dialdehydes (glyoxal, GO and malondialdehyde, MDA) and keto-aldehydes (4-oxo-nonenal, ONE) (Fig. 2) [18]. Another important class of LPO derived reactive carbonyl is that represented by a family of 64 highly reactive levuglandin-like γ-ketoaldehyde (isoketals, IsoK, or isolevuglandins, IsoLG) regio- and stereo-isomers which are are formed as the products of arachidonic acid oxidation. As shown in Fig. 2, isoK

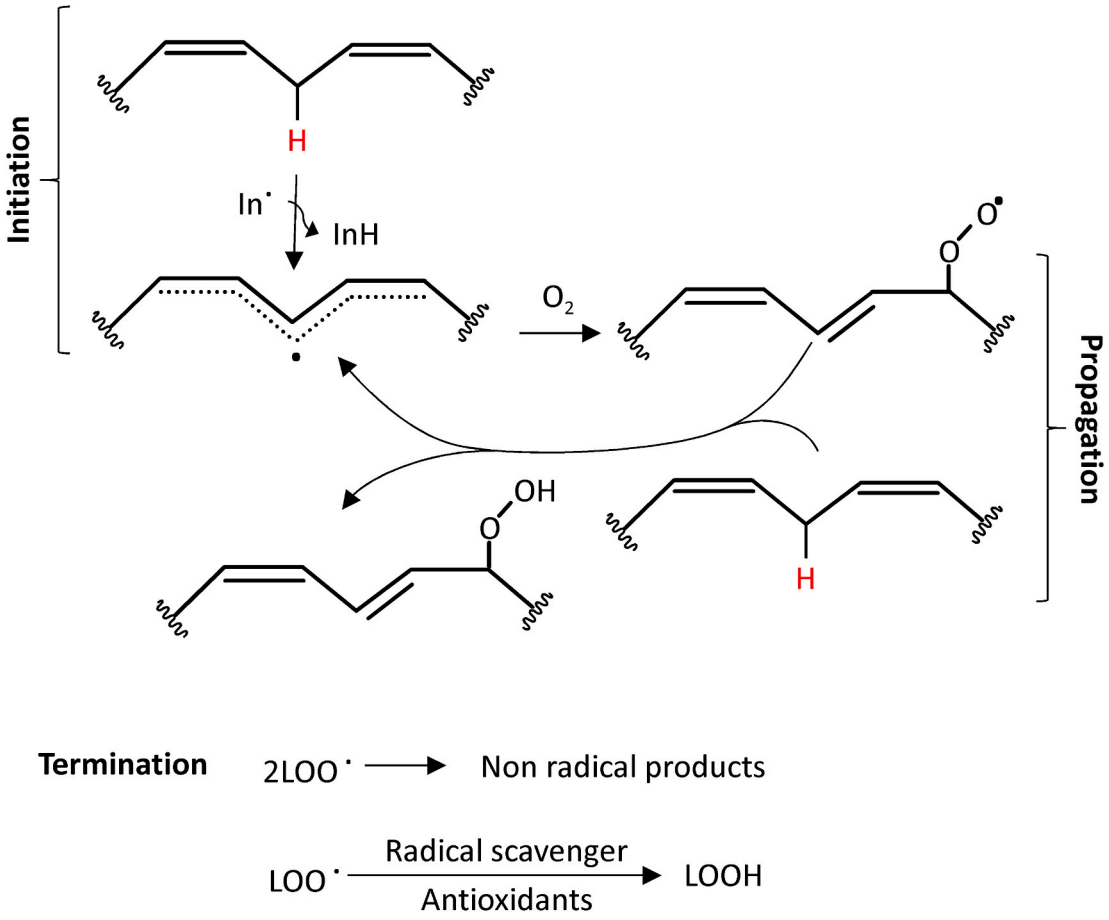

Fig. 1. Lipid chain autoxidation which consists of three stages: initiation, propagation and termination. Initiation: H-abstraction of bis-allylic hydrogens of PUFA by radical initiators (In$^{\bullet}$), such as hydroxyl radical and peroxynitrite, generates a lipid radical (L$^{\bullet}$) which in turn reacts with molecular oxygen to form a lipid peroxyl radical (LOO$^{\bullet}$). The LOO$^{\bullet}$ can abstract hydrogen from an adjacent fatty acid to produce a lipid hydroperoxide (LOOH) and a second lipid radical, thus propagating the reaction (LPO chain reaction, propagation phase). Termination of the chain reaction occurs when two LOO$^{\bullet}$s encounter one another to yield nonradical products or when the LOO$^{\bullet}$ abstracts an H atom from radical trapping antioxidants such as α-tocopherol.

derive from isoprostane endoperoxide, a peroxidatioin product of arachidonic acid which is subjected to rearrangement resulting in the generation of different reaction products, besides isoK, such as the non carbonyl by-products F_2-IsoP, E_2-isoP, D_2-IsoP, IsoTxA$_2$ (Fig. 2) [19]. Many methods based on the measurement of lipidperoxidation derived carbonyls based on a derivatization reaction targeting the carbonyl group have been proposed and still now they are popular and widely applied. Among others, the so-called thiobarbituric acid-reactive substances (TBARS) test for measuring MDA, which consists of heating the biological specimen with thiobarbituric acid in acid condition for 15–20 min. After cooling, the sample is centrifuged and the absorbance of the pink chromophore formed by the condensation reaction between 1 mol of MDA and 2 mol of thiobarbituric acid is measured spectrophotometrically [20]. The TBARS assay is very popular because it is simple and economic although it lacks selectivity because many substances interfere with the MDA, including carbohydrates, nucleic acids saturated and unsaturated aldehydes, sucrose, and urea [21,22]. The lack of selectivity causes a significant overestimation as demonstrated by comparing the TBARS values with those of MDA detected by more selective methods, such as those based on HPLC coupled with UV, fluorescence or MS detectors [23,24]. Moreover, the significant interindividual differences observed in plasma MDA levels as determined by hyphenated techniques were not apparent in plasma determinations by TBARS [23]. It is now accepted that the poor assay selectivity of the TBARS assay greatly limits its usefulness for detecting true differences in the level of LPO in clinical studies. Another general method is based on dinitrophenylhydrazine (DNPH) as a derivatizing agent of carbonyls followed by spectroscopic, immunological detection of the corresponding hydrazones as reaction products [25]. Hyphenated techniques such as HPLC-MS, HPLC-UV and HPLC with fluorescence detector have also been widely used for profiling DNPH derivatized carbonyls [26,27].

RCS are detected in free forms or as conjugated and non-conjugated metabolites. Conjugated metabolites can be formed either by enzymatic (phase II reactions) or non-enzymatic adduction.

RCS measurements as an index of LPO/oxidative stress is widely applied for several reasons. RCS are relatively abundant in respect to other oxidation products such as F2-isoPs and they are characterized by reactive groups so that derivatization reaction/purification steps can be used for their analysis/enrichment.

In the following chapters the most novel methods for profiling RCS and metabolites in free and conjugated forms are reported.

5. Measuring free RCS and enzymatic metabolites: methods and applications

5.1. Free RCS

Several MS techniques based on targeted or untargeted approaches have been reported for the measurement of free RCS in plasma and tissue and based on a derivatization process which targets the carbonyl moiety of RCS. Regarding the most recent papers, Tomono et al. [28] reported a

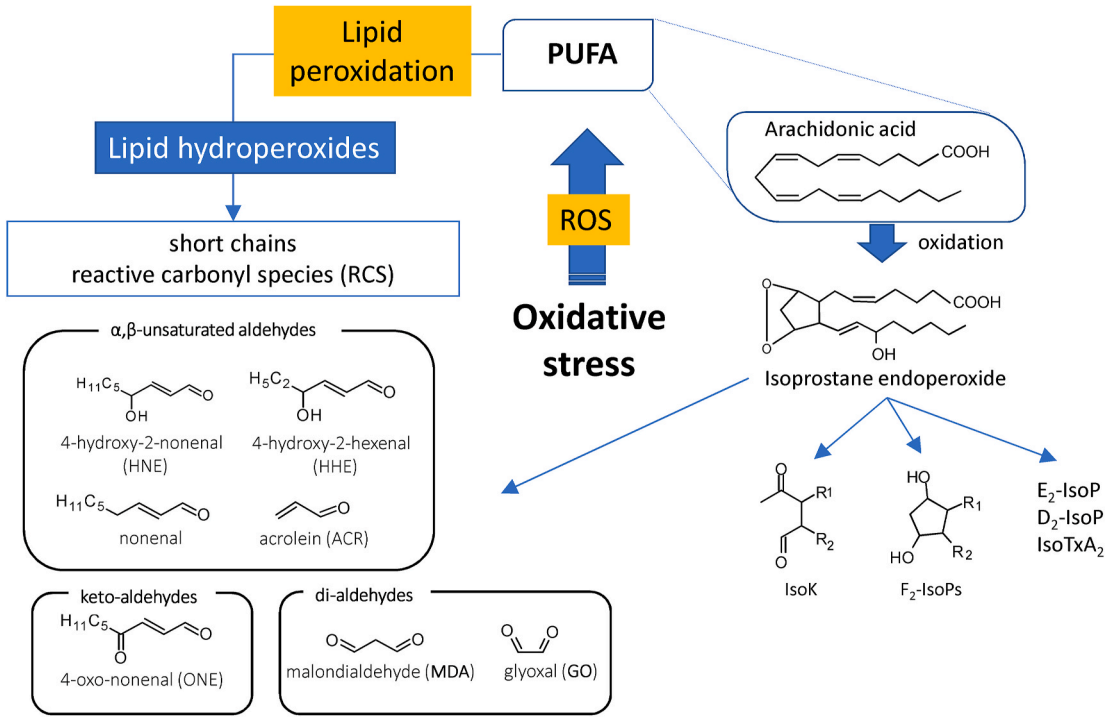

Fig. 2. Reactive carbonyl species (RCS) short chains and isoK as LPO by-products. Reactive carbonyl species (RCS) short chains are formed by the decomposition of lipid hydroperoxides deriving from the LPO of PUFA. Arachidonic acid LPO forms isoprostane endoperoxide which is subjected to rearrangement, resulting in the generation of reactive carbonyl species, including short RCS and isoK, and non carbonyl by-products such as F_2-IsoP, E_2-isoP, D_2-IsoP, IsoTxA_2.

comprehensive analysis of lipophilic RCS by using dansylhydrazine as derivatizing agent and HPLC-MS/MS. The method was used to analyze the RCS profiles of plasma samples obtained from C57BL/6J mice (10 weeks of age). A number of 405 peaks were detected in the plasma samples and assigned to free RCS or those liberated from unstable Schiff base adducts. Thirty-four of these RCS were identified by comparison with authentic RCS including alkanals (e.g., hexanal and hexadecanal), 2-alkenals (e.g., 2-nonenal) and 4-hydroxy-2-alkenals (e.g., HNE and ONE).

A shotgun analytical strategy based on the mass spectrometry (MS) and 7-(diethylamino)coumarin-3-carbohydrazide as derivatizing agent was reported, allowing simultaneous detection of aliphatic and lipid-bound carbonylated LPO products [29]. The method allowed the identification of 122 carbonyl compounds in a mixture of four PUFA and phosphatidylcholines (PC) oxidized in vitro.

Besides these untargeted methods, highly specific and sensitive methods for the measurement of selected aldehydes have been proposed (targeted approach). These methods are based on hyphenated techniques such as GC-MS as analyzer and isotopically-labelled internal standards [13,17]. By using such accurate techniques, Tsikas reported in plasma of patients suffering from cardiovascular diseases, concentrations of MDA and HNE ranging from 500 nM to 2500 nM, the MDA concentrations being about two-three times higher than the HNE concentrations [30]. The plasma concentration of total F2-IsoPs were more than three orders of magnitude lower compared to the plasma concentration of MDA and HNE (range, between 380 and 750 pM). Some studies based on RCS determined by GC-MS have reported an increase of the analytes in different conditions. As an example, Soulage et al. reported that the plasma levels of HHE (four and half-fold) and HNE (six-fold) were higher in chronic kidney disease patients than in controls [31].

In line of principle, the ideal LPO by-product to be considered as biomarker should fulfil these requirements: 1) be stable from both a chemical and metabolic point of view: this feature would permit the analyte not to undergo further enzymatic and non-enzymatic reactions and not to be degraded during sampling and conservation; 2) have a suitable structure or functional groups which permit it to be easily identified and quantified by current analytical methods.

It should be considered that F2-IsoPs partially fulfil the two requirements while RCS do not fulfil the first one. Even though more stable in respect to the parent oxidizing species, RCS are quite reactive and unstable in the biological matrices because of the presence of the carbonyl groups, and in some cases, of the unsaturation, making these compounds as substrates of phase I and II metabolism and able to react with other molecules such as lipids, proteins and nucleic acids. RCS half-life in the cell and in the extracellular environment is quite limited as reported in several papers in both cellular and extracellular milieus. When HNE at a concentration of 20 μM was incubated in plasma, it disappeared within 40 s because it forms adducts with proteins, in particular, with albumin [32]. Experiments involving cells and organs also report a time dependent consumption of HNE within minute [33]. Hence, free aldehydes do not accumulate but their steady-state content depends on the rate of their formation, metabolism and reaction with nucleophilic targets.

5.2. RCS enzymatic metabolites

RCS undergo an extensive metabolism and also a non-enzymatic adduction to nucleophilic substrates, so that the corresponding metabolites and adducts are considered as markers of oxidation. RCS can undergo enzymatic and non-enzymatic transformation, the former mediated by phase I and II enzymes, the latter involving nucleophilic substrates including proteins, lipids and nucleic acids. As an example, Fig. 3 reports the different enzymatic and non-enzymatic metabolic pathways of HNE, which is to date one of the most studied RCS. MS based methods have been already set-up and applied to profile both enzymatic and non-enzymatic metabolites as here reported.

Phase I metabolites: the main phase I metabolites are alcohol and carboxylic acid derivatives arising from reduction and oxidation of the carbonyl group mediated by alcohol dehydrogenase (ADH) or aldo-keto

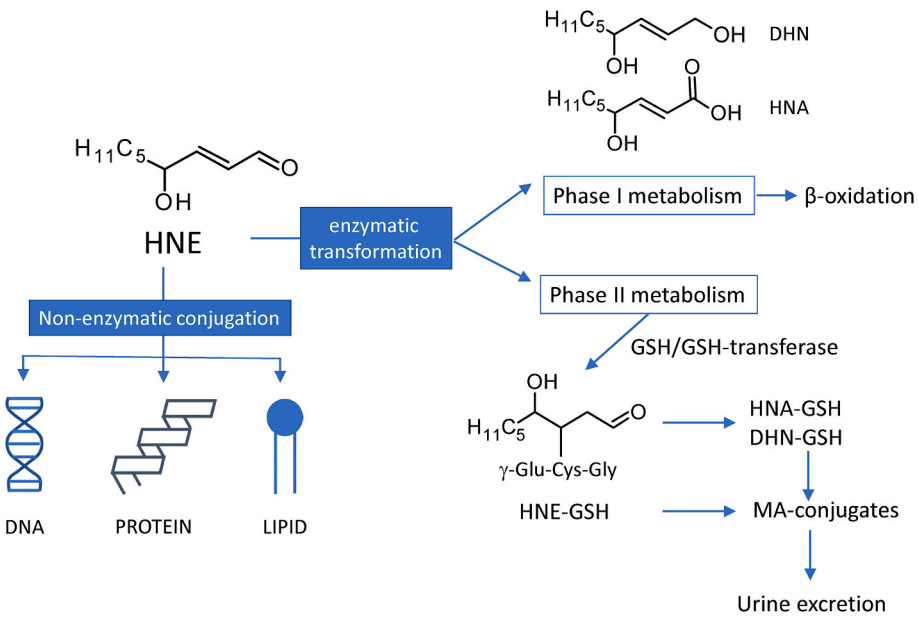

Fig. 3. Enzymatic and non-enzymatic metabolic pathways of HNE. HNE is detoxified by phase I and II metabolic reactions, the first targeting the carbonyl group which is reduced to alcohol (DHN) or oxidized to the corresponding acid (HNA) by alcohol dehydrogenase and by aldehyde dehydrogenase, respectively. Phase II metabolism is based on GSH conjugation through a Michael adduction, a reaction which is catalyzed by GSH-transferase. GSH-HNE conjugate is then metabolized to the reduced (DHN-GSH) and oxidized (HNA-GSH) forms which are then converted to the mercapturic acid conjugates (MA-conjugates) to be excreted in the urine.

reductase (AKR) enzymes and aldehyde dehydrogenases (ALDH), respectively [20]. The metabolism of HNE has been fully characterized and 1,4-dihydroxy-2-nonene (DHN) and carboxyl 4-hydroxy-2-nonenoic acid (HNA) are the two main phase I metabolites [34]. Phase I metabolites represent the main metabolic products and in the case of HNE, HNA is the main abundant metabolite being excreted for 27% [35]; however, the absence of reactive moieties and their reduced molecular weight limit the application of sensitive analytical and immunological methods for their detection. The main analytical methods used for their analysis are based on GC-MS. Furthermore, their content not only depends on the formation of RCS but more importantly on the metabolism, which is affected by several factors such as aging, as demonstrated by Siems et al. for HNE [36]. Furthermore, HNA is not the final metabolite being further metabolized by β-oxidation steps on carbon 1 and/or 9 [35]. Hence the content of these metabolites could vary not only on the basis of LPO but also on that of metabolic activity. So far, to our knowledge, no phase I metabolites have been proposed as an index of oxidative stress in ex vivo studies.

Phase II metabolites. GSH adducts and the urinary mercapturic acid (MA) derivatives are the main phase II metabolites of RCS. Mercapturic acid adducted with the aldehyde in reduced or oxidized forms, such as 3-(s-glutathionyl)-1,4-dihydroxynonane (GSH-DHN) and 3-(s-glutathio-nyl)-4-hydroxynonanoic acid (GS-HNA) for HNE, are the final metabolic products and hence fulfill the first requirements and also have a peculiar structure allowing them to be easily identified by use of a mass analyzer. HPLC-ESI-MS is the main analytical tool which is used for mercapturic acid adduct analysis and most recently an ELISA test has been proposed and validated [37]. So far, validated analytical methods for mercapturic acid of HNE and ACR analyses in urine have been set-up and also applied as an index of oxidative stress. As an example, the adducts between ACR and HNE with MA were found to be increased in smokers [38] and to decrease upon smoking cessation [39]. If such metabolites are positively related to the exposure of exogenous RCS from environment or food [40] their use as an index of endogenous formation of RCS is not so clear cut. Yoshida et al. measured the content of 3-hydroxypropyl mercapturic acid (3-HPMA), an acrolein-glutathione metabolite by liquid chromatography with tandem MS (HPLC-MS/MS) in urine taken from stroke patients and control subjects [41]. Previous studies had reported a significant increase of ACR determined as protein adduct in the plasma of stroke patients in respect to control [42]. Unexpectedly, the level of 3-HPMA was found to be lower in the urine of stroke patients than in the urine of control subjects so that the results were in contrast to the changes of ACR found at plasma level [41]. The Authors explained this apparent contradiction by considering a reduction of GSH in the locus of stroke, resulting in a reduced formation of conjugates metabolites and in the reaction of the not metabolized ACR act with proteins. The results underline the fact that the content of RCS-MA adducts, even though they are suitable as potential biomarker as they are metabolically stable and real end-products, is the result of the RCS formation and more importantly of GSH availability whose content is affected by the oxidative stress. Based on these aspects we can comment that the urinary amount of RCS-MA adducts can be considered as a valuable tool for measuring exogenous exposure to RCS but their content is not directly linked to the endogenous formation because they are affected by GSH availability and metabolism.

6. RCS non-enzymatic transformation

Upon formation, RCS can escape the enzymatic metabolism and react with nucleophilic targets and in particular with proteins, nucleic acids and lipids (Fig. 4). By using MS based techniques such adducts have been identified and characterized and applied as an index of LPO.

6.1. *DNA adducts*

LPO break down products and in particular RCS can diffuse into the nucleus or into the mitochondria where they could react with nucleophilic groups of DNA forming covalent adducts. In particular, soft and hard electrophiles LPO by-products can attack the free amino group of DNA bases e.g., the N1 or N3 of the deoxyguanosine (dG), leading to several exocyclic DNA adducts which can be classified into two groups: DNA adducts with substituted lipid side chains and unsubstituted etheno-DNA adducts [43,44]. Exocyclic DNA adducts are those in which an extra ring or rings have been added to the DNA base; some of the most common exocyclic adducts are the so-called etheno (ε) adducts, which contain an extra two carbons [45]. As an example, Fig. 4 shows the etheno adduct formed by the reaction of dG with α,β-unsaturated aldehydes. The various classes of exocyclic DNA adducts have attracted intensive research interest not only because they are considered potential biomarkers of oxidative stress-based diseases but also biomarkers of cancer risk since they can induce high mutagenicity during cell replication.

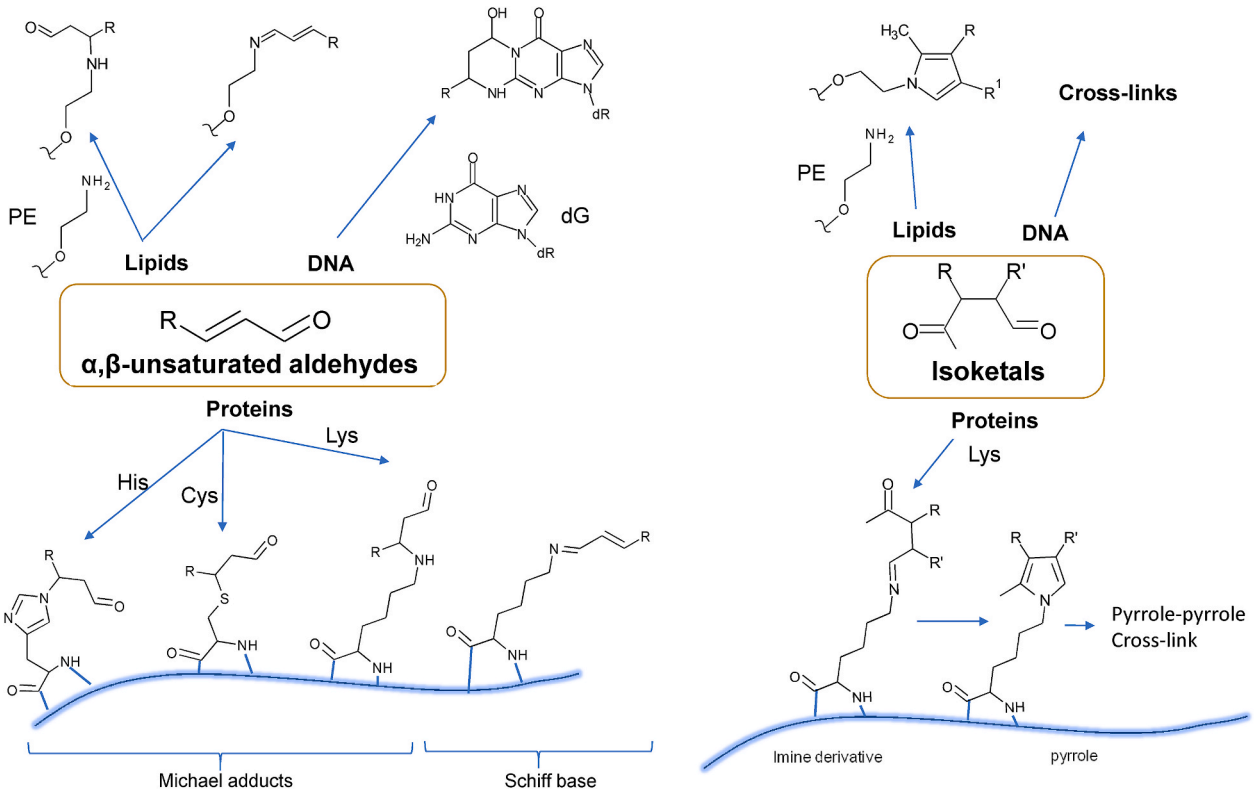

Fig. 4. RCS react with nucleophilic targets and in particular with proteins, nucleic acids and lipids. The panel on the left shows some covalent reactions of α,β-unsaturated aldehydes with His, Cys and Lys protein residues, with lipids (PE) and with deoxyguanosine. The reaction of α,β-unsaturated aldehydes with His and Cys leads to the formation of the corresponding Michael adducts while with Lys to the formation of the Michael adduct or of the Schiff base. The reaction of α,β-unsaturated aldehydes with the amino group of PE forms the Michael adduct or the Schiff base and the reaction with dG forms the exocyclic etheno adduct. The panel on the right shows the covalent reactions involving isoK. IsoK reacts with Lys forming the corresponding Schiff base which then rearranges to the pyrrole derivative and the cross-links. Reaction with PE generates the pyrrole derivatives and cross-links when reacting with DNA.

The reaction products of DNA with LPO derived RCS have been fully characterized. Characterization of exocyclic DNA adducts has been reported for both soft and hard electrophiles such as ACR, HNE and its epoxide 2,3-epoxy-4-hydroxynonanal, ONE, GO and MDA [43,46].

To date, several analytical methods have been developed and applied to identify and quantify DNA adducts in human tissue, blood, and urine, including (i) ^{32}P-postlabeling, (ii) gas chromatography coupled to MS (GC–MS), (iii) HPLC–MS (iv) capillary electrophoresis–MS, (v) fluorescence, (vi) immunoblot assay and (vii) immunohistochemistry. With the rapid growth of MS technologies, recent works have focused on the investigation of DNA adducts at a systems biology level, defining the field of "DNA adductomics" [43].

The technique using ^{32}P-postlabeling was initially proposed as a very sensitive and versatile method for the analysis of nucleoside-adducts and this has permitted the identification of a number of DNA adducts derived from exogenous sources and endogenous stimuli (LPO), including adducts with ACR and HNE [47,48]. The technique has been optimized by the integration of some enrichment procedures, i.e. immunoaffinity chromatography, HPLC, TLC, or solid phase extraction (SPE), an optimized quantitative protocol based on ^{32}P-postlabeling/SPE/HPLC which achieves a limit of detection of 0.1 fmol [49]. Despite its ultra-high sensitivity, the limitations of the ^{32}P -postlabeling technique include the risk of exposure to energetic radioisotope (^{32}P is a strong beta-emitter), poor selectivity and poor recovery efficiency both for the chromatographic resolution and the detection [43].

Later, in the mid-1990s, hyphenated techniques such as GC-MS and HPLC-MS were used and gained popularity thanks to their selectivity and the absence of radioactive materials. GC–MS was found to be highly selective and sensitive but limited because it required a chemical derivatization and purification step to make the DNA-adducts volatile and this may introduce artifacts [49]. HPLC-MS rapidly became the choice for DNA adduct analysis, especially thanks to the fact that no derivatization step was needed and that polar nucleosides suitably ionize in the electrospray ionization source (ESI). Measurement of modified nucleosides by HPLC-MS consists of the canonical steps: (i) sample collection, (ii) DNA isolation, (iii) enzymatic or thermal hydrolysis to obtain nucleoside monomers, (iv) enrichment, (v) clean-up, and finally (vi) separation and detection [49]. Several HPLC-MS methods based on tandem MS experiments have been set-up to simultaneously detect multiple DNA adducts and use single reaction monitoring (SRM) or multiple reaction monitoring (MRM) techniques. HPLC-ESI-MS sensitivity was further improved using nanoflow HPLC coupled with nanospray ionization tandem MS and the equivalent of only 4–6 μg of DNA samples were needed for this method [50].

Besides the quantification of known DNA adducts, tandem MS has been applied to identify unknown adducts. DNA adductomics has been developed to exploit bond cleavage using collision-activated low-energy dissociation, as all DNA adducts share a common structural feature with a sugar group bound to the nucleobase via a glycosidic bond. By monitoring the neutral loss of deoxy-ribose (dR) from positively ionized deoxynucleoside adducts, this methodology was able to detect several well-known adducts simultaneously and identify unknown ones [51,52].

Accurate quantitative analysis of DNA adducts by HPLC-MS are carried out using isotopically labelled internal standards which take into account the sample loss which can occur during the sample purification.

Great attention should be paid to the sample preparation in order to prevent artifacts which can arise from RCS formation or loss during DNA isolation, enzymatic digestion, and storage. Artifacts can explain the great variations of RCS-DNA adduct levels so far reported in the

literature, ranging from one adduct in 10^{10} nucleotides (nts) to one adduct in 10^5 nts [53]. The use of antioxidants and metal ion chelators is usually considered in order to prevent artifacts. Chen et al. [53] have conducted a systematic evaluation of artifact formation and the loss of LPO adducts during DNA isolation, enzymatic digestion, and storage steps with different tissue workup methods. The Authors have demonstrated that metal chelators and free radical scavengers do not completely prevent artifact formation, while the thiol compound β-mercaptoethanol efficiently mitigates RCS-induced DNA damage during sample workup and digestion of DNA.

Specific issues for analysts can be resumed as follows: (i) the trade-off between DNA input and limit of detection (ii) prevention of accidental adduct formation during hydrolysis and the following deproteinisation (iii) availability of isotope-labelled internal standards.

Moreover, since DNA adducts must be presented in relation to normal nucleoside content and all nucleosides could be altered during sample processing, pair-wise quantification of unmodified nucleosides needs to be performed with a proper method validation. Antonowicz et al. [51] presented a nucleoside adduct method (UPLC-ESI-MS/MS method) that is rapid, simple and accurate; in detail, the method was reported to quantify a great number of adducts belonging to diverse classes, including a validated method for paired normal nucleoside quantification, utilized deaminase inhibitors to avoid artifact formation, and had the fastest HPLC method time; also the sensitivity was comparable to or better than earlier methods (1 adduct per 10^8 normal bases with 20 μg DNA input for most targets).

Chen et al. have optimized and validated an ultrahigh-performance liquid chromatography electrospray ionization ion trap multistage MS (UHPLC/ESI-IT-MS^3) method to quantify LPO adducts, covering most of the known adduct types; as above described, the method was optimized to avoid artifact formation of adducts and was proposed to be used to examine the correlation between oxidative stress, inflammation, and LPO adduct levels in patients with benign prostatic hyperplasia and prostate cancer. Acrolein adducts were detected in all the patients while HNE and ONE were below the LOD [53].

Alamil et al. [54] have recently described the development and full validation of an analytical method on UHPLC-ESI-MS/MS to quantify simultaneously several exocyclic DNA adducts derived from aldehydes. After synthesis, identification and quantitation of these adducts and their $^{13}C_{10}$, $^{15}N_5$ isotopes homologues, the calibration curves were established ranging from 0.25 (LLOQ) to 250 ng/mL (ULOQ) of adducts in both water and DNA matrices. The validated UHPLC-MS/MS method was found to be sensitive and accurate enough for low-level quantification of 9 exocyclic DNA adducts derived from 8 main exogenous and endogenous aldehydes, namely formaldehyde, acetaldehyde, ACR, crotonaldehyde, MDA, HNE, GO and MGO. This method proved to be applicable to biological samples and in the future its application may be extended to the quantification of other adducts, generated from the external environment as well as endogenously.

In 2017 Chen et al. [55] published a work focused on the optimization of the first Stable Isotope Dilution (SID)-nanoHPLC−NSI/MS/MS method for the simultaneous detection and quantification of GO-induced DNA cross-linked adducts. The stable isotopomers of dG-GO-dC and dG-GO-dA were synthesized and used as internal standards to ensure that sensitivity and specificity were good enough to allow an accurate quantification of these two cross-linked adducts in leukocyte DNA from type 2 diabetes mellitus patients and nondiabetic subjects. In the literature there are few works on quantification of DNA−DNA cross-linked adducts in human tissue or biological fluids, probably because of the low adduct levels. The proposed SID nanoHPLC-NSI/MS/MS assay is highly sensitive and specific, and it requires a very low amount of leukocyte DNA isolated from blood to accurately quantify the two cross-linked adducts simultaneously. The proposed assay therefore provides a useful biomarker for assessing glyoxal-derived DNA damage.

Different repair pathways are engaged in the removal exocyclic DNA adducts from the DNA and depend on the size of exocyclic DNA adducts. Small, etheno-type DNA adducts are repaired by: (i) base excision repair; (ii) nucleotide incision repair and (iii) AlkB proteins, while bulky substituted propano- and substituted etheno-type adducts are repaired by nucleotide excision repair and homologous recombination [44].

Activity and expression of proteins engaged in repair of exocyclic DNA adducts is modulated during infection and inflammation, and may be related to the development of some human diseases like cancer, as well as aging [44]. Hence the amount of adducted DNA is the result of RCS formation as well as of the expression and activity of repair enzymes.

6.2. Lipid adducts

Aminophospholipids (APL) which include two classes of glycerophospholipids, namely phosphatidylethanolamine (PE) and phosphatidylserine (PS) are also targeted by lipid-derived RCS (including hydroxy-alkenals, keto-alkenals, gamma-keto-aldehydes and isolevuglandins) with which they form covalent adducts through the free amino groups of the polar heads [56–59]. It is well established that Michael adducts and Schiff bases together with their hemiacetal and pyrrole derivatives are the most common reaction products between APL and α,β-unsaturated aldehydes. As an example, Fig. 4 shows the reaction of α,β-unsaturated aldehydes with PE, forming the corresponding Michael adduct and Schiff base. A detailed study of the reaction mechanism between HNE or ONE with APL showed that for both the reactive aldehydes two types of chemical reactions are possible: formation of Michael adducts and Schiff bases. In the case of HNE, an initially formed Michael adduct can also undergo an additional cyclization step to a hemiacetal derivative, whereas no cyclization occurs in the case of ONE. A Schiff base product initially formed when HNE is added to PE lipid can also further cyclize to a pyrrole derivative in contrast to ONE, where only a Schiff base product is isolated [60]. The mechanism of adduction between isoLG and PE was also studied, showing that the reaction occurs with an initial formation of a Schiff base, which rapidly forms a stable pyrrole through cyclization (Fig. 4). Experimental evidence showed that the reaction speed of isoLG with PE was 4.4 times faster than the reaction with lysine [61]. The adduction reaction of the "smallest" aldehydes, such as MDA and ACR was also studied. Both the aldehydes react with the amino group of PE headgroup through the initial formation of SB, which then evolves in more complex products. For example, the predominant product of the incubation of MDA with PE was identified as dihydropyridine-PE [62], while two acroleins in reactions with PE formed a compound termed (3-formyl-4-hydroxy)-piperidine-PE [63].

The detection and characterization of modified APLs is mainly based on tandem MS and MS^n techniques and several papers have been published to this regard [61]; however, the negligible APL in vivo amount and the great structural complexity together with the low ionization efficiency of APL adducts represent the most prominent challenges for the analysis of modified APL. Ni et al., in 2015, tried to bypass the low ionization efficiencies of APL-carbonylated adducts by optimizing an experimental protocol involving a 7-(diethylamino)coumarin-3-carbohydrazide (CHH) derivatization step followed by static-positive ESI-MS analysis; a nano-UPLC system was coupled on-line to an LTQ Orbitrap XL ETD MS equipped with a nano-ESI source operating in positive ion mode. This work identified carbonylated glycerophospholipids belonging to six different classes, i.e. glycerophosphatidylcholine, PE, glycerophosphatidylglycerol, PS, glycerophosphatidic acids and phosphatidylinositiolphosphates, independently of the head groups [64].

Despite several papers having reported the formation of APL adducts with RCS in in vitro models, using artificial membranes, cells or tissues exposed to oxidative stress [59,65], very few studies have reported the detection of adducts in ex-vivo conditions.

Carboxymethylethanolamine was detected in mitochondrial

phospholipids by using a selected-ion-monitoring/gas-chromatography/MS [66]. Accumulation of aldehyde-modified PEs was found in atherosclerotic lesions [67] and isoK adducts with PE found to be increased in the liver of chronic ethanol-fed mice and in plasma from patients with age-related macular degeneration in respect to controls [68]. Bacot et al. by using a GC-MS approach have reported a 5.3-fold increase of the adduct between PE and HHE in diabetic rat retinas compared with controls. Also the adduct with HNE was found to be increased 3.2-fold [58].

Notwithstanding APL adducts with RCS having been fully characterized by MS strategies, the in vivo detection has found a very limited application. The metabolic fate of APL adducts is also not known.

6.3. RCS protein adducts (RCS-PA)

Even though the metabolic removal of RCS is efficient, 2–8% (calculated for HNE) in cells appears to form conjugates with proteins [36]. RCS form adducts with proteins through a non-enzymatic reaction which is based on the electrophilic reactivity of RCS and on the nucleophilicity of the protein sites. Fig. 4 shows the covalent adducts between α,β-unsaturated aldehydes and Lys, His and Cys residues (left panel) and those between isoK and Lys. Protein adduction by RCS has attracted a great interest because many of the biological activities of LPO and of RCS are mediated by such reactions [69]. RCS induced protein modification can induce a very large variety of biological effects through a direct (protein dysfunction) or indirect (cell signaling) mechanism [70]. Structural dysfunction is quite characteristic for long lived proteins such as collagen. Collagen RCS adducts form cross-links and further covalent modifications which can affect the regeneration and reorganization of tissues, instigating a loss of elasticity and disturbance in tissue remodeling, which contributes to the development of pathology within the organism [117].

Moreover, protein adducts have also been considered as markers of LPO. Their successful application is given by their immunological properties, leading to the development of immunological assays. Moreover, being characterized by a carbonyl group, these adducts can be easily detected or enriched by derivatization reactions followed by MS detection. Protein adducts are formed spontaneously, by a reaction between the electrophile and nucleophile, not enzymatically-mediated. Their formation depends on the amount of RCS and of proteins (the reaction is second order) and if it is considered that the protein target does not vary significantly, we can argue that the protein adduct is an index of RCS amount. However, the stability of protein adducts is an aspect to be considered and which will be expanded below.

In recent years the interest in RCS-protein adducts has greatly increased thanks to the advent and diffusion of HPLC-ESI-MS techniques which permit a sensitive and selective measurement of adducted proteins.

Three variables identify RCS-PA: the modified moiety which depends on the attacking RCS, the target protein and the adducted amino acid. The modified moiety depends both on the RCS and on the targeted amino acid. In line of principle any protein containing nucleophilic exposed amino acids can be targeted by the RCS. However, in a mixture containing proteins with a different concentration range, taking into account that the reaction between RCS and protein is second order [72], the RCS protein targets are in line of principle those which are the most abundant. Besides concentration, other parameters can affect the formation of the adducts. Bearing in mind that usually, few nucleophilic residues are carbonylated within each analyzed protein, protein carbonylation can be considered a selective process, based on a well-defined residue susceptibility which is regulated by the accessibility of the amino acid and by the nucleophilicity of the residue for basic amino acids such as Lys and by acidity for Cys. A more acid thiol of Cys corresponds to a higher fraction of the thiolate form and hence to a higher reactivity toward the electrophilic RCS. Acidity in the case of Cys is regulated by the neighboring amino acids which stabilize the thiolate thus affecting the thiol's acidity. In the case of Lys, its ability to form a Schiff base is mediated by the nucleophilicity of the amino group which is also modulated by the amino acid environment [72,73]. Protein susceptibility to carbonylation is also affected by protein conformation since it regulates amino acid accessibility and nucleophilicity/acidity by a neighboring effect. Hence a protein is targeted by RCS not necessarily because it is relatively more abundant in the proteome but because contains reactive amino acids whose reactivity is given by the peculiar protein structure. Proteomic studies have been carried out to identify the main protein and amino acid target in both in vitro and ex vivo experiments as reported below. Protein adducts as determined by MS have been profiled as an index of LPO.

7. RCS-PA: analytical methods approach

MS analytical strategies applied to identify RCS-PA can first be classified on the basis of a labelled or unlabeled approach. The labelled approach consists of derivatizing the adduct moiety, before or after the enzymatic digestion, with a derivatizing agent and then isolating it by an enrichment step. The unlabeled approach permits the identification of the protein adducts as such, without any chemical modification of the analyte.

7.1. Labeling approaches

One proposed strategy is based on combining biotin hydrazide and streptavidin capture methodology with HPLC–MS/MS. Adducts containing carbonyl moieties (Michael adducts) react with the hydrizide moieties forming the corresponding hydrazone, which can be readily reduced by borohydride to stable secondary amines. Derivatized protein adducts can then be captured by streptavidin [74]. By using this strategy, Codreanu et al. have profiled the HNE Michael protein adducts formed in human colorectal carcinoma cells exposed to increasing concentrations of electrophile (0, 50, and 100 μ M) for 1 h at 37 °C. Almost 420 HNE proteins adducted by HNE were identified over a total number of 1500 proteins identified [75].

Aldehyde/keto reactive probe (ARP) is another example of labeling agent based on a biotinylated hydroxyl amine probe, able to react with carbonyl moieties forming the corresponding aldoximes. The enrichment procedure can be carried out both at protein or peptide level and in the latter case the enrichment procedure is followed by tryptic digestion thus enabling the capture of adducted targets at the peptide level [76]. Using this technique, Tzeng et al. [77] profiled HNE protein adducts by exposing liver mitochondria to HNE. By examining the concentration dependence of the protein modifications, a distinct reactivity profile for HNE-protein adduction was found. Proteins associated with metabolic processes, including amino acid, fatty acid, and glyoxylate and dicarboxylate metabolism, bile acid synthesis and TCA cycle, showed enhanced reactivity to HNE adduction, whereas proteins associated with oxidative phosphorylation displayed retardation toward HNE adduction.

The main limit of biotin tag is that it reduces the ionization efficiency and alter the fragmentation pattern of the modified peptide causing a loss in signal intensity and in the quality of MS/MS spectra. To solve this limit the biotin tag should be removed before the MS analysis, as proposed by Coffey & Gronert, who have developed an analytical method utilizing an alkoxyamine affinity tag and streptavidin beads [78]. The cleavable tag, named EZ-Link Alkoxyamine-PEG4-SS-PEG4-biotin, contains a carbonyl reactive site (alkoxyamine), a cleavable disulfide, and a biotin moiety permitting enrichment with avidin products and easy incorporation into existing protocols. Other labeling approaches have been reported using different strategies as reviewed [79,80].

A recent labeling approach for ACR adducts based on an aniline-based probe and characterized by an improved sensitivity compared to the hydrazide and hydroxylamine based derivatizing agents has been reported [81]. The method consists of derivatizing the free aldehyde

group of the ACR protein adducts with the aniline based probe forming the corresponding Schiff base which is then subsequently reduced by sodium cyanoborohydride. The bio-orthogonal alkyne handle of the aniline probe enables either fluorescence-based visualization or biotin-based enrichment of ACR modifications depending on the type of the azide-functionalized reporter group. The method was then applied to cells exposed to ACR and has permitted the identification of >2300 proteins and >500 cysteine sites.

7.2. Unlabeled approaches

The unlabeled approach consists of identifying the protein adducts without any chemical derivatization. Several approaches have so far been reported and which can be classified as follows:

- **Full-targeted analysis**: known adduct/known protein/known targeted amino acid/
- **Semi-targeted analysis**: known adduct/known protein/unknown targeted amino acid/
- **Semi-untargeted analysis**: unknown adduct/unknown targeted amino acid/known protein
- **Full-untargeted analysis**: unknown adduct/unknown targeted amino acid/unknown protein

Full-targeted analysis: the approach is based on targeting a fully characterized modified peptide by a bottom-up approach. MS/MS transitions of the modified peptide are known and used to set-up the MRM method by selecting the precursor ion and the *y* and *b* fragment ions. The target adducted peptide is usually identified by using the approaches here below reported. Absolute or semi-quantitative (% of modification in respect to the non-adducted peptide) analyses can be carried out. A recent application has been proposed by Altomare et al.. By using a semi-untargeted analysis a set of Lys residues in albumin were found to be adducted as carboxymethyl residues in patients affected by heart failure in respect to age-control subjects. A MRM method to selectively measure the relative content of adducted vs non-adducted peptides was then set-up and used to measure their significant increase in HF patients (submitted manuscript).

Semi-targeted analysis: this approach consists in identifying the amino acids (unknown) of a selected protein which undergoes the covalent adduction of a selected RCS. The covalent moiety to be searched for as variable modification is known. This approach is usually applied to in vitro systems where the targeted protein is incubated with a selected RCS and the aim of the work is to search the amino acid sites undergoing known chemical modifications. The analytical approach consists of a bottom-up analysis: the protein sample, after incubation with the RCS, is analyzed in a data-dependent scan mode. Adducted peptides are then searched for by setting the known adducts as variable modifications. As an example, when HNE is tested as RCS, then Michael adducts (+156 Da or + 158 in reducing conditions) or Schiff base (+138 or +140 Da in reducing conditions) are set as variable modification of nucleophilic amino acids such as His, Lys and Cys for the Michel adduct and Lys for the Schiff base. Several in vitro experiments have been performed using this approach and using, as an example, albumin and apoA as protein targets and different RCS such as oxidized phospholipids, HNE, isoK, and ACR [82,83]. The target protein is usually selected on the basis of Western blot or proteomic studies. This approach has permitted confirmation of the reactivity of the tested proteins with RCS, and of the adducted moieties and of the most reactive amino acids undergoing the adduction. Functional studies are also carried-out to assess whether the covalent adduction impairs the protein activity. Taken together these in vitro studies have enabled to the creation of a database of potential adducts to search in ex vivo samples where the information usually regards the modified amino acid site, the peptide containing the modified amino acid, the accurate mass and the *y* and *b* fragmentation pattern of the adducted peptide. It should be considered that the adducted amino acid detected in semi-targeted analysis can potentially be different in respect to those detected when the protein is in this natural biological matrix because of the absence, in isolated proteins, of protein assemblies, protein ligand interactions and possibly of quaternary structures which affect the conformation. As described above, a conformation change can affect the reactivity of the amino acids.

Semi-untargeted analysis: This approach is usually applied to ex vivo samples and to limit the complexity of the search, a target protein is isolated and analyzed and any covalent adducts searched for. As an example, the approach has been applied to albumin [72,84] or apoA [82] which are abundant and reactive substrates to covalent modifications. The method consists of searching for any known covalent adducts to any residues and to do this usually a wide database of variable modifications is built from the literature and the peptides analyzed by MS/MS are then searched for.

Some methods have been proposed to search for variable and unknown modification by targeting Cys34 of albumin since it is the most reactive nucleophile site in plasma and very abundant. Precursor ion scanning has been proposed to search any variable modification of Cys34 [32,72]. The method consists of searching any variable modifications of a tryptic/chymotryptic peptide containing Cys34 by searching for the precursor ions which upon fragmentation forms the *y* and *b* ions not including the Cys34 moiety.

Rappaport et al. also reported an untargeted method to profile Cys34 adducts. The method proposed is based on the FS-SRM method (fixed-step SRM), that allows detection of essentially all HSA-Cys34 modifications over a specified range of mass increases (added masses). The approach does not require that exact masses of adducts be known in advance but rather uses a theoretical list of peptide-adduct *m/z* values separated by a fixed increment of 1.5 [85]. By using this method and others untargeted methods set-up by the group, different oxidation products of Cys34 such as sulfinic and sulfonic acids, mixed disulfides such as cysteinylated, homocysteinylated, glutathionylated forms were detected as were adducts with RCS such as ACR and crotonaldehyde [86].

Carlsson et al. have developed and applied an approach for the screening of unknown hemoglobin (Hb) adducts [87]. The methodology is based on the FIRE procedure, a modified Edman degradation reaction where adducts to N-terminals in Hb are selectively detached by the reagent fluorescein isothiocyanate and analyzed as fluorescein thiohydantoin derivatives by HPLC-MS/MS. Application of the method has permitted identification of four precursor electrophiles, namely glyoxal, methylglyoxal, acrylic acid and 1-octen-3-one [88].

Full-untargeted analysis: In line of principle this method permits the identification of unknown adducts. Hence it is not based on the search for known variable modifications as is the semi-targeted analysis. Different analytical strategies have been reported. Shibata et al. recently reported a histidine and lysine adductome analysis [89]. Samples are reduced with sodium borohydride ($NaBH_4$) to stabilize the unstable adducts, such as Schiff base adducts, and then hydrolyzed to free (modified) amino acids under the conventional acidic conditions. The resulting free amino acid mixture is then subjected to HPLC-ESI-MS/MS analyses. The untargeted strategy is based on the identification of any adducted His and Lys which are detected on the basis of the product ion at *m/z* 110.0 (immonium ion of histidine) from the positively ionized histidine adducts and *m/z* 84.0 (loss of NH_3 from the lysine immonium ion) from positively ionized lysine adducts by HPLC-ESI-MS/MS. The adductome analysis has been applied to the native and Cu^{2+}-oxidized LDL (oxLDL). Nε-(8-carboxyoctanyl)lysine was identified as the most abundant lysine adduct in the oxLDL together with at least six minor products of the lysine adducts with 2-alkenals such as ACR, 2-heptenal 2-hexenal, 2-octenal, and 2-noxenal [90]. An untargeted analysis based on immonium ion of histidine has been also applied to detect unknown adducts of histidine peptides in the urine of Zucker rats [91].

Gesslbauer et al. [118] described a method for the detection of lipid-modified proteins that does not require an a priori knowledge of

the chemical structure of RCSs or the identity of target proteins. The method is based on the change of electrophoretic mobility of lipid-modified proteins, which is induced by conformational changes and cross-linking with other proteins. The method does not identify adducted proteins but those which have a different electrophoretic mobility in respect to controls. The method was applied to study the effects of oxidized palmitoyl-arachidonoyl-phosphatidylcholine on endothelial cells and several adducted proteins both insoluble and membrane cell fractions were identified and their adduction confirmed by immunoblotting analysis.

Besides MS approaches, immunological based techniques have also been proposed and are widely used. Monoclonal and ploYclonal antibodies have been produced and are commercially available for the detection of protein adducts with most of the LPO break-down products. Immunological tests for the measurement of RCS-PA have some obvious advantages in respect to MS methods such as that they do not need such expensive instrumentation and specific technical skills as are required for MS measurements. Moreover, ELISA kits permit the processing of hundreds of samples, yielding results in only a few days and thus immunological techniques have been successfully used to measure oxidative stress and represent a popular method of analysis. However, ELISA tests have some limits in respect to MS methods and in particular: 1) they can only perform targeted analysis while MS can also identify unknown adducts; 2) they detect the type of adduct but in contrast to MS no information on the targeted amino acid or on the protein identity is given. Moreover, MS has a greater selectivity and sensitivity in respect to ELISA which permits the detection of lower differences among groups.

8. RCS-PA: identification and characterization in ex-vivo samples

In this paragraph a selection of the most recent (last two years) papers reporting the application of the above-mentioned approaches for the identification of RCS-protein adducts (RCS-PA) in ex vivo samples are described.

Gegotek et al. [92] used an unlabeled, semi-targeted approach to search for HNE−cysteine/lysine/histidine adducts set as a dynamic modifications in plasma from psoriatic patients and healthy controls (sex- and age-matched individuals). The analysis of HNE-protein adducts identified quantitative and qualitative differences between the plasma of psoriatic patients and healthy individuals, the levels of HNE-protein adducts being approximately 2.5-times higher in psoriatic samples compared to healthy individuals. From a qualitative point of view, omitting albumin, HNE-protein adducts identified in the plasma from healthy individuals were structural molecules and molecules involved in binding and catalytic activity. In the plasma of psoriatic patients, the number of structural molecules forming adducts with HNE decreased in favor of proteins with catalytic activity.

Rappaport Petrick et al. performed an untargeted adductomics in a population-based case-control study to identify HSA-Cys34 adducts associated with childhood leukemia. Acute lymphoblastic leukemia patients had higher abundances of adducts of RCS, suggestive of oxidative stress and LPO as potentially etiologic factors [93]. The method was then applied to detect Cys34 adducts in colorectal cancer cases and controls, two of these adducts were Cys34 modifications by methanethiol, a microbial–human cometabolite, and crotonaldehyde, a product of LPO [94].

Guerby et al. [95] reported the analysis of placentas from preeclampsia-affected patients (PE) and normal pregnancies, showing a significant increase in protein carbonyl content, and in particular the accumulation of ACR, HNE, and ONE adducts in PE placentas. Immunofluorescence and confocal experiments pointed out a colocalization of endothelial nitric oxide synthase (eNOS) with ONE-Lys adducts, whereas eNOS was not modified in normal placentas. By using a semi-targeted analysis, it was found that recombinant eNOS pre-incubated with ONE, allowed the identification of several ONE-modified Lys-containing peptides, confirming that eNOS may undergo post-translational modification by LPO products. No data were reported on the detection of eNOS by ONE in ex-vivo samples.

By using a targeted approach, May-Zhang reported an increased level of ONE protein adducts in HDL in patients with familial hypercholesterolemia compared with healthy control volunteers. ONE-adducts were determined as 4-ketoamide adducts by HPLC-MS/MS [96]. By using a targeted approach the same research group found that IsoLG-adducted HDL is increased in conditions associated with hypercholesterolemia and atherosclerosis and that such modification significantly affects the HDL structure–function [97].

By using the biotin hydrazide enrichment strategy Shearn et al. reported that chronic ethanol intake significantly increased protein carbonylation, including carbonylation of ribosomal proteins: only 3 ribosomal proteins were detected as carbonylated in chow-fed animals, compared to 43 in the ethanol-exposed groups. Additional HPLC-MS/MS studies were then performed to identify the types and sites of carbonylation [98].

9. RCS-PA metabolic and chemical stability

RCS-PA, including Michael adducts, are not the final reaction products of lipid-peroxidation break-down products and probably they further undergo an extensive chemical/metabolic transformation besides a proteosomal degradation, this last caused by protein unfolding and exposure of hydrophobic regions, normally buried in native form [99]. The exposed hydrophobic patches could serve as signals for molecular chaperones and proteolytic systems, and result in the refolding or degradation of target proteins. A majority of studies indicate that oxidized proteins are mainly degraded by the 20S proteasome, while there is also evidence suggesting that the ubiquitin dependent 26S proteasome, immunoproteasome and lysosome are also involved in the degradation of oxidized proteins [100]. Besides proteasomal degradation, RCS-PA undergoes a metabolic transformation which rapidly degrades these adducts. Yang et al. [101] by using a chemoproteomic approach as above described, reported the stability of HNE protein adducts in cells. In particular they identified and quantified 398 HNE protein adducts after 2 h of HNE incubation and then measured the relative content after one and 4 h of incubation in an HNE free medium. Analysis of HNE adducts revealed a surprisingly high degree of adduct loss at one and 4 h of recovery. In total, ~87% of quantifiable HNE alkylating events showed at least a two-fold decrease over the course of 4 h. Nevertheless, several individual HNE alkylations were quite stable. Stability was independent of the type of target amino acid but was found to depend on the site of the amino acid. Experiments using proteasome inhibitors suggested that site specific HNE alkylation dynamics in cells is mediated by some unknown repair or reversion processes, rather than by global protein degradation. Furthermore, metabolic (intact cells) versus chemical (lysates) stability was tested. The protein adducts were relatively stable in cell lysates in respect to intact cells indicating that HNE adduct instability is mediated by factors present in intact, metabolically competent cells and is not due to simple chemical instability.

Besides being rapidly degraded, RCS-PA are not static and localized in the site of formation but undergo a rapid dynamic path as recently reported by Lincoln et al. [102]. By using fluorogenic probe and super-resolution fluorescence microscopy, the Authors reported the spatiotemporal localization of protein carbonylation at the cellular level. In particular the Authors reported a non-emissive BODIPY-acrolein fluorogenic probe that was used to map protein alkylation by ACR within the cellular lipid milieu. Experiments demonstrated that protein adducts are not localized but follow a dynamic path. In particular, upon probe bolus, alkylation was predominantly observed within mitochondria from which protein adducts are rapidly excreted, sorting within the Golgi apparatus and trafficking along microtubules and through the cell endo- and exocytic pathways. These fluo techniques permit the visualization of the tagged proteins but not the identity of the adducts and

whether the electrophilic moiety is transferred to other proteins/peptides.

Using a dynamic model of nitroxidative stress, Griesser et al. [103] have demonstrated rapid changes in biomolecule carbonylation in rat cardiomyocytes. Levels of carbonylated species increased as early as 15 min upon treatment with the peroxynitrite donor, 3-morpholinosydnonimine (SIN-1), and decreased to values close to control after 16 h and using inhibitors of proteasomal and autophagy/lysosomal degradation pathways a significant role of the proteasome in the degradation of carbonylated proteins was observed.

From the above-mentioned paper, it is clear that protein adducts in cells undergo a rapid spatial and metabolic change which is partially independent of proteosomal degradation. We currently do not know the fate of carbonylated proteins and whether a decarbonylation reaction also occurs, thus saving the protein as suggested by some Authors. In particular it has been proposed that protein carbonyls can be removed by thiol-dependent reduction. This process is called "protein decarbonylation". Notably, Wong et al. have found, in rat heart homogenates, that protein carbonyls can be reduced by cysteine and glutathione [104]. Moreover, it has been proposed thioredoxin, a thiol-based antioxidant enzyme, is involved in cellular protein decarbonylation [105] and this confirmed by reducing the decarbonylation process by inhibiting thioredoxin reductase [106]. However, the decarbonylation mechanism needs further work to be confirmed and elucidated.

Few data are available about the stability of protein carbonyls in plasma and their metabolic fate or tissue transfer. Thakore et al. [107] used radiolabeled albumin in native and ACR adducted form injected into rats and radioactivity measured over time in plasma and organs. The radioactivity was cleared from the circulating plasma more rapidly in the radiolabeled ACR-albumin-treated group (32% of the injected radioactivity remained) than the radiolabeled-albumin group (52%). At 33 h after the injection, 22% of the injected radioactivity remained in the plasma in the albumin ACR adduct-treated group as compared to 32% in the albumin-treated group. The whole homogenates of liver and kidney showed higher radioactivity in the ACR albumin-treated group as compared to the albumin-treated group. These results indicated that the albumin-acrolein adduct was removed more rapidly from the circulation than the native albumin and degraded more rapidly by the liver and kidney.

10. Perspectives

Some aspects need to be further clarified with a view to setting RCS and their metabolites as a validated index of lipid-peroxidation. The first aspect is to complete the elucidation of the metabolic pathway of RCS in particular when they adduct proteins. Besides proteosomal degradation, there are other pathways such as the decarbonylation process which deserve further studies. There are novel techniques which would permit a better understanding of this topic, such as novel fluorogenic probes applied to super-resolution fluorescence microscopy which permit a spatiotemporal localization of protein carbonylation at the cellular level [102]. A great advance of this approach would be to implement the method with suitable labelling agents with the aim of a structural resolution of protein carbonyl metabolites by MS approaches. Another important analytical strategy which could permit a better understanding is that reported by the group of Aye who have developed a protein-specific electrophile modification strategy that functions in cells and whole organisms, called targetable reactive electrophiles and oxidants [108]. By using this technique, the stability and metabolic fate of specific protein adducts could be evaluated in a specific way.

The stability of protein adducts in plasma is another important topic since many ex-vivo studies use plasma as a biological matrix to be analyzed. In this compartment protein carbonyl stability could be evaluated by injecting adducted albumin in animal models and monitoring the time-dependent decay by HPLC-ESI-MS.

Another aspect that deserves to be investigated is the sample stability of RCS and adducts at the different temperatures used for sample conservation. A decay of endogenous carbonyls together with the formation of other species caused by the artifactual oxidation during the sample conservation could greatly affect the results even for a fully validated analytical approach. To date, some evidence of protein carbonyls instability/artifactual formation has been reported [109,110] but no systemic studies using MS methods, to our knowledge, have been reported. Moreover, procedures to avoid protein carbonyl loss and their artifactual formation need to be set-up and applied.

11. Conclusion

RCS formed by lipid-peroxidation as free forms or as conjugates are widely used as an index of oxidative stress. Besides general measurements based on derivatizing reactions, such as those based on thiobarbituric acid and DNPH, still popular but limited due to the lack of selectivity, more selective and sensitive MS based analyses have been proposed in the last decade. Untargeted and targeted methods for the measurement of free RCS have been reported and their applications have led to that besides the most known RCS, such as HNE, HHE, MDA and GO, many other circulating species are present, as reported by Tomono who reported hundreds of carbonyl species in murine plasma and only few of them were identified [28]. Validated techniques have also permitted establishment of the steady state circulating levels of HNE and MDA ranging from 0.5 to 2.5 μM which are an order of magnitude higher than F2-isoprostanes and which represent a promising ex vivo index of oxidative stress [30]. Analytical techniques have also fully characterized phase I and II metabolites of RCS, at least for the most studied species, such as HNE, but the use of these metabolites as markers of endogenous lipid oxidation are limited due to the fact that their formation is greatly affected by the metabolic activity which depends on individual variabilities. Moreover, the yield of phase II metabolites (GSH adducts) depends not only on the RCS amount but also on the GSH availability in the site of oxidation which is affected by the same oxidative stress so that the amount of adducts can paradoxically decrease due to the consumption of GSH because of the oxidant reactions. In any case, mercapturic acid of RCS seems a good marker of exposure to exogenous RCS and ELISA methods have been proposed which will permit a greater application of the method [37].

Regarding DNA and APL adducts as an index of oxidative stress, it should be considered that many analytical studies have so far been performed for their structural characterization and in the case of DNA adducts also to acquire accurate quantitative measurements by isotopic dilution methods. However, ex vivo studies are very limited for both the analytes due to their low content which requires very sensitive methods and suitable sample preparations thus limiting a wide application. Moreover, the metabolic fate of lipid adducts has not yet been addressed while for DNA adducts it is regulated by enzymatic repair so that their in vivo content is the result of RCS formation as well as of the expression and activity of repair enzymes.

In recent years several methods have been proposed for profiling RCS protein adducts. This growing interest can be explained by considering the diffusion of HPLC-MS instrumentations which are the most suitable analytical techniques for the measurement of protein covalent adducts. Moreover, protein adducts are now recognized as being involved in the damaging action of oxidative stress so that their measurement is performed not only to obtain an index of LPO but also to gain a deeper insight into the molecular mechanisms of oxidative stress [6]. Many label and label-free as well as targeted and untargeted methods have been proposed and applied. A certain number of plasma and cell proteins have been identified in oxidative stress conditions as protein targets as have several adducted sites and regarding the RCS moieties, those mostly identified derive form HNE, ONE, ACR or GO. It should be noted that so far, although it is likely that some specific proteins exhibit highly stereo- and chemoselective electrophile sensing [6] and that some groups of proteins are more sensitive, no specific protein target/adduct

has been identified to be constantly formed in ex vivo or even in vitro conditions. In some cases, in vitro studies focusing on the same target protein have even reported different modification sites, as found for albumin [3,111,112]. It seems that protein carbonylation affects so many protein targets and that some of them are involved in a random process whose modifications greatly depend on the experimental conditions. The result is that the attacking RCS does not react with a specific protein in a consistent way so that it could be used as specific marker/s. This aspect is sustained by in vitro studies which have identified hundreds of proteins when cells or tissues are exposed to a bolus of RCS. An exception can be given by considering Cys34 of albumin which on the basis of its reactivity (acidity) and concentration, seems to act as a primary site of RCS trapping in plasma. So far several adducts involving Cys34 in ex vivo conditions have been reported. However, if from a qualitative point of view Cys34 adduction could be a promising marker, its quantitative amount as an index of LPO can be limited by the availability of the free form of Cys which is reduced in oxidative based conditions by the formation of the cysteinylated form or oxidized forms (sulfinic, sulfonic residues) [113–115]. Hence in oxidative stress conditions, a decrease of RCS adducts with Cys can paradoxically be detected even if there is an increase of RCS because of the oxidation which reduces the availability of Cys34.

Another aspect to be taken into consideration is the metabolic/chemical stability of protein carbonyls. Some papers report that protein RCS adducts are not stable end products but more likely transient (spatially and temporally) [101,103,116] and that several metabolic processes regulate their stability, not only mediated by a proteasomal degradation but also by a putative enzymatically driven de-carbonylation process [105,106]. This aspect needs to be fully clarified not only to understand the fate of protein carbonyls but also with a view to search for some eventual metabolic final products which could be used as stable biomarkers. Finally, from an analytical point of view, stability studies of protein carbonyls at different temperatures need to be carried out so that in the case of significant instability a suitable sample preparation (for instance $NaBH_4$ reduction) should be considered before storage.

In conclusion HPLC-MS/MS methods for measuring protein carbonyls have made a significant progress in the recent years and represent a valuable tool to understand the molecular mechanisms of lipid-oxidation mediated damage. Besides MS methods, immunological techniques are also very popular for the measurement of RCS conjugates with DNA and proteins. However, if MS instrumentation is available and the MS methods are well set-up and validated, MS methods are preferred in respect to ELISA thanks to their greater sensitivity, selectivity, accuracy and precision and for the possibility to develop untargeted analyses. However, the immunological tests represent a valid alternative when MS is not available and are also useful as an orthogonal method to validate/confirm MS results. However, despite much analytical progress in profiling RCS and their enzymatic and non-enzymatic metabolites, their use as validated biomarkers of lipid-peroxidation and oxidative stress is still a goal and a better understanding of their metabolic and chemical stability is needed.

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Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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