# WILEY
## Online Proofing System Instructions

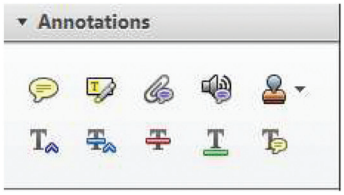
The Wiley Online Proofing System allows proof reviewers to review PDF proofs, mark corrections, respond to queries, upload replacement figures, and submit these changes directly from the locally saved PDF proof.

**1.** For the best experience reviewing your proof in the Wiley Online Proofing System ensure you are connected to the internet. This will allow the PDF proof to connect to the central Wiley Online Proofing System server. If you are connected to the Wiley Online Proofing System server you should see a green check mark icon above in the yellow banner.


Connected


Disconnected

**2.** Please review the article proof on the following pages and mark any corrections, changes, and query responses using the Annotation Tools outlined on the next 2 pages.




**3.** Save your proof corrections by clicking the "Publish Comments" button in the yellow banner above. Corrections don't have to be marked in one sitting. You can publish comments and log back in at a later time to add and publish more comments before you click the "Complete Proof Review" button below.


**4.** If you need to supply additional or replacement files bigger than 5 Megabytes (MB) do not attach them directly to the PDF Proof, please click the "Upload Files" button to upload files:


Click Here

**5.** When your proof review is complete and all corrections have been published to the server by clicking the "Publish Comments" button, please click the "Complete Proof Review" button below:

**IMPORTANT:** Did you reply to all queries listed on the Author Query Form appearing before your proof?
**IMPORTANT:** Did you click the "Publish Comments" button to save all your corrections? Any unpublished comments will be lost.
**IMPORTANT:** Once you click "Complete Proof Review" you will not be able to add or publish additional corrections.

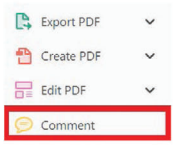

Click Here

For technical questions about reviewing your proof contact Aptara_ops_support@aptaracorp.com

## USING e-ANNOTATION TOOLS FOR ELECTRONIC PROOF CORRECTION

**Required software to e-Annotate PDFs: Adobe Acrobat Professional or Adobe Reader (version 11 or above). (Note that this document uses screenshots from Adobe Reader DC.)**
**The latest version of Acrobat Reader can be downloaded for free at: http://get.adobe.com/reader/**

Once you have Acrobat Reader open on your computer, click on the Comment tab (right-hand panel or under the Tools menu).



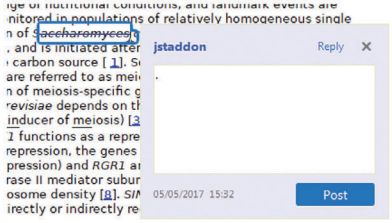

This will open up a ribbon panel at the top of the document. Using a tool will place a comment in the right-hand panel. The tools you will use for annotating your proof are shown below:


### 1. Replace (Ins) Tool – for replacing text.

Strikes a line through text and opens up a text box where replacement text can be entered.

**How to use it:**

- Highlight a word or sentence.
- Click on .
- Type the replacement text into the blue box that appears.

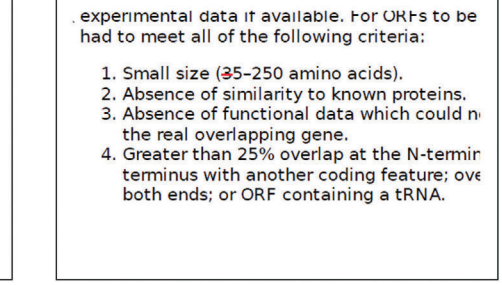



### 2. Strikethrough (Del) Tool – for deleting text.

Strikes a red line through text that is to be deleted.

**How to use it:**

- Highlight a word or sentence.
- Click on ..
- The text will be struck out in red.

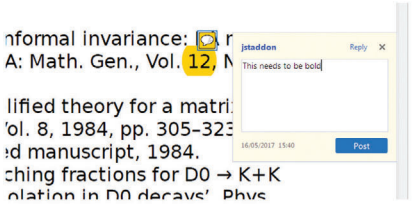



### 3. Commenting Tool – for highlighting a section to be changed to bold or italic or for general comments.

Use these 2 tools to highlight the text where a comment is then made.

**How to use it:**

- Click on .
- Click and drag over the text you need to highlight for the comment you will add.
- Click on .
- Click close to the text you just highlighted.
- Type any instructions regarding the text to be altered into the box that appears.

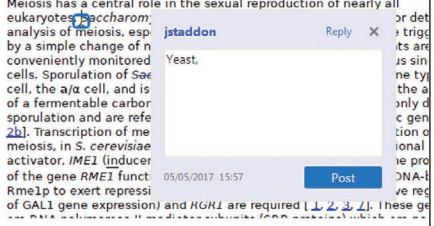



### 4. Insert Tool – for inserting missing text at specific points in the text.

Marks an insertion point in the text and opens up a text box where comments can be entered.

**How to use it:**

- Click on .
- Click at the point in the proof where the comment should be inserted.
- Type the comment into the box that appears.



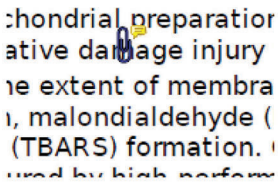
**5. Attach File Tool – for inserting large amounts of text or replacement figures.**

Inserts an icon linking to the attached file in the appropriate place in the text.

**How to use it:**

- Click on .
- Click on the proof to where you'd like the attached file to be linked.
- Select the file to be attached from your computer or network.
- Select the colour and type of icon that will appear in the proof. Click OK.

The attachment appears in the right-hand panel.

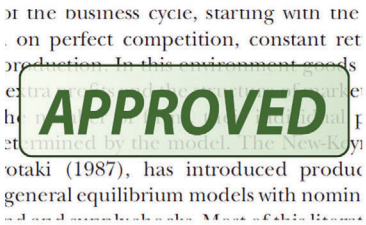


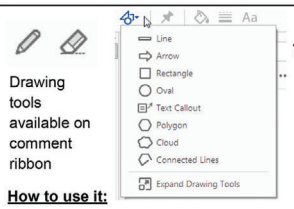

**6. Add stamp Tool – for approving a proof if no corrections are required.**

Inserts a selected stamp onto an appropriate place in the proof.

**How to use it:**

- Click on .
- Select the stamp you want to use. (The Approved stamp is usually available directly in the menu that appears. Others are shown under *Dynamic*, *Sign Here*, *Standard Business*).
- Fill in any details and then click on the proof where you'd like the stamp to appear. (Where a proof is to be approved as it is, this would normally be on the first page).

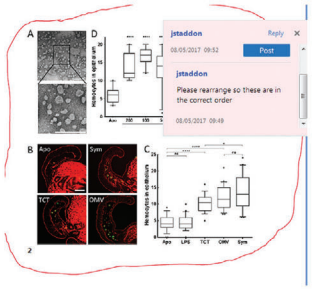



Drawing tools available on comment ribbon

**7. Drawing Markups Tools – for drawing shapes, lines, and freeform annotations on proofs and commenting on these marks.**

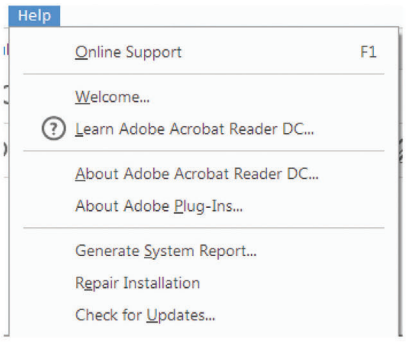
Allows shapes, lines, and freeform annotations to be drawn on proofs and for comments to be made on these marks.




**How to use it:**

- Click on one of the shapes in the Drawing Markups section.
- Click on the proof at the relevant point and draw the selected shape with the cursor.
- To add a comment to the drawn shape, right-click on shape and select *Open Pop-up Note.*
- Type any text in the red box that appears.

**For further information on how to annotate proofs, click on the Help menu to reveal a list of further options:**



WILEY

# Author Query Form

Journal: JSSC

Article: jssc201900804

Dear Author,

During the copyediting of your manuscript the following queries arose.

Please refer to the query reference callout numbers in the page proofs and respond to each by marking the necessary comments using the PDF annotation tools.

Please remember illegible or unclear comments and corrections may delay publication.

Many thanks for your assistance.

| Query No. | Description | Remarks |
|---|---|---|
| Q1 | Please confirm that forenames/given names (blue) and surnames/family names (vermilion) have been identified correctly. | |
| Q2 | Please verify that the linked ORCID identifiers are correct for each author. | |

**Please confirm that Funding Information has been identified correctly.**

Please confirm that the funding sponsor list below was correctly extracted from your article: that it includes all funders and that the text has been matched to the correct FundRef Registry organization names. If a name was not found in the FundRef registry, it may not be the canonical name form, it may be a program name rather than an organization name, or it may be an organization not yet included in FundRef Registry. If you know of another name form or a parent organization name for a "not found" item on this list below, please share that information.

| FundRef Name | FundRef Organization Name |
|---|---|
| Ministero dell'Istruzione, dell'Università e della Ricerca | |
| IRCCS Fondazione Santa Lucia | |
| Regione Autonoma Sardegna | |
| Ministero della Salute | Ministero della Salute |
| Università Cattolica del Sacro Cuore | Università Cattolica del Sacro Cuore |
| Università di Cagliari | Università di Cagliari |
| Consiglio Nazionale delle Ricerche | Consiglio Nazionale delle Ricerche |

Received: 21 August 2019 | Revised: 23 September 2019 | Accepted: 17 October 2019

DOI: 10.1002/jssc.201900804

JOURNAL OF SEPARATION SCIENCE

REVIEW ARTICLE

# Enrichments of post-translational modifications in proteomic studies

Luisa Pieroni[1]* | Federica Iavarone[2,3]* | Alessandra Olianas[4]* | Viviana Greco[2,3] | Claudia Desiderio[5] | Claudia Martelli[6] | Barbara Manconi[4] | Maria Teresa Sanna[4] | Irene Messana[5] | Massimo Castagnola[1] | Tiziana Cabras[4]

[1]Laboratorio di Proteomica e Metabolomica, Centro Europeo di Ricerca sul Cervello, IRCCS Fondazione Santa Lucia, Rome, Italy

[2]Istituto di Biochimica e Biochimica Clinica, Facoltà di Medicina, Università Cattolica del Sacro Cuore, Rome, Italy

[3]IRCCS Fondazione Policlinico Universitario Agostino Gemelli, Rome, Italy

[4]Dipartimento di Scienze della Vita e dell'Ambiente, Università di Cagliari, Cagliari, Italy

[5]Istituto di Chimica del Riconoscimento Molecolare, Consiglio Nazionale delle Ricerche, Rome, Italy

[6]Department of Biology, Institute of Molecular Systems Biology, ETH Zurich, Zurich, Switzerland

**Correspondence**
Massimo Castagnola, Laboratorio di Proteomica e Metabolomica, Centro Europeo di Ricerca sul Cervello, IRCCS Fondazione Santa Lucia, Via del Fosso di Fiorano, 64. I-00143, Rome, Italy.
Email: massimo.castagnola@icrm.cnr.it

**Funding information**
Ministero dell'Istruzione, dell'Università e della Ricerca; IRCCS Fondazione Santa Lucia; Regione Autonoma Sardegna; Ministero della Salute; Università Cattolica del Sacro Cuore; Università di Cagliari; Consiglio Nazionale delle Ricerche

More than 300 different protein post-translational modifications are currently known, but only a few have been extensively investigated because modified proteoforms are commonly present in sub-stoichiometry amount. For this reason, improvement of specific enrichment techniques is particularly useful for the proteomic characterization of post-translationally modified proteins. Enrichment proteomic strategies could help the researcher in the challenging issue to decipher the complex molecular cross-talk existing between the different factors influencing the cellular pathways. In this review the state of art of the platforms applied for the enrichment of specific and most common post-translational modifications, such as glycosylation and glycation, phosphorylation, sulfation, redox modifications (i.e. sulphydration and nitrosylation), methylation, acetylation, and ubiquitinylation, are described. Enrichments strategies applied to characterize less studied post-translational modifications are also briefly discussed.

**KEYWORDS**
enrichment, post-translational modifications, proteomics

## 1 | INTRODUCTION

Proteomics is probably the most complex omic science because from the same amino acid sequence, encoded by specific genes, numerous proteoforms can be generated by more than 300 post-translational modifications (PTMs) occurring after protein synthesis and over time. This is due to the interaction with the changing biochemical environment during development and under physiological and pathological conditions. It should be emphasized that many proteins and peptides can exert different roles in different biological

Article Related Abbreviations: AGE, advanced glycation end product; CHD, cleavable hydrophobic derivatization; COFRADIC, COmbined FRActional DIagonal Chromatography; DTT, dithiothreitol; Hb, haemoglobin; HB, histidine-biotin; IAA, iodoacetamide; ICAT, isotope-coded affinity tag; IMAC, immobilized metal affinity chromatography; K-suc, lysine succinylation; LWAC, lectin weak affinity chromatography; MMTS, methyl methanethiosulfonate; NP, nanoparticle; PARP, ADP-ribosyl-transferase; PTM, post-translational modification; ROS, reactive oxygen species; SH2, Src homology 2; SrtA, sortase A; SUMO, small ubiquitin-like modifier; Ub, ubiquitin; WB, Western Blot.

*These authors contributed equally to this work.



contexts also depending on PTMs affecting and modulating their function [1,2]. In order to understand the functional information hidden in the protein structure well, the physiological and pathological molecular transformations occurring during protein life-span have to be clarified. In most cases a sub-stoichiometric amount of the protein is submitted to specific PTMs. Thus many modifications, even relevant for their functional meaning, may remain unrevealed. For this reason, from the beginning of the proteomic era, strategies to enrich specific PTMs have been developed. In this review the current pipelines for the enrichment of the most relevant PTMs in proteomics are discussed. According to the method, utilized enrichment platforms may be classified as: a) antibody-based affinity (or immuno-selection), b) lectin-based, c) tagging by chemical, d) ionic interaction-based, and e) enzyme-based [3].

In the present review, the description of the diverse enrichment methods has been organized according to a list of various PTMs, because more than one platform can be utilized for each of them.

In the first section, the enrichment methods for non-enzymatic glycation and glycosylation are discussed. In the second section, platforms to enrich the phosphoproteome are described and compared with those applied to the sulfoproteome, being them isobaric modifications. A section is devoted to the strategies optimized to enrich redox modifications with particular concern for sulphydration and nitrosylation. Acetylation and methylation are thereafter discussed. A section is dealing with the techniques employed to enrich proteins modified by ubiquitin and ubiquitin-like peptides and the enrichments of few less studied PTMs are finally described. The present review reports an update of the different methods applied for PTMs enrichment, based on selected references mainly chosen among the most cited and significant in this field, certainly with some omissions, with the aim of underlining how the enrichment of specific PTMs can improve the information on their important biological functions.

## 2 | GLYCATION AND GLYCOSYLATION

It is well known that the reaction of sugars with proteins can be either non-enzymatic, commonly called glycation, or enzymatic, the classical protein glycosylation mainly occurring during the Golgi transit.

### 2.1 | Glycation (non-enzymatic glycosylation)

Glycation is the result of the nucleophilic addition of the free amino groups of protein (N-terminal or lysines and arginines lateral chain) to the carbonyl group of reducing sugars present in biological fluids. Due to its concentration in bodily fluids, glucose is the main sugar involved in the reaction. The reaction of glucose generates at first a labile aldimine, which can undergo a reversible dissociation. The aldimine can be slowly and irreversibly converted, throughout an Amadori rearrangement, in a ketoamine which forms a stable pyranosic ring (Figure 1) [4]. Other sugars and phospho-sugars can be involved in glycation reactions, as demonstrated few years ago on hemoglobin (Hb) [5,6]. The first products of the glycation reaction, i.e. fructosamine, are usually called "early glycation products". They can be transformed throughout reactions of elimination, condensation, and oxidation in other products, such as glucosone, ribosone, erythrosone, glyoxal, methylglyoxal, and methyfurfural, to cite a few, which are collectively termed "advanced glycation end-products" or AGE [7]. Dicarbonyl compounds are highly reactive. For instance, methyl-glyoxal is 20 000 folds more reactive than glucose, even because only a small percentage of glucose is present in the open reactive form (Figure 1). Therefore, dicarbonyls are not only products deriving from degradation of fructosamine, but also reactive intermediates originating from lipid peroxidation, which can react with arginine and lysine residue [7,8].

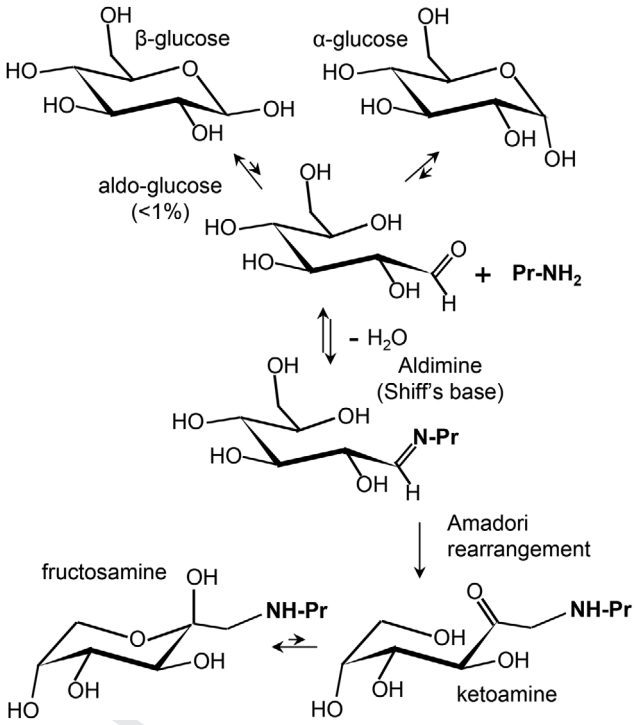



**FIGURE 1** Reactions between glucose and the amino-group of proteins leading, throughout an Amadori rearrangement, to the formation of a stable fructosamine, which, as suggested in [4], is cyclized in a piranosidic ring. Only the open aldehyde structure of glucose is able to react with the amino group

Glycation is thereby a slow reaction and the percentage of protein glycation is related to sugar concentration and to the life-span of the protein. The life-span of human erythrocytes is

about 120 days, and no protein synthesis and turnover occur in mature erythrocytes. Thereby, Hb is slowly glycated during its lifespan by glucose on N-terminal valine residues of β chains, and to a lesser extent on the C-terminal valine residues of α chains, which for steric hindrance are less exposed than β chains, as well as on the amino group of some exposed lateral chains of lysines [9]. The level of glycation is therefore higher in old than in young erythrocytes [10], and the percentage of glycated Hb is always a mean value related to the mean blood glucose concentration two-three months before the clinical analysis. Currently, the measurement of the extent of Hb specifically glycated at the N-terminal valine residue of the β chains, also called $HbA_{1c}$, is most commonly performed by cation-exchange chromatography, which takes advantage of the charge modification of the amino group of valine following glycation. HbA1c elutes therefore faster than the unmodified Hb ($HbA_0$) or than the Hb glycated on the lateral amino group of lysine, which elutes in the front of $HbA_0$ peak. $HbA_{1c}$ is also the most abundant fast Hb form [6]. In normal subjects the mean percentage of $HbA_{1c}$ is in the range 4–6%, but in diabetic patients this value can exceed 15% [11,12]. The choice of $HbA_{1c}$ as a marker of long term glycemic status was dictated by the high Hb content within the erythrocytes. Other proteins inside the erythrocytes are potential markers of glycation levels, as well as all the proteins of biological fluids in contact with free glucose (or other reducing sugars). Serum proteins, for instance, are prone to extensive glycation, but due to their reduced life-span with respect to Hb, measurement of the glycation level of serum proteins may give information on exposure to circulating glycaemia for a shorter period, which may be beneficial to assess prompt metabolic alterations or changes in diabetes treatment [13].

A general method for the enrichment of early glycation protein products is based on the specific reaction of boronate with *cis*-diols (Figure 2). Even though several sugars, as mannose and galactose, present in the antennary structure of enzymatically glycosylated proteins, contain *cis*-diol groups, the use of opportune experimental conditions grant specificity of boronate affinity chromatography towards early (non-enzymatically) glycated products [14,15]. In the next section, the use of boronate chromatography for the enrichment of enzymatically glycosylated proteins will be described. Although boronate affinity chromatography can be utilized for the enrichment of intact glycated proteins (top-down proteomics), enrichments carried out on the peptide digest after chemical or enzymatic hydrolysis (bottom-up proteomics) provided the detection of more extensive glycoproteomes [16,17]. Muralidharan and colleagues [18] isolated the glycated proteome devoid of hemoglobin from control and diabetic erythrocyte samples by boronate affinity chromatography. Proteomic analysis using nano-LC/ESI-MS platform identified and characterized 37 glycated proteins in subjects with $HbA_{1c}$ of 6, 8, 12, and 16%. Results showed

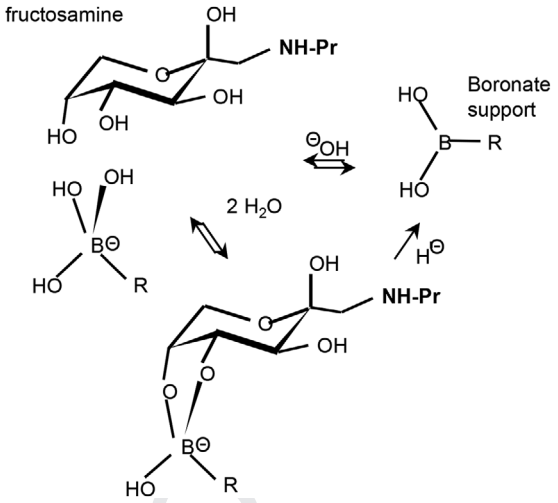



**FIGURE 2** Interaction between a boronate chromatographic support and *cis*-diols of fructosamine in basic environment

that both extent and site specific modification of proteins increased according to $HbA_{1c}$ increasing levels. The study also demonstrated that residue-specific modifications of catalase, peroxiredoxin, carbonic anhydrase, lactate dehydrogenase B, and delta-aminolevulinic acid dehydratase could affect their functions proportionally to the percentage of glycation.

The number of AGEs detectable by mass spectrometry is continuously increasing, as recently reviewed by D'Aronco and colleagues [7]. Due to their elevated structural heterogeneity, AGEs enrichment is a challenging task and for some classes it was achieved by affinity chromatography with immobilized specific attractors, such as receptor for advanced glycation endproducts (RAGEs), which are the receptors of AGEs and other proteins such as, for example, DIAPH1 and some members of the S100 family [19]. The N-terminal domain of RAGE, the V domain, is the major site of AGEs binding and it is stabilized by the adjacent C1 domain. Recently, Degani and colleagues [20] developed an affinity assay relying on the highly specific interaction of AGEs for the VC1 domain. A recombinant glycosylated form of VC1 domain, produced in the yeast *Pichia pastoris*, was attached to magnetic beads to capture specific AGEs modified tryptic peptides obtained after digestion of AGE-modified serum albumin prepared in vitro, incubating the protein with glucose or ribose. The PTMs were characterized by MS. Interestingly, the method also led to the isolation of advanced lipoxidation end products generated by malon-dialdehyde treatment of albumin. According to the authors, this enrichment strategy might lead to the identification of new RAGE ligands potentially involved in pro-inflammatory and pro-fibrotic responses independently from their structures or physical properties and without the use of any covalent derivatization process [20]. The observation that lysozyme linked to

sepharose-4B was able to bind $N^{\varepsilon}$-(carboxymethyl)lysine, $N^{\varepsilon}$-(carboxyethyl)lysine, and pentosidine, induced Mitsuhashi and colleagues to use this chromatographic stationary phase to deplete AGEs from diabetic sera [21]. However, as far as we are aware, until now nobody used this stationary phase for the proteomic enrichment of specific AGEs. Specific enrichment of peptides containing $N^{\varepsilon}$-(carboxymethyl)lysine, $N^{\varepsilon}$-(carboxyethyl)lysine, various dycarbonyl-derived hydroimidazolones, imidazolone B, and other AGE-modified peptides was recently carried out by Cu(II)-immobilized metal affinity chromatography (Cu(II)-IMAC) [22].

## 2.2 | Enzymatic glycosylation

Many studies on the enrichment of glycosylated proteins have been recently reviewed [23–25]. Enzymatic glycosylation is one of the most common and prominent PTMs, modulating the activity of cellular and extracellular proteins, implicated in many and articulate physiological processes [26,27] such as cell adhesion, signaling and cell-to-cell interaction, to cite a few. The two principal types of protein glycosylation in humans are N- and O-glycosylation, while C-mannosylation occurs less frequently [28].

N-glycosylation occurs as a co-translational modification on the Asn residue in the sequon Asn-X-Ser/Thr, even though some recent glycoproteomic studies evidenced that several N-glycosylation sites do not adhere to this canonical motif [29]. The N-linked glycans present a common pentasaccharide core to which different types of monosaccharide residues can be linked in different positions, generating diverse classes of branched glycans [30]. O-glycosylation occurs on either Ser or Thr residue exposed on the protein surface. The O-linked glycans can be very simple and are constituted by one or few monosaccharide residues, such as N-acetyl-galactosamine, mannose, glucose, and fucose. C-mannosylation is an unusual type of protein glycosylation occurring on Trp residues, with function still not well understood [31].

Glycoproteins are characterized by high microheterogeneity, because each glycoprotein may exist as a complex family of glycoforms sharing the amino acid sequence and differing for various features: (a) occupancy of the multiple glycosites; (b) type of glycan (only N-linked, only O-linked, N- and O-linked on the same protein); (c) composition and stereoisomery of the glycans that can change on the same glycosite.

Altered glycoprotein expression and glycosylation aberrations, consisting in increased glycan size, extra branching of glycan chains, over-sialylation, or over-fucosylation, were associated to many diseases, especially cancer and neurological disorders [32,33]. Therefore, glycoproteomic studies are of great interest for the development of disease biomarkers. Nonetheless, they are a complex and challenging field of research, requiring the evaluation of the glycoprotein profiling of a biological sample in physiological conditions and the characterization of the qualitative and quantitative changes related to a specific pathological condition [33,34].

Characterization of glycoproteins is challenging due to the low abundance and the low ionization efficiency of the glycopeptides with respect to the not glycosylated peptides present in the same sample that suppress the glycopeptide MS signals [24]. Furthermore, specific MS analysis methods and expert bioinformatic tools devoted to glycoproteomics are missing [35].

Glycoproteomic enrichments were based principally on bottom-up approaches implicating the MS characterization of glycopeptides generated by proteolysis of glycoproteins [36], although integrated top-down and bottom-up approaches were also applied [37–39]. The proteolysis could be coupled or not with a deglycosylation treatment and the glycosites could be localized and characterized by MS/MS analysis of the deglycosylated peptides. The three main enrichments methods used were lectin-, hydrazide-, and boronic acid-based capturing techniques [37]. The lectin-based capturing technique is a powerful tool to enrich specific glycoproteins from a complex biological sample. Indeed, several lectins are available to capture glycoproteins carrying a specific glycan structure, such as those recognizing fucosylated, high-mannose, branching glycans, or lactosamine moiety of the glycans [37]. For this reason, the lectin-based method was largely used to study disease-associated aberrant protein glycoforms, especially cancer related, and discover glycoprotein biomarkers [40]. A high-recovery lectin-based capturing approach consisted in the use of a multiple lectin stationary phase to capture simultaneously various glycoproteins from the same sample [41,42]. A more selective approach was the lectin-channel monitoring based on a multiplex and parallel lectin-capturing and fractionation system, allowing to bind and to enrich separately many different glycoproteins from the same biological sample [43].

The hydrazide-based capturing involves a chemical reaction of the *cis*-diols present in the glycan moieties of the glycoproteins. They are oxidized into aldehydes by sodium periodate and then covalently bound to hydrazide immobilized either on a solid support [44] or on magnetic beads [45] (Figure 3). Gold or silica nanoparticles were also utilized to this aim [37]. Captured glycoproteins were digested in situ by trypsin and N-glycopeptides were released by PNGase F. The site of N-glycoylation was recognized by MS analysis of deglycosylated peptides where Asn of the sequon AsnXSer/Thr was transformed in Asp by the action of PNGase F. In the case of sialylated glycoproteins, acid hydrolysis was used to release bound glycopeptides [46]. For O-linked glycopeptides a treatment with hydroxylamine was utilized [47]. Recently, Bai and co-workers developed a method to implement the efficiency of hydrazide-based capturing [48] based on the use of new thermosensitive

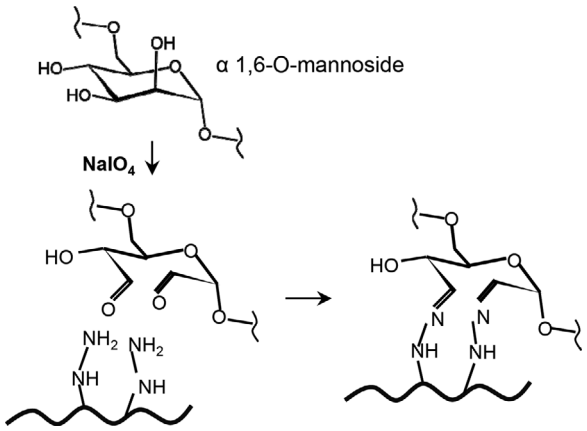



**FIGURE 3** Scheme showing the reactions involved in the hydrazide-based glycosylation enrichment

soluble polymers to replace the solid-phase systems. The disadvantages of the common SPE systems are characterized by the high steric hindrance and the large interfacial mass transfer resistance interfering with the hydrazide, the oxidized glycan moiety and the removal of not specifically adsorbed proteins. They could be overcome by soluble polymers which form a homogeneous liquid-phase system that facilitates the interactions with low-abundant glycoproteins [48]. With this strategy it was possible to enrich 180 N-glycoproteins and to identify 329 N-glycosites from human plasma exosomes of glioma patients. The hydrazide-based capturing is a high-throughput method to enrich glycoproteins, but, differently from the lectin-based capturing, it neither allows the recovery of intact glycopeptides, nor provides information on the glycan structure, and therefore, it cannot be applied to study aberrant protein glycoforms associated to pathological conditions.

As described in the previous section, capturing by boronic acid was based on its affinity for the *cis*-diols present in the monosaccharides of the glycan moieties (Figure 2) [49]. The technique was successfully applied also to glycopeptide enrichment [37]. Captured glycoproteins were released by an elution buffer containing high concentrations of monosaccharides or by switching the pH to lower values. Boronate affinity capturing of glycoproteins was also exploited in capillary liquid chromatography with monolithic columns [50]. The boronate-based capturing is a low cost and MS-compatible technique to enrich glycoproteins from complex biological samples. However, as the hydrazide-based capturing, does not allow the selective capturing of specific glycan structures. Moreover, the usual boronate affinity material showed low affinity towards *cis*-diols glycans [51]. To overcome this problem several research groups developed different materials for the boronate-based capturing, such as boronate MIPs, boronic acid Mn-doped ZnS quantum dots, and boronate avidity materials [52]. In the avidity materials the binding strength was enhanced by highly branched poly-(amidoamine) where the boronic acid moieties were amplified [52]. Another option, attractive for its low cost, chain flexibility, and hydrophilic properties, was to bind boronic acid to branched poly-ethyleneimine. Silica nanoparticles functionalized with branched poly-ethyleneimine-assisted boronic acid, when compared with other boronic acid functionalized materials, exhibited optimal performance in capturing the low concentrated glycoproteins present in human saliva [51].

Compared to N-glycosylation, protein O-glycosylation was less explored mainly due to its low abundance. The most widely used enrichment strategy for O-glycoproteins and O-glycopeptides was lectin weak affinity chromatography (LWAC), which used different kinds of lectins to bind different sugar moieties and surrounding amino acid sequences [53]. LWAC enrichment method has been successfully combined with HILIC for extensive coverage of O-GlcNA modified proteins/peptides [54]. Shen and colleagues synthesized a novel kind of HILIC material (pPEGMA-SMs) that specifically enriched O-GlcNAc-modified peptides after the removal of N-linked and O-GalNAc glycans by enzymatic reactions, identifying 474 O-GlcNAc-modified peptides corresponding to 457 proteins in human urine [55]. Steentoft and colleagues developed a new technology for O-GalNAc analysis called "SimpleCell" in which a zinc-finger nuclease was used to interfere with the O-glycan elongation pathway obtaining more homogeneous O-GalNAc on the glycosite, facilitating their enrichment by LWAC [56]. Boronic acid-based enrichment for O-glycosylation was rarely used, probably for the low affinity of the support. An improvement of interaction was obtained with boronic acid derivatives, among them benzoboroxolo was found to be the most efficient [57]. Indeed, conjugation of benzoboroxolo with dendrimer beads has been used synergistically to selectively interact with O-GlcNac glycopeptides [57].

Yang and colleagues developed a chemo-enzymatic solid-phase approach for deciphering complex O-glycosylation [58]. In this method, called O-glycopeptide immobilization for O-glycosylated peptide enrichment, tryptic glycopeptides have been immobilized in a solid aldehyde-activated support via reductive amination on peptide N-termini and released from the support by O-protease OpeRATOR digestion.

Zhang and colleagues recently obtained an efficient simultaneous enrichment of O-/N-glycopeptides using a novel self-assembling 4-mercaptobenzene boronic acid functionalized and Au-doped straticulate $C_3N_4$. The hydrophilic properties of 4-mercaptobenzene boronic acid functionalized and Au-doped straticulate $C_3N_4$ enabled the glycopeptides enrichment on its surface in hydro-organic solution (water/ACN) [59].

To the best of our knowledge, no specific enrichment for C-mannosylated proteins has been reported to date.

# 3 | PHOSPHORYLATION AND SULFATION

## 3.1 | Phosphorylation

Phosphorylation is one of the most common PTMs in nature, and it has been estimated that at least one third of the whole proteome of a eukaryotic cell can be phosphorylated on single or multiple sites and at different time of the cellular cycle [60]. More than 800 protein kinases are responsible for this reversible PTM, adding a phosphate group commonly on Ser and Thr residues, less on Tyr residues (O-phosphorylation). Rarely phosphorylation involves basic amino acid residues, i.e His, Lys, and Arg (N-phosphorylation), an important but often understimated PTM [61]. As it is well known, phosphatases are the enzymes responsible for the removal of the phosphate group.

Due to its deep impact on protein function, phosphoproteome enrichment has been widely reviewed [62,63] and here we report only several selected studies.

The addition of a phosphate group to an amino acid produces a mass shift of 80/*z*. However, since sulfation is isobaric to phosphorylation, this mass shift cannot ensure for a phospho-modification [64]. Commonly, the treatment with phosphatase allows to evidence the presence of phosphorylated sites and to discriminate them with respect to sulfosites. Neutral losses in MS/MS experiments also differentiate phosphate from sulphate group; while the phosphate is lost as a molecule of phosphoric acid (98/*z*, $H_3PO_4$), sulphate is lost as sulphur trioxide (80/*z*, $SO_3$) [65]. Nonetheless, the presence of confounding factors in the detection of phosphorylation, joined to the existence of many phosphorylation sites at low sub-stoichiometric levels [66], stimulated the development of different pipelines for the enrichment of phosphoproteome.

IMAC was the method of election to enrich phosphorylated proteins and peptides. It is based on the affinity between negatively charged phosphorylated peptides and positively charged metal ions such as $Fe^{3+}$, $Ga^{3+}$, $Al^{3+}$, $Zr^{4+}$, and $Ti^{4+}$. However, also highly acidic non-phosphorylated peptides show affinity for metal ions. Low pH can reduce the binding of non-phosphorylated peptides, but can also decrease specific phospho-peptides binding [67]. In the last years the metal oxide chromatography, based on microcolumns of titanium dioxide has been developed in order to improve the efficiency of IMAC, whilst reducing the time of the chromatographic separation. Thingholm and colleagues showed that the selection toward phospho-peptides was achieved by loading the sample onto a $TiO_2$ column using a TFA/ACN solution containing 2,5-hydroxybenzoic acid or phthalic acid. Even though enrichment could be achieved using TFA/ACN alone, selectivity was dramatically enhanced by adding one of these two acids, because they, in conjunction with the low pH, prevent binding of non-phosphorylated peptides to $TiO_2$ column [68]. Recently, Wang and colleagues investigated the enrichment of the phospho-tyrosine proteome using different experimental pipelines. An enrichment factor of more than 38 000 times was obtained by combining initial immune-affinity purification with phospho-tyrosine antibodies with $TiO_2$ chromatography [69].

Satisfactory enrichment strategies for the phosphoproteome were achieved exploiting the increase of polarity subsequent to the negative charge of the phosphate group in various chromatographic methods, such as IEC, HILIC, and electrostatic repulsion HILIC [63]. On this vein, Engholm-Keller and colleagues developed a multidimensional phospho-proteomic strategy, applicable to limited sample amounts [70,71]. They combined a highly specific pre-enrichment $TiO_2$-based with sequential elution from immobilized metal affinity chromatography. Mono- and multi-phosphorylated peptides were sequencially eluted and mono-phosphorylated peptides further fractionated by capillary HILIC. The AssayMap Bravo platform allowed to conduct protein digestion, peptide desalting, samples fractionation with C18 followed by phosphopeptide enrichment, based on Fe(III)-IMAC-phosphorylation protocol, as an automated workflow on minute amounts of malignant melanoma tissues. The workflow was efficient in terms of sensitivity, reproducibility, and phospho-site localization. From only 12.5 μg of sample, more than 1000 phospho-peptides were identified [72].

The majority of enrichment pipelines for phosphoproteomics have been devoted to bottom-up platforms, because the improvement of top-down MS-based platforms is challenging due to the difficulty in enriching low abundance intact phosphoproteins as well as separating and detecting the enriched phosphoproteins from complex mixtures [3]. Nonetheless, recently Chen and colleagues have designed and synthesized a next generation functionalized superparamagnetic cobalt ferrite ($CoFe_2O_4$) nanoparticles (NPs), and have developed a top-down phosphoproteomics strategy coupling phospho-protein enrichment based on $CoFe_2O_4$ NPs with online LC-MS/MS. The mixture of proteins extracted from a swine heart tissue lysate spiked with β-casein was submitted to enrichment by functionalized CoFe2O4 NPs, and phosphoproteins bound on magnetic NPs were then pulled down for subsequent elution. This arrangement allowed the specific enrichment of spiked β-casein as well as the detection and the determination of the relative percentage of three phosphorylated β-casein genetic variants [73].

The specific enrichment of thyrosine phosphorylation has been successfully achieved utilizing Src homology 2 (SH2) domains. SH2 domains are small protein modules specifically interacting with tyrosine-phosphorylated peptides. There are more than 100 SH2 domains in the human genome, and different SH2 domains interact with different classes of tyrosine-phosphorylated ligands. These domains have a relevant role

in the transmission of cell signals, mediating the complex formation of various proteins in response to changes in tyrosine phosphorylation. Machida and colleagues utilized a battery of SH2 domains to probe the global state of tyrosine phosphorylation [74]. Later, taking advantage of the sequence-independent high affinity of SH2 superbinder toward pTyr residues, a simplified one-step pTyr peptide enrichment method using immobilized SH2 superbinder linked to a monolithic capillary chromatographic column was optimized for a sensitive analysis of the tyrosine phospho-proteome [75]. It was found that a synthetic pTyr peptide could be specifically enriched from a peptide mixture prepared by spiking of the synthetic peptide into the tryptic digests of α-casein and β-casein with molar ratios of 1:1000:1000 [75]. This platform, applied to enrich pTyr peptides from pervanadate-treated HeLa cell digests, was able to identify 796 unique pTyr sites. When applied to analyze the pTyr in the Shc1 immunopurified protein complex, it was able to identify 15 pTyr sites, demonstrating its suitability to detect pTyr residues in minute amount of samples [75]. Indeed, the platform, applied to Jurkat cells at resting state, where the tyrosine phosphorylation level is low, was able to identify 511 non-redundant pTyr peptides, corresponding to 403 high confidence pTyr sites [76]. Application of this pipeline to two human breast cancer cell lines, BT474 and HCC1954, before and after epidermal growth factor stimulation, demonstrated that it could be a powerful tool for illustrating pTyr-dependent signaling network controlling cellular behaviour such as drug resistance [76].

For its structural flexibility and acid lability, the identification of phosphorylated lysine (pLys) peptides is a great challenge. However, recently a cleavable hydrophobic derivatization (CHD) strategy for the enrichment and identification of pLys peptides has been developed. First, 2,5-dioxopyrrolidin-1-yl-3-(decyldisulfanyl)propanoate was synthesized to react with dephosphorylated lysine peptides, and then the derived peptides were captured by a $C_{18}$ column, followed by cleavage of the hydrophobic chain, with the specific label left on the target peptides for further identification. By CHD, the enrichment of pLys peptides from interfering peptides (1:1000 mass ratio) was achieved. Furthermore, CHD was applied to screen the pLys targets from *Escherichia coli* lysates, and 39 pLys sites from 35 proteins were identified [77]. As we are aware, no enrichment procedure for other N-phosphorylation has been described so far.

## 3.2 | Sulfation

Sulfation is a protein PTM less common than phosphorylation and it is usually an irreversible modification (efficient sulfatases have not been found yet). Inorganic sulfate, to be utilized by organisms, needs to be activated and transformed in adenosine-5′-phosphosulfate and subsequently in 3′-phosphoadenosine-5′-phosphosulfate. The activated sulfate of 3′-phosphoadenosine-5′-phosphosulfate can be transferred to different biomolecules, comprising hormones, neurotransmitters, carbohydrates, and proteins, by the action of several sulfotransferases. Tyrosylprotein sulfotransferase, a *trans*-Golgi membrane enzyme, is involved in protein tyrosine sulfation, a PTM revealed for the first time in fibrinogen [78]. Several studies demonstrated that protein tyrosine sulfation plays an important role in enhancing protein-protein interaction [79]. Tyrosine-sulfation is involved in blood coagulation, optimum rolling of leukocytes on endothelial cells, chemokine receptor ligand binding, chemokine CCR5 binding to HIV-1 gp120 and entry of virus into cells, hormone binding to receptors, and protein interactions [79]. It is relevant to outline that the majority of the proteins submitted to sulfation are membrane proteins, and this makes the characterization of new sulfated proteins even more difficult.

As already described in the previous section, mass modifications following tyrosine sulfation and tyrosine phosphorylation are around 80/$z$ Da (sulfation: 79.9568 Da; phosphorylation: 79.9663 Da), and the small mass variation of 9.5 mDa that distinguishes tyrosine sulfation from phosphorylation needs to be revealed by ultra-high accuracy mass measurements. To increase the ability to distinguish the two PTMs several efforts have been done. For instance, the simultaneous identification of tyrosine phosphorylation and sulfation sites has been achieved utilizing tyrosine-specific bromination [80]. Free tyrosine residues (and amine groups) of a tryptic digest of albumin were acetylated by sulfo-succinimidyl acetate and imidazole as a catalyst. Then the sample was divided in two fractions to be treated either by alkaline phosphatase or sulfatase followed by incubation in HBr to brominate the free tyrosine residues generated by hydrolysis. The identification of the different modified tyrosine residues was finally achieved by MALDI-TOF MS [80] under strong ionization conditions. Free tyrosines, phosphotyrosines and sulfotyrosines were detected as acetyl-tyrosine, phospho-tyrosine and bromo-tyrosine, respectively, in the sample treated by sulfatase, while they were detected as acetyl-tyrosine, bromo-tyrosine and free-tyrosine in the sample treated by phosphatase. It should be indeed outlined that sulfation of tyrosine is more difficult to be detected by MS, being this modification less stable than phosphorylation [65,81] in acidic environment. Often, during the MALDI ionization process, sulfate is lost by in source neutral loss of sulfur trioxide. Because the mass of $SO_3$ is 80 Da, sulfation of tyrosine can sometimes pass unobserved during the analysis [65]. A similar subtractive strategy was applied by Yu and colleagues for the determination of the sulfation sites of mouse lumican and human vibronectin [82].

Medzihradszky and colleagues reported in 2004, for the first time, the characterization of O-sulfation of serine and threonine residues in several proteins. The modification was

detected and characterized by on-line HPLC tandem mass spectrometry from proteins isolated by SDS-PAGE [83].

The use of specific antibodies for the recognition of sulfated proteins/peptides, and eventually for enrichment protocols, is limited, since only one anti-sulfotyrosine antibody, named PSG2, was reported in the literature [84]. It has been demonstrated that PSG2 binds sulfo-tyrosine residues with high affinity independently of sequence context [84], but its specificity or its co-recognition of phospho-tyrosine has not been established. Furthermore, the ability of commercial PSG2 antibody to recognize sulfated proteins in native samples has not been established yet.

Immobilized metal ion affinity chromatography (IMAC-Ga), one of the platforms used for enrichment of phosphorylated proteins, can be also applied to the analysis of sulfated tyrosine. Indeed, sulfo-peptides were enriched from a complex mixture of peptides extracted from skin secretions of *Pachymedusa dacnicolor* frog by using IMAC-Ga microcolumns [85]. The enriched fraction, analyzed by LC coupled to high resolution ESI-MS and MALDI-TOF/TOF, contained different sulfated and non-sulfated peptides belonging to the caerulin and bradykinin families. Phosphate adducts of dermaseptins and pyridoxal phosphate attached to a protease inhibitor were also characterized, together with some unexpected dermaseptin antimicrobial peptides. The study demonstrated that IMAC-Ga enrichment was a fast and useful method for high-throughput analysis of sulfated peptides [85].

Recently, weak anion exchange has been used for the enrichment of sulfopeptides that have been modified via carbamylation to convert all primary amines to less basic carbamates, thus enhancing the ability of carbamylated sulfopeptides to bind to the resin. The method was applied to a tryptic digest of bovine coagulation factor V, and electrospray ionization in the negative mode and ultraviolet photodissociation peptide sequencing allowed the identification of $Tyr_{1513}$ sulfation [86].

## 4 | REDOX MODIFICATIONS (SULFHYDRATION, NITROSYLATION, AND OTHER LESS COMMON S-CYSTEINE MODIFICATIONS)

Proteins are main targets of the action of free radicals such as reactive oxygen species (ROS) and reactive nitrogen species which induce their oxidation associated with numerous pathological conditions [87]. Redox proteomics, supported by the progress of MS, is the branch of proteomics aimed at detecting the oxidized proteins to investigate the proteome adaptation to mechanisms of redox unbalance and oxidative stress [87]. The amino acid residues most sensitive to oxidation are cysteine and methionine, but almost all the other protein amino acids are susceptible to this modification. Oxidation of several amino acid residues can result in the alteration of protein conformation and unfolding which stimulate turnover, toxic aggregation, and alteration of cell signaling which, in turn, can activate cell death pathways [88,89]. The three oxidation states of sulphur atom of the Cys thiol group, sulfenic (-SOH), sulfinic ($-SO_2H$), and sulfonic acid ($-SO_3H$), may change in response to numerous alterations in the redox cell state [90] strongly affecting the activity of modified proteins [91]. The identification of the specific oxidation status of cysteines is a big challenge due to the low amount of cysteine residues in the human proteome (~2.3% of all amino acids) and the frequent changes of the different redox oxidation states [92]. As we are aware, specific enrichment protocols to detect different oxidation states of sulphur amino acid have not been developed yet. However, several general redox proteomics methods have been improved, including gel-based and non gel-based approaches. Among the gel-based, the "diagonal" 2D-SDS PAGE [93] and redox DIGE [94] were developed to investigate the global disulfide proteome. The non-gel based proteomic approaches, mainly supported by MS, overcame some limitations of the gel-based methods and allowed to identify and quantify redox modulated proteins/peptides in complex biological samples. One method, named "oxICAT", combined the isotope-coded affinity tag (ICAT) analysis with a thiol trapping method in order to identify in four steps affected proteins by stress conditions and define their redox-sensitive cysteines. Proteins existing in either reduced or oxidized forms are incubated under denaturing conditions to expose all of their cysteine side chains. In the first step, isotopically light $^{12}$C-ICAT reagent was added to irreversibly modify all reduced cysteines. In the second step, all oxidized cysteines are reduced with tris-(2-carboxyethyl)-phosphine and subsequently modified with isotopically heavy $^{13}$C- ICAT reagent. In the third step, the protein mixture was digested and ICAT-labeled peptides wer purified by using a biotin-affinity tag. In the fourth step, quantitative MS of the protein mixture revealed the extent of thiol modification in any given peptide. Peptide sequence and identification of the modified cysteine was achieved by MS/MS. [95]. Another method, called oxidative multiple reaction monitoring (OxMRM), combines protein purification and differential alkylation with stable isotopes coupled to MRM identification of targeted cysteine residues, enabling analysis of sulfinic and sulfonic acid oxidation levels [96].

### 4.1 | S-sulfhydration

Hydrogen sulphide ($H_2S$), a further gas-transmitter in addition to NO and CO, generated in vivo by three different enzymes, i.e. cystathionine gamma-lyase, cystathionine beta synthase, and mercapto-sulfur transferase, modulates various biological processes and participates in multiple signaling

pathways involved in both health and disease conditions. $H_2S$, modifies proteins by yielding a hydropersulfide moiety (-SSH) through a process termed sulfhydration (or persulfhydration). Sulfhydration usually occurs at specific cysteine residues with a low pKa. Indeed, the cysteine thiolate anions at physiological conditions are more susceptible to the modification. Sulfhydration is recognized as a new oxidative PTM (oxPTM), included in a more general class of modification called RSS (reactive sulphur species) together with ROS and reactive nitrogen species [97].

Many proteins have been demonstrated to be the targets for S-sulfhydration such as Keap1, phospholamban, NF-κB, parkin, p66Shc [98]. Moreover, sulfhydration is one of the mechanisms through which $H_2S$ modulates several cellular physiologic pathways and aberrant alteration of its levels might be responsible for the appearance of pathological conditions [99], with a particular concern to neurological disorders [100,101]. Selective recognition of hydropersulfide is a main target for the detection of S-sulfhydration. The identifications of proteins containing persulfides remains a big challenge [98]. The main issue is due to distinguish the persulfide group from free thiols. Persulfides show a similar (or even better) reactivity than thiols to electrophilic substances and can participate to the formation of disulfide bonds [97]. Moreover, $H_2S$ can easily interact with sulfenic acid, generated by the oxidation ROS-mediated of cysteine, to form a persulfide bond.

Several methods have been developed to distinguish persulfide groups from free thiols. Biotin-switch assay is based on the use of the electrophilic reagent methyl methanethiosulfonate (MMTS), which is capable to react with the −SH group and to selectively remove thiols and enrich persulfides (S-SH) [102]. After removal of MMTS, persulfides were labelled with N-[6-(biotinamido) hexyl]-3′-(2′-pyridyldithio) propionamide (biotin-HPDP), then separated and eluted by streptavidin agarose beads for western blot (WB) analysis or MS. Many S-sulfhydrated proteins have been found by this approach [103]. Pan and Carroll evidenced a limitation of this method, because MMTS may not block and alkylate all free thiols resulting in a large number of false positives [104].

The cysteinyl labeling assay is based on the use of biotin-linked IAA (iodoacetamide) which reacts with both peptides and proteins containing persulfide (-SSH) and free thiols [105] subtracting them from the mixture. The subsequent use of dithiothreitol (DTT) reduced and detached only the -SSH peptides modified by IAA (and not -SH modified peptides), which were eluted from the column (or detached from magnetic beads) and they could be subsequently detected by WB or MS (Figure 4).

Using a similar scheme, Doka and colleagues developed a method called protein persulfide detection protocol (ProPerDP) [106]. In the first step, the alkylating agent EZ-Link Iodoacetyl-PEG2-Biotin (IAB) was used to alkylate both thiol and persulfide containing proteins, which were enriched using streptavidin-coated magnetic beads. Reduction by tris-(2-carboxyethyl)-phosphine or DTT released only the persulfide modified peptides (or proteins).

Sen et al. developed a similar method based on the use of Alexa Fluor 680 conjugated C2 maleimide (Red Maleimide). Red maleimide labeled selectively both sulfhydrated as well as un-sulfhydrated free cysteines. The samples were treated with DTT, which selectively cleaves disulfide bonds and so will detach the red fluorescent signal from sulfhydrated but not from unsulfhydrated protein, resulting in decreased fluorescence. The principle of this method is that the fluorescence signal decreases when the sample contains persulfides, hence the decreased ratio of the fluorescence signal, before and after adding DTT, may be a quantitative index of the presence of persulfides [107].

Taking advantage of the possibility to discriminate -SSR derivative of persulfide modified from the –SR modified peptides by a simple reduction step always, Gao and colleagues developed an interesting quantitative method using maleimide-PEG2-biotin (NM-Biotin) to alkylate both cysteine residues or sulfhydrated cysteine after trypsin digestion. An avidin column was used to capture the NM-biotinilated peptides [108]. The quantitative modifications generated by oxidative stress were established after reduction with DTT and a further reaction of the -SH group released from sulfidrated peptides after DTT reduction was carried out with heavy ($D_5$) (treated sample) and light ($H_5$) (control sample) N-ethyl-maleimide. LC-MS/MS determination of the H/L ratios of the individual pair-labeled cysteines in the identified peptides established that in pancreatic β cells exposed to endoplasmic reticulum stress, elevated $H_2S$ promotes the sulfhydration of enzymes involved in energy metabolism and stimulates glycolytic flux. This study showed that transcriptional and translational reprogramming by the integrated stress response in pancreatic β cells is coupled to metabolic alternations triggered by sulfhydration of key enzymes in intermediary metabolism.

An advantage of the previous described methods was that nitrosylated or oxidized cysteines did not interfere with the enrichment because they did not react either with IAA or with maleimide (Figure 4). Nonetheless, it is not possible to avoid interference by other disulfide.

The tag switch assay is based on the high nucleophilic reactivity of persulfide adducts compared to common disulfides in proteins. The scheme is based on the reaction of methylsulfonyl-benzothiazole with both free thiol and persulfide, which generated a high reactive persulfide adduct. Methyl-cyanoacetate was found as the high reactive and specific nucleophile able to react only with the persulfide adduct, and therefore biotinylated cyanoacetic acid was used to enrich only persulfide proteins which were subsequently characterized by various methods [109]. Finally, anti-SSH

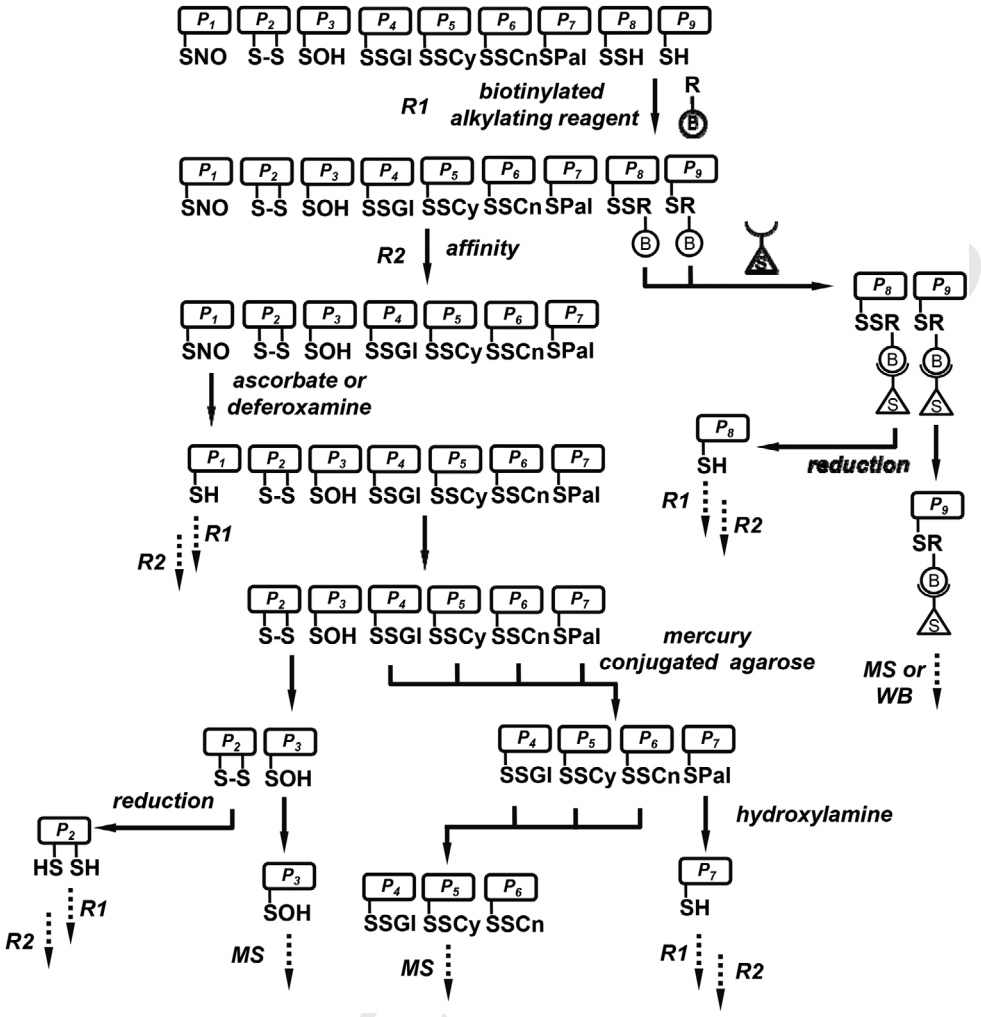



**FIGURE 4** Scheme summarizing the possible enrichments of different S-cysteine PTMs according to several pipelines reported in Sections 4.1, 4.2 and 4.3. In the first line (top) nine different peptides (or proteins) carrying varios S-cysteine PTMs are reported. (P)SH and (P)S-S: free thiol and disulfide; (P)SSH: persulfide; (P)SOH: sulfenic acid; (P)SNO: S-nitrosylated; (P)SSGl, (P)SSCy, (P)SSCn: S-gluthationylated, S-cysteinylated, S-cysteaminylated, respectively; (P)SPal: S-palmitoylated. The key reactions of the scheme are the reaction (***R1***) which consists in the attachment of a biotinylated alkylating reagent (**R-B**) to (P)SH (or (P)SSH) (i.e. maleimide-modified or iodoacetamide-modified biotin) followed by an affinity step (***R2***) with avidin (**S**) agarose or magnetic beads [105,106,108]. The peptides (or proteins) collected by the avidin affinity steps may be recognized by MS or WB. These two steps are at first responsible for the capture of (P)SH and (P)SSH from the other (P)-modified peptides or proteins. Because a reduction (i.e. DTT) can convert (P)SSR(B), but not (P)SR(B) in (P)SH, a new application of ***R1*** and ***R2*** is able to discriminate the (P)SSH modified peptides from (P)SH. Similarly, treatment with ascorbate or deferoxamine is able to transform (P)SNO in (P)SH [97]. An affinity chromatography with mercury-conjugated agarose is able to capture S-thiolated and S-acylated (S-palmitoylated) peptides [118,119]. (P)SPal can be trasformed in (P)SH by hydroxyamine [120–122]

cysteine antibodies have been designed and developed to enrich and investigate several specific proteins such as S-sulfhydrated GAPDH [97].

## 4.2 | S-nitrosylation

NO reacts with specific cysteine residues leading to S-nitrosylation. Protein S-nitrosylation showed features similar to protein S-sulfhydration, both occurring on cysteine residues. Like $H_2S$ for S-sulfhydration NO acts modulating the activities of target proteins involved in different molecular and cellular processes [110]. Different methods have been developed to study protein S-nitrosylation. In the method described by Ju and colleagues, the free thiol groups in proteins were first blocked with MMTS. SNO group was decomposed and reduced to free thiol with ascorbate or deferoxamine and labeled with a thiol specific biotinylating reagent [97] (Figure 4).

Total S-nitrosylated proteins could also be detected with anti-biotin antibody by immunoblotting or MS/MS analysis [97]. Indeed, anti-S-nitrosocysteine antibodies are commercially available for detection of protein

S-nitrosylation [97] and they have been used to detect S-nitrosylated proteins in serum of patients with multiple sclerosis, that were considered a potential predictive marker for demyelination [111].

### 4.3 | Further cysteine modifications

Due to their peculiar characteristics, cysteine residues are prone to other PTMs. For instance, free cysteines can react with glutathione, cysteamine, as well as with another cysteine residue to generate S-gluathionyl, S-cysteaminyl, and S-cysteinyl derivatives, respectively. These modifications are collectively termed S-thiolation and play interesting roles in signal transduction [112,113].

For instance, S-thiolation has been described for S100A9 and cystatin B playing a role in inflammation and development [114–116]. Cysteine can also undergo S-acylation reactions and post-translational modification consisting in the covalent attachment of an acyl chain to a cysteine residue of the target protein. Because palmitic acid is the most common reactant, this modification is also referred as S-palmitoylation. The lability of the resulting thioester bond gives to S-acylation the peculiar characteristic of reversibility. Therefore, S-acylation dynamically regulates different aspects in the life of a protein, including stability, localization, interactions, function and, thus, plays critical roles in cellular physiology. For long, both S-thiolation and S-acylation have been neglected and its potential as a regulatory mechanism for protein function underestimated [117].

Taking advantage of the strong interaction of the sulfur lone pairs with mercury, different research groups developed methods to enrich cysteine PTMs based on the use of organic mercury-conjugated agarose resin for affinity chromatography [118,119]. The method, initially designed for the enrichment of S-nitrosylated proteins, with minimal modifications was able to capture also S-glutathionylated and S-acylated proteins (Figure 4). To date, by this procedure, more than 2150 sites on 1446 proteins have been identified enabling a more comprehensive picture of the redox proteome landscape. Nonetheless, it was not established if this method can capture either S-cysteaminyl or S-cysteinyl modified proteins too.

Specific large-scale enrichment of S-palmitoylated proteins has been obtained utilizing a technique called acyl-biotin exchange. This method first requires to cap free cysteines with thiol-reactive reagents such as N-ethyl-maleimide. Palmitic acid linked by a thioester bond is subsequently removed from modified cysteines using hydroxylamine, leaving the previous S-palmitoylated cysteines available for reaction with a thiol-reactive biotin moiety for affinity enrichment followed by MS or WB identification (Figure 4). Acyl-biotin exchange protocols have been successfully used in yeast and mammalian systems, revealing numerous new S-palmitoylated proteins that were not predicted by existing algorithms, comprising immunity-associated proteins [120], various neuronal proteins [121], and histone H3 variants [122], contributing to the increase of the complexity of the histone code. Figure 4 shows a general scheme summarizing some protocols for the enrichment of the S-modifications described in the previous sections.

## 5 | METHYLATION AND ACETYLATION

### 5.1 | Methylation

Methylation is an important PTM commonly occurring in proteins and involved in the regulation of key cellular processes. This PTM, formerly described as a modification of the N-terminal tail of histones, was recognized lately on non-histone proteins. Alterations of methylation pathways deeply modify the interactome and can be implicated in various diseases comprised tumorigenesis processes [123,124]. Protein methylation was identified more than 50 years ago on both arginine [125] and lysine [126], even if it also occurs on methionine, cysteine, histidine, glutamine, asparagine, glutamic, and aspartic acids, although by far with lower frequency [127]. The regulation of biological process by protein methylation, mainly involving nuclear proteins implicated in gene transcription and nucleic acids metabolism, can be exerted by either direct or indirect action, the latter through the so called "readers", effector proteins which recognize methyl marks [127]. Multiple cofactors, intermediates, and enzymes, can strongly influence protein methylation. In fact, protein arginine methyltransferases and lysine methyltransferases use S-adenosyl-methionine as methyl donor of the guanidino nitrogens of arginine or of the ε-amino group of lysine. The complexity of protein methylation is enhanced by the fact that both methyl-arginine and methyl-lysine can occur in three distinct forms. Lysine can be methylated generating mono-, di-, or trimethyl forms. Arginine exists in mono-methyl, as well as in asymmetric or symmetric dimethyl forms, with dynamic interplay in cells [128]. Protein arginine methylation is an important regulator of protein function [129], signal transduction [130], cell cycle [131], and transcriptional control [132,133].

One of the reasons why the study of protein methylation has been delayed compared to other PTMs was the lack of robust and efficient enrichment strategies [134], due to the amino acid residues differently methylated that should be targeted by antibodies [127]. Methylation does not add significant steric bulk or change in the amino acid charge state. Therefore, enrichment of methyl peptides is more difficult than many other PTMs. Advances in methyl peptide enrichment through MS have been made using immunoaffinity enrichment. The challenge was the development of specific antibodies able

to discriminate the different methylation states of arginine and lysine residues. Immunoprecipitation by specifically designed pan-specific anti-lysine antibodies, was successfully employed to a first comprehensive analysis of lysine methylome in HeLa cells, disclosing 413 distinct human proteins in cell lysates from 493 different methylated peptides, 552 distinct lysine methylation sites, including 323, 127, and 102, mono-, di-, three-methylation sites, respectively [135]. Afterward, the protocol was further improved and applied to the analysis of protein lysine methylation increasing the number of methylation sites of non-histones protein up to 1500 in a single experiment, evidencing the prevalence of mono-methylated lysine [135]. Specific antibodies have been developed and validated for the selective enrichment of the diverse methyl forms both of arginine and lysine amino acid residues and applied for LC-MS characterization of methylome in human cells and mouse brain tissue [136]. The analysis characterized about 1000 mono-methylated and 300–400 di-methylated arginine sites in human HTC116 cells. Apart from histones, few further sites of lysine methylation were characterized. A new platform based on the specific chemical propionylation of mono-methylated lysine followed by immunoaffinity enrichment of the tryptic peptides using a pan anti-propionyl mono-methyl-lysine antibody has been recently applied to investigate the lysine mono-methylome of hepatocytes. LC-MS/MS analysis of the captured peptides, allowed 446 lysine mono-methylation sites in 398 proteins to be identified [137]. The combination of peptide fractionation prior to immuno-affinity enrichment with antibody against mono-methyl-arginine allowed increasing the identification of methylation sites in human embryonic kidney 293 cells up to 8000 in 3300 proteins [138].

Due to its intrinsic characteristics, heavy methyl stable isotope labelling by amino acid in cell culture (hmSILAC) is particularly suitable for the study of lysine and arginine methylation, and several enrichments of methylome were developed exploiting this platform. A pilot study applied hmSILAC in coupling with the use of antibodies targeted to methyl arginine residues for characterization and quantification of arginine methylated proteins by HPLC-MS, disclosing numerous protein methylation sites, many of them previously unknown [139]. Similarly, hmSILAC based strategy was able to discover about 400 non-histone distinct lysine and arginine sites on 139 unique proteins in HeLaS3 cells [140]. Taking into account the intrinsic high basicity and hydrophilicity of arginine methylated tryptic peptides, hm-SILAC platform was coupled to different enrichment protocols based on cation exchange chromatography, isoelectric focusing and HILIC, to investigate the T-cell methylome. The HILIC enrichment was the most effective and identified 249 arginine methylation sites in 131 proteins, including newly discovered methylated proteins involved in cytoskeleton rearrangement at the immunological synapse and in endosomal trafficking [141]. The use of multiple enzymes for protein digestion in coupling with cation exchange chromatography at high pH avoided the potential miscleavage of trypsin proteolysis, thus enlarging methyl peptides identifications by hm-SILAC amino acid labelling and LC-MS/MS analysis in HepG2 cells [142]. To overcome the interference by the co-enrichment of unmodified hydrophylic peptides and the poor cleavage by trypsin of methylated proteins, a new antibody free approach based on a novel de-glyco-assisted methylation site identification strategy was developed. The method involved hmSILAC in A549 cells, followed by cell lysates digestion with trypsin or LysargiNase. A subsequent PNGase F treatment, HILIC-tip enrichment and RP-tip fractionation prior to LC-MS/MS analysis allowed the identification of 573 methylation sites in 270 proteins, [143]. Recently, an hmSILAC coupled to high resolution MS proteomic platform was applied to characterize the methylome of *Escherichia coli*, *Saccharomyces cerevisiae,* and HeLa cells. In this study, out of the 234 methylated sites identified, 94 were newly characterized on nine different amino acids [144].

Within the diverse techniques for methylated peptides enrichment, the approaches to study methylome as product of methyl transferase enzymes activity or through the study of methyl transferases substrates are also enclosed. An example was the application of chemical methods of labelling, such as a chemical biology approach based on copper-catalyzed azide-alkyne cycloaddition chemistry [145]. As recently reviewed, bio-ortogonal chemistry approaches were also applied to allow in vivo methylated protein selective enrichment by the introduction of selective reactive group in the proteins, such as clickable S-adenosyl-methionine analogues [134]. More specifically for arginine methylation, the different pattern of fragment ions produced in MS/MS analysis can be used to distinguish the diverse peptides methylated forms carrying symmetric or asymmetric dimethylated arginine residues [134]. A recent review summarizes the diverse methodologies used for the characterization of the methylated arginine forms [146]. Enrichment of proteins modified by lysine methylation have been obtained utilizing specific domains able to recognize methyl-lysine residues, such as the triple malignant brain tumor domains of L3mbtl1 which bound mono- and di-methylated lysine [147] and the chromodomain of heterochromatin 1β which recognized several histone residues in a methylation dependent manner. Combining hmSILAC with enrichment by this chromodomain and multiple reaction monitoring MS it was possible to demonstrate that lysine methylation underwent widespread changes in response to DNA damage in HEK293T cells [148].

## 5.2 | Acetylation

Acetylation is a PTM that commonly affects Nα-terminal peptide residues (Nt-acetylation) or the ε-$NH_2$ of lysine (lysine

acetylation), and these two acetylation types are collectively defined as N-acetylation. Apart from the O-acetylation of sugars, protein O-acetylation can involve serine and threonine residues [149] but the role of this PTM has not been well recognized yet and, as far we are aware, the literature on the enrichment and proteomic investigation of acetylated proteins on these specific amino acids are sparse.

Nt-acetylation is one of the most frequent PTMs in eukaryotes and occurs in ≤80% of the cellular proteins [150]. Proteins are acetylated at their N-terminus by ribosome-associated N-terminal acetyltransferases in a co-translational event. This modification influences significantly protein global structure and, as a consequence, protein-protein interactions and subcellular targeting. Nt-acetylation influences a broad number of cellular mechanisms such as cell growth and proliferation, apoptosis, protein degradation, and transcriptional control [151], and it has been shown as it is correlated in several disorders [152].

Acetylation of the ε-amino group of lysine residues is included among the major regulatory dynamic PTMs and it is involved in a variety of physiological processes including gene expression (through actin remodeling), protein-protein interactions, protein subcellular localization, cell cycle, splicing, and nuclear transport. Recently, lysine acetylation has been found to enhance the binding of Atg3 protein to phosphatidylethanolamine-containing liposome, thereby promoting the lipidation process [153]. In cells, lysine residues are acetylated by histone/lysine acetyltransferases and deacetylated by histone/lysine deacetylases [154]. In the mitochondrial matrix, the acetylation can also occur by a nonenzymatic mechanism, through the attack of the lysine amino group on acetyl-CoA and acetyl phosphate esters [155].

Due to the low stoichiometry of peptide acetylation, antibody-based enrichment strategies were normally applied. To quantify the different acetylome between two cellular states, labeling strategies, such as stable isotope labeling (i.e. SILAC) [156,157] followed by antibody-based enrichment strategies and chemical labeling were utilized. The pipelines enable to determine the stoichiometry of acetylation at individual sites by measuring by MS analysis the abundance of the endogenously acetylated group (carrying a naturally abundant $^{12}C_2$-acetyl group) in comparison to a chemically introduced acetyl group (carrying an isotopically labeled $^{13}C_2$-acetyl group) [158]. For the identification of acetylation sites in tissues, Lundby and colleagues generated an organ-wide map of lysine acetylation sites from 16 rat tissues identifying 15 474 modification sites on 4541 proteins. These results were obtained by the combination of agarose-conjugated acetyl lysine antibody enrichment followed by label-free MS analysis using a top-10 higher-energy collisional dissociation data fragmentation method [159]. Schilling and colleagues implemented the tissue analysis by further optimizing the affinity enrichment (adding a small-scale acetyl-peptide desalting step with C18 Stage-Tips prior to LC-MS) in combination with label-free data-independent acquisition-MS [160]. This protocol could accommodate relatively low amounts of starting material and lead to the determination of protein acetylation regulation using a quantitative approach such as data-independent acquisition-$MS^2$ quantification.

Although these strategies were efficient for relative quantification studies across different conditions, they did not provide any information about the stoichiometry of individual acetylation sites within a single protein. Baeza and colleagues developed an experimental workflow for the direct quantification of single site-specific acetylation without a prior enrichment step [161]. This approach consisted in the protein denaturation (through cysteine reduction and alkylation) followed by chemical acetylation of lysine residues with acetic anhydride-$d_6$ (or acetic anhydride-$^{13}C_4$,$d_6$) and trypsin digestion. The reaction of the free lysine residues allowed to identify the endogenous acetylation sites with respect to the artificial sites and, therefore, to enable the calculation of the acetylation stoichiometry. Recently, N-acetoxy-succinimide *d3* (NAS-*d3*), for its higher stability and absence of side reactions, was used instead of acetic anhydride to label the untargeted lysine residues [162]. Furthermore, the filter aided sample preparation, in-gel sample preparation, and in-solution sample preparation have been tested for acetylated peptides analysis. So far, the in-solution sample preparation method exhibited the better recoveries, even though complementary sets of identified acetyl-lysine peptides with the three preparation methods were obtained.

Lysine acetylome enrichment enabled also the purification of Nt-acetyl lysine residues. However, due to the presence of many acetyl-lysine in multiple peptide sites, the sensitivity of this approach was lower for the examination of the Nt-acetylation alone. Therefore, other strategies were available for improving the enrichment of this PMT. One of the most recent strategies applied included the use of N-terminal COmbined FRActional DIagonal Chromatography (COFRADIC) [163]. The pipeline consisted in a derivatization of primary amines (i.e. by stable isotope labeling) before trypsin digestion, in order to distinguish the acetylation events (occurred in the sample) and to specifically cleave arginine residues. Later, strong cation exchange chromatography was used to remove most of the internal peptides. The resulting peptides could be directly quantified by mass spectrometry (Stable-Isotope Protein N-terminal Acetylation Quantification, SILProNAQ) [164] or further purified with COFRADIC. Another method utilizing a COFRADIC strategy was based on the treatment of the fractions obtained in a first HPLC separation with 2,4,6-trinitrobenzenesulfonic acid to chemically modify the α-amines of C-terminal and internal peptides. This treatment dramatically increased the hydrophobicity of the reactive peptides. The second RP-HPLC fractionation allowed to separate the 2,4,6-trinitrobenzenesulfonic

acid-modified from the comparatively less hydrophobic acetylated N-termini, which were forwarded toMS/MS analysis [165].

## 6 | UBIQUITINATION (AND UBIQUITIN-LIKE MODIFICATIONS)

It is well known that modification of proteins by ubiquitin (Ub) is one of the most stimulating and challenging field of biochemistry of the cell to date [166,167]. Ubiquitination (or ubiquitylation) consists in a complex enzymatic conjugation process that culminates in the formation of an isopeptide bond between the C-terminal glycine ($Gly_{76}$) of Ub and the amino group of a lysine residue of the substrate protein. Ub itself presents in its structure a total of seven lysine residues ($K_6$, $K_{11}$, $K_{27}$, $K_{29}$, $K_{33}$, $K_{48}$, $K_{63}$). Thus, the C-terminus of another Ub can be ligated to the N-terminus ($M_1$) or to a ε-NH2 of the seven lysine residues of the previously ligated Ub, generating poly-Ub chains. If the subsequent ubiquitination event occurs at the N-terminus of the first Ub, a linear poly-Ub is generated. Otherwise, the conjugation to one of the lysine residues in the Ub sequence brings to various and complex branched poly-Ub chains. Similar to a bar code, the various Ub modifications adopt distinct conformations and lead to different outcomes of the ubiquitinated proteins in cells. Only poly-ubiquitination on defined lysine residues, mostly $K_{48}$ and $K_{29}$, leads to proteasome degradation of the target protein (called "molecular kiss of death") [168]. Other poly-ubiquitination (e.g. on $K_6$, $K_{11}$, $K_{63}$, and $M_1$) and mono-ubiquitination modifications may alter cellular location of the target protein, influence its activity, promote or prevent interactions with other molecular partners, affect endocytic trafficking, inflammation, translation and DNA repair. Ubiquitination is a reversible process and the ubiquitin scaffold can be demolished by steps or completely by a family of de-ubiquitinating enzymes. The Ub code is further complicated by the large and growing family of Ub-like peptides. The family includes the small Ub-like modifier (SUMO, four different paralogues in humans), the Ub cross-reactive protein (UCRP, also known as interferon-stimulated gene-15 ISG15), the Ub-related modifier-1 (URM1), and the neuronal-precursor-cell-expressed developmentally downregulated protein-8 (NEDD8), to cite a few [169]. Even though several mixed linear and branched poly-Ub chains have been reported, one major difference between modification by Ub and its relatives is that they commonly do not form polymeric structures, with the exception of NEDD8 and SUMO, with poly-SUMO chains playing a secondary role in overall SUMO signaling [170,171]. Development of capturing platforms for Ub and Ub-like modified proteins is really demanding, however the complexity of the modifications makes any improvement a challenging task. The first approaches to enrich Ub modified proteins were based on immuno-affinity chromatography [172]. By this platform, Matsumoto and colleagues were able to identify 670 distinct proteins [173]. MS analysis was able to evidence ubiquitination of many ribosomal subunits, suggesting that ubiquitination might play an important role in the regulation and/or quality control of ribosomal proteins.

The use of Ub-specific antibodies has been hindered by its low affinity and high background. The development of N-terminally epitope-tagged Ub as a probe was quickly identified as an interesting platform for ubiquitination studies. Tagged Ub could be performed under strongly denaturing conditions and allowed for reliable and extensive purifications [174]. Cells transformed with tagged Ub formed the basis for several studies in different organisms [175]. For instance, the four copies of the Ub gene were replaced by an Ub gene carrying a 6-His tag at the N-terminus. $Ni^{2+}$-chelate chromatography was able to recognize selectively the His-tag present on the Ub and to enrich the ubiquitinated proteome, which was subsequently digested by trypsin allowing the detection of 110 ubiquitination sites present in 72 ubiquitin-protein conjugates. Tandem affinity strategies based on histidine-biotin (HB) tag were utilized for two-step purification under denaturing conditions. The HB tag consisted of a 6-His tag and a bacterially derived in vivo biotinylation signal peptide that induced biotin attachment to the HB tag in yeast and mammalian cells. HB-tagged proteins could be sequentially purified under fully denaturing conditions, such as 8 M urea, by $Ni^{2+}$ chelate chromatography and binding to streptavidin resins. These tandem ubiquitin-binding entities were employed to study ubiquitination because greatly reduced nonspecific background increased up to thousand times the affinity for poly-Ub chains. Moreover, the denaturing conditions inhibit hydrolase activity and protect ubiquitinated proteins from the action of the proteasome and de-ubiquitinating enzyme enzymes [176,177]. Various alternative tags have been developed and reviewed [178]. The C-terminus of Ub ends in the amino acid sequence -RGG. Therefore, treatment with trypsin leaves a glycine dipeptide as a characteristic signal on the side-chain of modified lysines. Taking advantage of this characteristic, immunoaffinity reagents have been developed capable of capturing K-GG peptides from Ub and its thousands of cellular substrates [179]. This platform led to the detection of about 19 000 ubiquitination sites in about 5000 proteins in HCT116 cells, evidencing that this PTM competes with phosphorylation for a number of substrates. The homology of the C-terminus of Ub and Ub-like peptides can lead to ambiguous identifications. Indeed, the tryptic digestion of Nedd8 and ISG15 modified substrates generated a glycine dipeptide on the lysine acceptor site, making them indistinguishable from ubiquitination by standard methods [180]. On the basis of experiments carried out utilizing ubiquitin specific protease 2 and inhibition of NEDD8-activating enzyme

dependent neddylation, it was possible to conclude that more than 94% of the identified sites presented conjugation to Ub, as opposed to NEDD8 or ISG15. The authors concluded that this was likely an underestimation, given that under conditions of proteasome inhibition and Ub depletion NEDD8 could be utilized by the Ub machinery and was likely transferred to canonical ubiquitination targets [180]. A similar study was able to detect 1786 K-GG sites in 921 synaptic proteins of rat brain demonstrating that a large number of neuronal proteins were modified by ubiquitination. In this study it was estimated that interference by NEDD8 and ISG15 was less than 2% [181].

Even though less extensively than those for Ub, several studies were carried out to capture SUMOylated proteins. In mammals, there are four reported SUMO proteoforms named SUMO-1/-4 [182]. SUMO-1 is the most prominently conjugated isoform under normal conditions (50%). The conjugated forms of SUMO-2 and SUMO-3 only differ in three N-terminal residues and for this high similarity sometimes they are reported as SUMO-2/3. Although virtually all of the SUMO-1 is engaged in conjugations, SUMO-2/3 appea to be preferentially conjugated to proteins under stress conditions [183]. SUMO-2/3 has a conserved acceptor lysine and has the capability to form polySUMO chains, while SUMO-1 lacks this consensus and is often used as a poly-SUMO chain terminator [184]. SUMO-4 remains enigmatic as it has a restricted tissue distribution and appears to be insensitive to SUMO-specific proteases because $Pro_{90}$ prevents its conjugation [185]. In similarity with Ub, four different strategies were used to enrich SUMO conjugates. They included single or double tagged versions of SUMO, anti-SUMO antibodies, and SUMO traps. The tagged forms of SUMO were largely utilized, and various epitope tags in combination with affinity matrices and/or tag antibodies allowed for the capture of SUMOylated proteins in an unbiased manner. As for ubiquitin, the His-tags and biotin tags enabled the enrichment of SUMOylated proteins under fully denaturing conditions, with the contemporaneous inactivation of SUMO-specific proteases [186]. Alternatively, HA, protein-A and Myc-tagged versions of SUMO may be utilized for a large identification of SUMO conjugates [187,188]. SUMOylated proteins can be also directly purified by immunoprecipitation with antibodies directed against SUMO, under native conditions, which allow for the capture of poly-SUMOylated proteins [189]. The different platforms utilized for SUMOylation enrichment have been nicely described in a recent review and the reader can refer to it for more detailed information [190].

## 7 | MISCELLANEOUS PTMs

In the previous section, lysine appeared as an amino acid residue largely prone to PTMs. Recent studies have surprisingly demonstrated the existence of newly discovered lysine acylations including propionylation [191], malonylation [192], succinylation [193,194], and glutarylation [195], emphasizing the influence of post-translational lysine acylations on various cellular functions. Among them, lysine succinylation (K-suc) [196] deserves a special attention. K-suc transfers a structural moiety larger than that occurring in lysine methylation or acetylation. This can change the charge status (+1 to −1) at physiological pH levels [196]. Hence, K-suc may lead to profound changes in protein structure regulating its function in various types of cells such as mouse embryonic fibroblasts [197] and HeLa cells [198]. Over the past few years, lysine succinylation has also been shown to be a relevant PTM in diverse microrganisms such as *Escherichia coli* [199], *Bacillus subtilis* [200], *Mycobacterium tuberculosis* [193,194], *Vibrio parahaemolyticus* [201], and *Toxoplasma gondii* [202].

Immune-affinity enrichment using an anti-succinyl-lysine antibody is the elective method to enrich K-suc peptides for mass spectrometry investigations. In a recent study, this approach was used to provide a comprehensive analysis of the succinylome of *Histoplasma capsulatum*, the causative agent of lung disease histoplasmosis, and successfully identify 463 unique lysine succinylation sites corresponding to 202 succinylated proteins with diverse biological functions and cellular localizations [203]. Recently, a similar approach was used to map succinylation sites in five bacterial species providing insights on common regulation mechanisms utilizing this PTM [204]. Also in the gram-negative anaerobe *Porphyromonas gingivalis* the analysis of K-suc suggested that this modification may be relevant in modulating the virulence, adaptation, and fitness of the pathogen with new molecular mechanisms [205].

Another widespread PTM with crucial functions in many cellular processes is ADP-ribosylation. Protein ADP-ribosylation occurs when members of ADP-ribosyltransferases (or PARPs) covalently link the ADP-ribose moieties, derived from $NAD^+$, to the side chains of several putative amino acid residues (glutamate, aspartate, arginine, lysine, cysteine, serine) on target proteins [206,207]. This PTM can generate either a monomer (mono-ADP-ribosylation), where a single ADP-ribose moiety is linked to the target residue, or a polymer (poly-ADP-ribosylation) in which a branched polymeric chain of ADP-ribose units originates from the target residue, disclosing the existence of an ADP-ribosylation code.

Therefore, PARPs are a group of nuclear proteins with DNA-dependent mono (PARP-3) or poly (PARPs 1 and 2) ADP-ribosyl transferase activities involved in DNA repair, chromosome maintenance, chromatin regulation, and gene expression [206,207].

The proteome-wide investigation of ADP-ribosylation functions has been limited due to numerous technical

challenges including the complexity of the poly(ADP-ribose) chains, low abundance of the modification, and lack of sensitive enrichment methods. Various studies using immune-based enrichment, different affinity resins, and protein microarrays were carried out to identify the targets of ADP-ribosylation lacked specificity for individual PARP family members [208].

In particular, MS-based proteomics studies of ADP-ribosylation have been limited due to the numerous technical challenges. These include low specificity and/or affinity when using recombinant macro domains [209], *cis*-diol binding boronate beads [210], or enzymatic strategies [211].

Gibson and colleagues [212] reported a robust $NAD^+$ analog-sensitive approach for PARPs, which allowed PARP-specific ADP-ribosylation of substrates suitable for subsequent copper-catalyzed azide-alkyne cycloaddition reactions. Using this approach, the authors mapped hundreds sites of ADP-ribosylation for PARPs 1, 2, and 3 in HeLa cells. A recent work analyzed the labelling efficiency of 2-alkyne adenosine (2YnAd), using tandem mass tag (TMT)-based quantitative proteomics in mammalian cells allowing a more comprehensive assessment of the putative ADP-ribosylome [213]. A recent study obtained a comprehensive profile of the human ADP-ribosylome in HeLa cells (CCL-2). The samples were digested with either Lys-C or trypsin before enrichment of ADP-ribosylated peptides by using the macrodomain Af1521 and the potential of electron transfer dissociation (ETD) fragmentation for localizing the ADP-ribose to the correct amino acid residue [214].

N-myristoylation corresponds to the covalent binding of the saturated fatty acid myristate to the N-terminal glycine of specific protein substrates catalyzed by *N*-myristoyltransferase. This modification plays an important role in protein regulation by controlling localization, stability, and interactions of the protein target [215]. Recently, Goya Grocin and colleagues [216] were able to develop a method for whole-proteome profiling of free N-terminal glycines through labeling with *Staphylococcus aureus* sortase A (SrtA) and used it for assessment of target engagement by an *N*-myristoyltransferase inhibitor. Analysis of the SrtA-labeling pattern with an engineered biotinylated depsipeptide SrtA substrate enabled whole proteome identification and quantification of de novo generated N-terminal Gly proteins in response to *N*-myristoyltransferase inhibition by nano-LC-MS/MS proteomics. The modification was confirmed for specific substrates across multiple cell lines by gel-based analyses and ELISA. To achieve optimal signal over background noise, a novel and generally applicable improvement to the biotin/avidin affinity enrichment step by chemically dimethylating commercial NeutrAvidin resin was carried out. The combination of this modification with two-step LysC on-bead and trypsin off-bead digestion, effectively eliminated avidin-derived tryptic peptides and enhanced identification of enriched myristoylated peptides [216].

## 8 | CONCLUDING REMARKS

Purpose of this review was to give an overview of the general aspects of PTMs enrichment in proteomic studies, evidencing some selected researches. It highlighted, when possible, the most salient aspects addressed towards the comprehension of the molecular events connected to a particular modification, in order to underline how and why specific enrichment protocols have been developed. In the effort to elicit all the proteins involved in a specific PTM, enrichment protocols have been often formulated in order to reach the highest selectivity. Nonetheless, enrichment platforms able to capture simultaneously more than one PTM are demanding to better understand the cross-talk or the relationship between different molecular codes. In this respect, some amino acid residues, such as lysine and cysteine, are prone to different PTMs and are therefore at the center of a complex network responsible for the regulation of the most important molecular pathways. It is thereby noteworthy to conceive the enrichment platforms as a relevant tool to investigate the proteomic signatures governing cell cycle and physiological status as well as cellular stress and/or pathological conditions. It is indeed evident along this review that almost all the PTMs reported, such as ubiquitination and ubiquitin-like modification, methylation and acetylation, ADP-ribosylation, S-sulphydration and S-thiolation utilize precise and distinct molecular conformation resembling a complex code, able to address the cellular machinery towards different and defined outcomes. It is therefore exciting to verify the connections existing among different PTMs, as recently reported, for instance, between the N-terminal acetylation of the lipid droplet protein PLIN2 and its ubiquitin mediated degradation pathway [217]. This and many other examples indeed show how complex can be the interplay between different concomitant PTMs and the cross-talk between different molecular pathways in the government of the cellular life. Enrichment proteomic strategies could surely help in the demanding and challenging issue to decipher these complex molecular languages, but they require the development of new robust methods, new high performance instruments and especially of new expert specific software able to help the researchers in the management of the huge amount of data.

## ACKNOWLEDGMENTS

The authors acknowledge the financial support of the IRCCS-Fondazione Santa Lucia, (5 × 1000, 2017 from Ministero della Salute), of the Cagliari University [PRID 2015], of the Catholic University of Rome [D1-2016 R4124500372], of

the MIUR [60% 2016], of the Regione Autonoma Sardegna [TENDERG25I-140000-70002] and of the Italian Consiglio Nazionale delle Ricerche.

## CONFLICT OF INTEREST

The authors have declared no conflict of interest.

## ORCID

*Massimo Castagnola*
https://orcid.org/0000-0002-0959-7259

## REFERENCES

1. Giardina, B., Messana, I., Scatena, R., Castagnola, M., The multiple function of hemoglobin. *Crit. Rev. Biochem. Mol. Biol.* 1995, *30*, 165–196.
2. Iavarone, F., Desiderio, C., Vitali, A., Messana, I., Martelli, C., Castagnola, M., Cabras, T., Cryptides: latent peptides everywhere. *Crit. Rev. Biochem. Mol. Biol.* 2018, *53*, 246–263.
3. Messana, I., Cabras, T., Iavarone, F., Vincenzoni, F., Urbani, A., Castagnola M., Unraveling the different proteomic platforms. *J. Sep. Sci.* 2013, *36*, 128–139.
4. De Rosa, M. C., Sanna, M. T., Messana, I., Castagnola, M., Galtieri, A., Tellone, E., Scatena, R., Botta, B., Botta, M., Giardina, B., Glycated human hemoglobin ($HbA_{1c}$): functional characteristics and molecular modeling studies. *Biophys. Chem.*, 1998, *72*, 323–335.
5. McDonald, M. J., Shapiro, R., Bleichman, M., Solway, J., Bunn, H. F., Glycosylated minor components of human adult hemoglobin. Purification, identification, and partial structural analysis. *J. Biol. Chem.* 1978, *253*, 2327–2332.
6. Castagnola, M., Caradonna, P., Salvi, M. L., Rossetti, D. V., Investigation of the heterogeneity of hemoglobin by cation exchange chromatography on Bio-Rex 70. *J. Chromatogr.* 1983, *272*, 51–65.
7. D'Aronco, S., Crotti, S., Agostini, M., Traldi, P., Chilelli, N. C., Lapolla, A., The role of mass spectrometry in studies of glycation processes and diabetes management. *Mass Spectrom. Rev.* 2019, *38*, 112–146.
8. Thornalley, P. J., Dicarbonyl intermediates in the Maillard reaction. *Ann. N.Y. Acad. Sci.* 2005, *1043*, 111–117.
9. Shapiro, R., McManus, M. J., Zalut, C., Bunn, H. F., Sites of nonenzymatic glycosylation of human hemoglobin A. *J. Biol. Chem.* 1980, *255*, 3120–3127.
10. Beltran Del Rio, M., Tiwari, M., Amodu, L. I., Cagliani, J., Rodriguez Lido, H. L., Glycated hemoglobin, plasma glucose, and erythrocyte aging. *J. Diabetes Sci. Technol.* 2016, *10*, 1303–1307.
11. Campbell, L., Pepper, T., Shipman, K., HbA1c: a review of non-glycaemic variables. *J. Clin. Pathol.* 2019, *72*, 12–19.
12. Ding, L., Xu, Y., Liu, S., Bi, Y., Xu, Y., Hemoglobin A1c and diagnosis of diabetes. *J. Diabetes* 2018, *10*, 365–372.
13. Ribeiro, R. T., Macado, M. P., Raposo, J. F., HbA1c, fructosamine, and glycated albumin in the detection of dysglycaemic conditions. *Curr. Diabetes Rev.* 2016, *12*, 14–19.
14. Gould, B. J., Hall, P. M., m-Aminophenylboronate affinity ligands distinguish between nonenzymically glycosylated proteins and glycoproteins. *Clin. Chim. Acta* 1987, *163*, 225–230.
15. Zhang, Q., Tang, N., Brock, J. W. C., Mottaz, H. M., Ames, J. M., Baynes, J. W., Smith, R. D., Metz, T. O., Enrichment and analysis of non-enzymatically glycated peptides: boronate affinity chromatography coupled with electron transfer dissociation mass spectrometry. *J. Proteome Res.* 2007, *6*, 2323–2330.
16. Zhang, Q., Monroe, M. E., Schepmoes, A. A., Clauss, T. R., Gritsenko, M. A., Meng, D., Petyuk, V. A., Smith, R. D., Metz, T. O., Comprehensive identification of glycated peptides and their glycation motifs in plasma and erythrocytes of control and diabetic subjects. *J. Proteome Res.* 2011, *10*, 3076–3088.
17. Frolov, A., Hoffmann, R., Analysis of amadori peptides enriched by boronic acid affinity chromatography. *Ann. N. Y. Acad. Sci.* 2008, *1126*, 253–256.
18. Muralidharan, M., Bhat, V., Bind glycemic index in diabetes mellitus. *Anal. Biochem.* 2019, *573*, 37–43.
19. MacLean, M., Derk, J., Ruiz, H. H., Juranek, J. K., Ramasamy, R., Schmidt, A. M., The receptor for advanced glycation end products (RAGE) and DIAPH1: Implications for vascular and neuroinflammatory dysfunction in disorders of the central nervous system. *Neurochem. Int.* 2019, *126*, 154–164.
20. Degani, G., Altomare, A. A., Colzani, M., Martino, C., Mazzolari, A., Fritz, G., Vistoli, G., Popolo, L., Aldini, G. A., Capture method based on the VC1 domain reveals new binding properties of the human receptor for advanced glycation end products (RAGE). *Redox Biol*. 2017, *11*, 275–285.
21. Mitsuhashi, T., Li, Y. M., Fishbane, S., Vlassara, H., Depletion of reactive advanced glycation endproducts from diabetic uremic sera using a lysozyme-linked matrix. *J. Clin. Invest.* 1997, *100*, 847–854.
22. Prasanna, R. R., Venkatraman, K., Vijayalakshmi, M. A., Pseudoaffinity chromatography enrichment of glycated peptides for monitoring advanced glycation end products (AGEs) in metabolic disorders. *J. Proteins Proteom.* 2016, *7*, 167–176.
23. Chen, W. X., Smeekens, J. M., Wu, R. H., Comprehensive analysis of protein *N*-glycosylation sites by combining chemical deglycosylation with LC-MS. *J. Proteome Res*. 2014, *13*, 1466–1473.
24. Yu, A., Zhao, J., Peng, W., Banazadeh, A., Williamson, S. D., Goli, M., Huang, Y., Mechref, Y., Advances in mass spectrometry-based glycoproteomics. *Electrophoresis* 2018, *39*, 3104–3122.
25. Xiao, H., Sun, F., Suttapitugsakul, S., Wu, R., Global and site-specific analysis of protein glycosylation in complex biological systems with mass spectrometry. *Mass Spectrom. Rev.* 2019, *38*, 356–379..
26. Helenius, A., Aebi, M., Intracellular functions of N-linked glycans. *Science* 2001, *291*, 2364–2369.
27. Xu, C., Nq, D. T., Glycosylation-directed quality control of protein folding. *Nat. Rev. Mol. Cell Biol.* 2015, *16*, 742–752.
28. Yang, Y., Franc, V., Heck, A. J. R., Glycoproteomic: a balance between high throughput and in depth analysis. *Trends Biotechnol.* 2017, *35*, 598–609.
29. Franc, V., Yang, Y., Heck, A. J., Proteoform profile mapping of the human serum complement component C9 revealing unexpected new features of N-, O-, and C-glycosylation. *Anal. Chem.* 2017, *89*, 3483–3491.
30. Morelle, W., Canis, K., Chirat, F., Faid, V., Michalski, J. C., The use of mass spectrometry for the proteomic analysis of glycosylation. *Proteomics* 2006, *6*, 3993–4015.

31. Niwa, Y., Nakano, Y., Suzuki, T., Yamagishi, M., Otani, K., Dohmae, N., Simizu, S., Topological analysis of DPY19L3, a human C-mannosyltransferase. *FEBS J.* 2018, *285*, 1162–1174.
32. Robertson, L. A., Moya, K. L., Breen, K. C., The potential role of tau protein O-glycosylation in Alzheimer's disease. *J. Alzheimers Dis.* 2004, *6*, 489–495.
33. Pinho, S. S., Reis, C. A., Glycosylation in cancer: mechanisms and clinical implications. *Nat. Rev. Cancer.* 2015, *15*, 540–555.
34. Yang, Y., Franc, V., Heck, A. J. R., Glycoproteomics: a balance between high-throughput and in-depth analysis. *Trends Biotechnol*. 2017, *35*, 598–609.
35. Cao, L., Qu, Y., Zhang, Z, Wang, Z, Prytkova, I., Wu, S., Intact glycopeptide characterization using mass spectrometry. *Expert Rev. Proteomics* 2016, *13*, 513–522.
36. Jiang, B., Huang, J., Yu, Z., Wu, M., Liu, M., Yao, J., Zhao, H., Yan, G., Ying, W., Cao, W., Yang, P., A multi-parallel N-glycopeptide enrichment strategy for high-throughput and in-depth mapping of the N-glycoproteome in metastatic human hepatocellular carcinoma cell lines. *Talanta* 2019, *199*, 254–261.
37. Ahn, Y. H., Kim, J. Y., Yoo, J. S., Quantitative mass spectrometric analysis of glycoproteins combined with enrichment methods. *Mass Spec. Rev.* 2015, *34*, 148–165.
38. Cabras, T., Boi, R., Pisano, E., Iavarone, F., Fanali, C., Nemolato, S., Faa, G., Castagnola, M, Messana, I., HPLC-ESI-MS and MS/MS structural characterization of multifucosylated N-glycoforms of the basic proline-rich protein IB-8a CON1$^{(+)}$ in human saliva. *J. Sep. Sci.* 2012, *35*, 1079–1086.
39. Manconi, B., Cabras, T., Sanna, M., Piras, V., Liori, B., Pisano, E., Iavarone, F., Vincenzoni, F., Cordaro, M., Faa, G., Castagnola, M., Messana, I., N- and O-linked glycosylation site profiling of the human basic salivary proline-rich protein 3M. *J. Sep. Sci.* 2016, *39*, 1987–1997.
40. Dai, Z., Zhou, J., Qiu, S. J., Liu, Y. K., Fan, J., Lectin-based glycoproteomics to explore and analyze hepatocellular carcinoma-related glycoprotein markers. *Electrophoresis* 2009, *30*, 2957–2966.
41. Heo, S. H., Lee, S. J., Ryoo, H. M., Park, J. Y., Cho, J. Y., Identification of putative serum glycoprotein biomarkers for human lung adenocarcinoma by multilectin affinity chromatography and LC-MS/MS. *Proteomics* 2007, *7*, 4292–4302.
42. Zeng, Z., Hincapie, M., Pitteri, S. J., Hanash, S., Schalkwijk, J., Hogan, J. M., Wang, H., Hancock, W. S. A., Proteomics platform combining depletion, multi-lectin affinity chromatography (M-LAC), and isoelectric focusing to study the breast cancer proteome. *Anal. Chem.* 2011, *83*, 4845–4854.
43. Ahn, Y. H., Ji, E. S., Shin, P. M., Kim, K. H., Kim, Y. S., Ko, J. H., Yoo, J. S., A multiplex lectin-channel monitoring method for human serum glycoproteins by quantitative mass spectrometry. *Analyst* 2012, *137*, 691–703.
44. Zhang, H., Li, X. J., Martin, D. B., Aebersold, R., Identification and quantification of N-linked glycoproteins using hydrazide chemistry, stable isotope labeling and mass spectrometry. *Nat. Biotechnol.* 2003, *21*, 660–666.
45. Berven, F. S., Ahmad, R., Clauser, K. R., Carr, S. A., Optimizing performance of glycopeptide capture for plasma proteomics. *J. Proteome Res.* 2010, *9*, 1706–1715.
46. Nilsson, J., Rüetschi, U., Halim, A., Hesse, C., Carlsohn, E., Brinkmalm, G., Larson, G., Enrichment of glycopeptides for glycan structure and attachment site identification. *Nat. Methods* 2009, *6*, 809–811.
47. Klement, E., Lipinszki, Z., Kupihár, Z., Udvardy, A., Medzihradszky, K. F., Enrichment of O-GlcNAc modified proteins by the periodate oxidation-hydrazide resin capture approach. *J. Proteome Res.* 2010, *9*, 2200–2206.
48. Bai, H., Pan, Y., Qi, L., Liu, L., Zhao, X., Dong, H., Cheng, X., Qin, W., Wang, X., Development a hydrazide-functionalized thermosensitive polymer based homogeneous system for highly efficient *N*-glycoprotein/glycopeptide enrichment from human plasma exosome. *Talanta* 2018, *186*, 513–520.
49. Lee, J. H., Kim, Y., Ha, M. Y., Lee, E. K., Choo, J., Immobilization of aminophenylboronic acid on magnetic beads for the direct determination of glycoproteins by matrix assisted laser desorption ionization mass spectrometry. *J. Am. Soc. Mass Spectrom.* 2005, *16*, 1456–1460.
50. Chen, M., Lu, Y., Ma, Q., Guo, L., Feng, Y. Q., Boronate affinity monolith for highly selective enrichment of glycopeptides and glycoproteins. *Analyst* 2009, *134*. 2158–2164.
51. Li, D., Xia, H., Wang, L., Branched polyethyleneimine-assisted boronic acid-functionalized silica nanoparticles for the selective enrichment of trace glycoproteins. *Talanta* 2018, *184*, 235–243.
52. Wang, H. Y., Bie, Z. j., Lu, C. C., Liu, Z., Magnetic nanoparticles with dendrimer-assisted boronate avidity for the selective enrichment of trace glycoproteins. *Chem. Sci.* 2013, *4*, 4298–4304.
53. Madariaga, D., Martinez-Saez, N., Somovilla, V. J., Garcia-Garcia, L., Berbis, M. A., Valero-Gonzalez, J., Martin-Santamaria, S., Hurtado-Guerrero, R., Asensio, J. L., Jimenez-Barbero, J., Avenoza, A., Busto, J. H., Corzana, F., Peregrina, J. M., Serine versus threonine glycosylation with alpha-O-GalNAc: unexpected selectivity in their molecular recognition with lectins. *Chemistry* 2014, *20*, 12616–12627.
54. Darula, Z., Sherman, J., Medzihradszky, K. F., How to dig deeper? Improved enrichment methods for mucin core-1 type glycopeptides. *Mol. Cell. Proteomics* 2012, *11*, O111.016774.
55. Shen, B., Zhang, W., Shi, Z., Tian, F., Deng, Y., Sun, C., Wang, G., Qin, W., Qian, X., A novel strategy for global mapping of OGlcNAc proteins and peptides using selective enzymatic deglycosylation, HILIC enrichment and mass spectrometry identification. *Talanta* 2017, *169*, 195–202.
56. Steentoft, C., Vakhrushev, S. Y., Joshi, H. J., Kong, Y., Vester-Christensen, M. B., Schjoldager, K. T., Lavrsen, K., Dabelsteen, S., Pedersen, N. B., Marcos-Silva, L., Gupta, R., Bennett, E. P., Mandel, U., Brunak, S., Wandall, H. H., Levery, S. B., Clausen, H., Precision mapping of the human O-GalNAc glycoproteome through simple cell technology. *EMBO J.* 2013, *32*, 1478–1488.
57. Xiao, H., Chen, W., Smeekens, J. M., Wu, R., An enrichment method based on synergistic and reversible covalent interactions for large-scale analysis of glycoproteins. *Nat. Commun.* 2018, *9*, 1692.
58. Yang, S., Onigman, P., Wu, W. W., Sjogren, J., Nyhlen, H., Shen, R. F., Cipollo, J., Deciphering protein O-glycosylation: solid-phase chemoenzymatic cleavage and enrichment. *Anal. Chem.* 2018, *90*, 8261–8269.
59. Zhang, Y., Jing, H., Wen, T., Wang, Y., Zhao, Y., Wang, X., Qian, X., Ying, W., Phenylboronic acid functionalized $C_3N_4$ facultative hydrophilic materials for enhanced enrichment of glycopeptides. *Talanta* 2019, *191*, 509–518.

60. Humphrey, S. J., James, D. E., Mann, M., Protein phosphorylation: a major switch mechanism for metabolic regulation. *Trends Endocrinol. Metab.* 2015, *26*, 676–687.
61. Cieśla, J., Frączyk, T., Rode, W., Phosphorylation of basic amino acid residues in proteins: important but easily missed. *Acta Biochim. Pol.* 2011, *58*, 137–148.
62. Arrington, J. V., Hsu, C. C., Elder, S. G., Andy Tao, W., Recent advances in phosphoproteomics and application to neurological diseases. *Analyst* 2017, *142*, 4373–4387.
63. Leitner, A., Enrichment strategies in phosphoproteomics. *Methods Mol. Biol.* 2016, *1355*, 105–121.
64. Messana, I., Inzitari, R., Fanali, C., Cabras, T., Castagnola, M., Facts and artifacts in proteomics of body fluids. What proteomics of saliva is telling us? *J. Sep. Sci.* 2008, *31*, 1948–1963.
65. Cabras, T., Fanali, C., Monteiro, J. A., Amado, F., Inzitari, R., Desiderio, C., Giardina, B., Castagnola, M., Messana, I., Tyrosine polysulfation of human salivary histatin 1. A post-translational modification specific of the submandibular gland. *J. Proteome Res.* 2007, *6*, 2472–2480.
66. Larsen, M. R., Thingholm, T. E., Jensen, O. N., Roepstorff, P., Jorgensen, T. J., Highly selective enrichment of phosphorylated peptides from peptide mixtures using titanium dioxide microcolumns. *Mol. Cell. Proteomics* 2005, *4*, 873–886.
67. Thingholm, T. E., Larsen, M. R., Phosphopeptide enrichment by immobilized metal affinity chromatography. *Methods Mol. Biol.* 2016, *1355*, 123–133.
68. Thingholm, T. E., Jorgensen, T. J., Jensen, O. N., Larsen, M. R., Highly selective enrichment of phosphorylated peptides using titanium dioxide. *Nat. Protoc.* 2006, *1*, 1929–1935.
69. Wang, M. C., Lee, Y. H., Liao, P. C., Optimization of titanium dioxide and immunoaffinity-based enrichment procedures for tyrosine phosphopeptide using matrix-assisted laser desorption/ionization time-of-flight mass spectrometry. *Anal. Bioanal. Chem.* 2015, *407*, 1343–1356.
70. Engholm-Keller, K., Birck, P., Storling, J., Pociot, F., Mandrup-Poulsen, T., Larsen, M. R., TiSH–a robust and sensitive global phosphoproteomics strategy employing a combination of TiO2, SIMAC, and HILIC. *J. Proteomics* 2012, *75*, 5749–5761.
71. Engholm-Keller, K., Larsen, M. R., Improving the phosphoproteome coverage for limited sample amounts using TiO2-SIMAC-HILIC (TiSH) phosphopeptide enrichment and fractionation. *Methods Mol. Biol.* 2016, *1355*, 161–177.
72. Murillo, J. R., Kuras, M., Rezeli, M., Miliotis, T., Betancourt, L., Marko-Varga, G., Automated phosphopeptide enrichment from minute quantities of frozen malignant melanoma tissue. *PLoS One* 2018, *13*, e0208562.
73. Chen, B., Hwang, L., Ochowicz, W., Lin, Z., Guardado-Alvarez, T. M., Cai, W., Xiu, L., Dani, K., Colah, C., Jin, S., Ge, Y., Coupling functionalized cobalt ferrite nanoparticle enrichment with online LC/MS/MS for top-down phosphoproteomics. *Chem. Sci.* 2017, *8*, 4306–4311.
74. Machida, K., Mayer, B. J., Nollau, P., Profiling the global tyrosine phosphorylation state. *Mol. Cell. Proteomics* 2003, *2*, 215–233.
75. Yao, Y., Bian, Y., Dong, M., Wang, Y., Lv, J., Chen, L., Wang, H., Mao, J., Dong, J., Ye, M., SH2 superbinder modified monolithic capillary column for the sensitive analysis of protein tyrosine phosphorylation. *J. Proteome Res.* 2018, *17*, 243–251.
76. Yao, Y., Wang, Y., Wang, S., Liu, X., Liu, Z., Li, Y., Fang, Z., Mao, J., Zheng, Y, Ye, M., One-step SH2 superbinder-based approach for sensitive analysis of tyrosine phosphoproteome. *J. Proteome Res.* 2019, *18*, 1870–1879.
77. Hu, Y., Li, Y., Gao, H., Jiang, B., Zhang, X., Li, X., Wu, Q., Liang, Z., Zhang, L., Zhang, Y., Cleavable hydrophobic derivatization strategy for enrichment and identification of phosphorylated lysine peptides. *Anal. Bioanal. Chem.* 2019, *411*, 4159–4166.
78. Bettelheim, F. R., Tyrosine-O-sulfate in a peptide from fibrinogen. *J. Am. Chem. Soc.* 1954, *76*, 2838–2839.
79. Kanan, Y., Al Ubaidi, M. R., Detection of tyrosine sulfation on proteins. *Curr. Protoc. Protein Sci.* 2015, *80*, 1–20.
80. Kim, J. S., Song, S. U., Kim, H. J., Simultaneous identification of tyrosine phosphorylation and sulfation sites utilizing tyrosine-specific bromination. *J. Am. Soc. Mass Spectrom.* 2011, *22*, 1916–1925.
81. Mann, M., Jensen, O. N., Proteomic analysis of post-translational modifications. *Nat. Biotechnol.* 2003, *21*, 255–261.
82. Yu, Y., Hoffhines, A. J., Moore, K. L., Leary, J. A., Determination of the sites of tyrosine O-sulfation in peptides and proteins. *Nat. Methods* 2007, *4*, 583–588.
83. Medzihradszky, K. F., Darula, Z., Perlson, E., Fainzilber, M., Chalkley, R. J., Ball, H., Greenbaum, D., Bogyo, M., Tyson, D. R., Bradshaw, R.A., Burlingame, A. L., O-sulfonation of serine and threonine: mass spectrometric detection and characterization of a new post-translational modification in diverse proteins throughout the eukaryotes. *Mol. Cell. Proteomics* 2004, *3*, 429–440.
84. Hoffhines, A. J., Damoc, E., Bridges, K. G., Leary, J. A., Moore, K. L., Detection and purification of tyrosine-sulfated proteins using a novel anti-sulfotyrosine monoclonal antibody. *J. Biol. Chem.* 2006, *281*, 37877–37887.
85. Demesa Balderrama, G., Meneses, E. P., Hernández Orihuela, L., Villa Hernández, O., Castro Franco, R., Pando Robles, V., Ferreira Batista, C. V., Analysis of sulfated peptides from the skin secretion of the *Pachymedusa dacnicolor* frog using IMAC-Ga enrichment and high-resolution mass spectrometry. *Rapid Commun. Mass Spectrom.* 2011, *30*, 1017–1027.
86. Robinson, M. R., Brodbelt, J. S., Integrating weak anion exchange and ultraviolet photodissociation mass spectrometry with strategic modulation of peptide basicity for the enrichment of sulfopeptides. *Anal. Chem.* 2016, *88*, 11037–11045.
87. Sheehan, D., McDonagh, B., Bárcena, J. A., Redox proteomics. *Expert Rev. Proteomics* 2010, *7*, 1–4.
88. Butterfield, D. A., Hensley, K., Cole, P., Subramaniam, R., Aksenov, M., Aksenova, M., Bummer, P. M., Haley, B. E., Carney, J. M., Oxidatively induced structural alteration of glutamine synthetase assessed by analysis of spin label incorporation kinetics: relevance to Alzheimer's disease. *J. Neurochem.* 1997, *68*, 2451–2457.
89. Butterfield, D. A., Perluigi, M., Reed, T., Muharib, T., Hughes, C. P., Robinson, R. A., Sultana, R., Redox proteomics in selected neurodegenerative disorders: from its infancy to future applications. *Antioxid. Redox Signal.* 2012, *17*, 1610–1655.
90. Butterfield, D. A., Perluigi, M., Redox proteomics: a key tool for new insights into protein modification with relevance to disease. *Antioxid. Redox Signal.* 2017, *26*, 277–279.
91. Pieragostino, D., Del Boccio, P., Di Ioia, M., Pieroni, L., Greco, V., De Luca, G., D'Aguanno, S., Rossi, C., Franciotta, D., Centonze, D., Oxidative modifications of cerebral transthyretin are associated with multiple sclerosis. *Proteomics* 2013, *13*, 1002–1009.

92. Lennicke, C., Rahn, J., Heimer, N., Lichtenfels, R., Wessjohann, L. A., Seliger B., Redox proteomics: methods for the identification and enrichment of redox-modified proteins and their applications. *Proteomics* 2016, *16*, 197–213.
93. McDonagh, B., Martínez-Acedo, P., Vázquez, J., Padilla, C. A., Sheehan, D., Bárcena, J. A., Application of iTRAQ reagents to relatively quantify the reversible redox state of cysteine residues. *Int. J. Proteomics* 2012, *2012*, 514847.
94. Hurd, T. R., Prime, T. A., Harbour, M. E., Lilley, K. S., Murphy, M. P., Detection of reactive oxygen species-sensitive thiol proteins by redox difference gel electrophoresis implications for mitochondrial redox signaling. *J. Biol. Chem.* 2007, *282*, 22040–22051.
95. Leichert, L. I., Gehrke, F., Gudiseva, H. V., Blackwell, T., Ilbert, M., Walker, A. K., Strahler, J. R., Andrews, P. C., Jakob, U., Quantifying changes in the thiol redox proteome upon oxidative stress in vivo. *Proc. Natl. Acad. Sci. U.S.A.* 2008, *105*, 8197–8202.
96. Held, J. M., Danielson, S. R., Behring, J. B., Atsriku, C., Britton, D. J., Puckett, R. L., Schilling, B., Campisi, J., Benz, C. C., Gibson, B. W., Targeted quantitation of site-specific cysteine oxidation in endogenous proteins using a differential alkylation and multiple reaction monitoring mass spectrometry approach. *Mol. Cell. Proteomics* 2010, *9*, 1400–1410.
97. Ju, Y., Fu, M., Wu, L., Yang, G., Strategies and tools for detection of protein S-nitrosylation and S-sulfhydration. *Biochem. Anal. Biochem.* 2015, *4*, 224.
98. Zhang, D., Du, J., Tang, C., Huang, Y., Jin, H., HS-induced sulfhydration: biological function and detection methodology. *Front. Pharmacol.* 2017, *8*, 608.
99. Longen, S., Beck, K.-F., Pfeilschifter, J., HS-induced thiol-based redox switches: biochemistry and functional relevance for inflammatory diseases. *Pharmacol. Res.* 2016, *111*, 642–651.
100. Vandiver, M. S., Snyder, S. H., Hydrogen sulfide: a gasotransmitter of clinical relevance. *J. Mol. Med.* 2012, *90*, 255–263.
101. Greco, V., Spalloni, A., Corasolla Carregari, V., Pieroni, L., Persichilli, S., Mercuri, N., Urbani, A., Longone, P., Proteomics and toxicity analysis of spinal-cord primary cultures upon hydrogen sulfide treatment. *Antioxidants* 2018, *7*, 87.
102. Mustafa, A. K., Gadalla, M. M., Sen, N., Kim, S., Mu, W., Gazi, S. K., Barrow, R. K., Yang, G., Wang, R., Snyder, S. H., HS signals through protein S-sulfhydration. *Sci. Signal.* 2009, *2*, ra72.
103. Módis, K., Ju, Y., Ahmad, A., Untereiner, A. A., Altaany, Z., Wu, L., Szabo, C., Wang, R., S-sulfhydration of ATP synthase by hydrogen sulfide stimulates mitochondrial bioenergetics. *Pharmacol. Res.* 2016, *113*, 116–124.
104. Pan, J., Carroll, K. S., Persulfide reactivity in the detection of protein S-sulfhydration. *ACS Chem. Boil.* 2013, *8*, 1110–1116.
105. Krishnan, N., Fu, C., Pappin, D. J., Tonks, N. K., $H_2S$-Induced sulfhydration of the phosphatase PTP1B and its role in the endoplasmic reticulum stress response. *Sci. Signal.* 2011, *4*, ra86.
106. Dóka, É., Pader, I., Bíró, A., Johansson, K., Cheng, Q., Ballagó, K., Prigge, J. R., Pastor-Flores, D., Dick, T. P., Schmidt, E. E., A novel persulfide detection method reveals protein persulfide-and polysulfide-reducing functions of thioredoxin and glutathione systems. *Sci. Adv.* 2016, *2*, e1500968.
107. Sen, N., Paul, B. D., Gadalla, M. M., Mustafa, A. K., Sen, T., Xu, R., Kim, S., Snyder, S. H., Hydrogen sulfide-linked sulfhydration of NF-κB mediates its antiapoptotic actions. *Mol. Cell* 2012, *45*, 13–24.
108. Gao, X.-H., Krokowski, D., Guan, B.-J., Bederman, I., Majumder, M., Parisien, M., Diatchenko, L., Kabil, O., Willard, B., Banerjee, R., Wang, B., Bebek, G., Evans, C. R., Fox, P. L., Gerson, S. L., Hoppel, C. L., Liu, M., Arvan, P., Hatzoglou, M. Quantitative $H_2S$-mediated protein sulfhydration reveals metabolic reprogramming during the integrated stress response. *Elife* 2015, *4*, e10067.
109. Zhang, D., Macinkovic, I., Devarie-Baez, N. O., Pan, J., Park, C. M., Carroll, K. S., Filipovic, M. R., Xian, M., Detection of protein S-sulfhydration by a tag-switch technique. *Angew. Chem. Int. Ed. Eng.* 2014, *53*, 575–581.
110. Altaany, Z., Ju, Y., Yang, G., Wang, R., The coordination of S-sulfhydration, S-nitrosylation, and phosphorylation of endothelial nitric oxide synthase by hydrogen sulfide. *Sci. Signal* 2014, *7*, ra87. https://doi.org/10.1126/scisignal.2005478.
111. Boullerne, A. I., Rodrıguez, J. J., Touil, T., Brochet, B., Schmidt, S., Abrous, N. D., Le Moal, M., Pua, J. R., Jensen, M. A., Mayo W., Anti-S-nitrosocysteine antibodies are a predictive marker for demyelination in experimental autoimmune encephalomyelitis: implications for multiple sclerosis. *J. Neurosci.* 2002, *22*, 123–132.
112. O'Brian, C. A., Chu, F., Post-translational disulfide modifications in cell signaling–role of inter-protein, intra-protein, S-glutathionyl, and S-cysteaminyl disulfide modifications in signal transmission. *Free Radic. Res.* 2005, *39*, 471–480.
113. Grek, C. L, Zhang, J., Manevich, Y., Townsend, D. M., Tew, K. D., Causes and consequences of cysteine S-glutathionylation. *J. Biol. Chem.* 2013, *288*, 26497–26504.
114. Lim, S. Y., Raftery, M. J., Goyette, J., Geczy, C. L., S-glutathionylation regulates inflammatory activities of S100A9. *J. Biol. Chem.* 2010, *285*, 14377–14388.
115. Cabras, T., Manconi, B., Iavarone, F., Fanali, C., Nemolato, S., Fiorita, A., Scarano, E., Passali, G. C., Manni, A., Cordaro, M., Paludetti, G., Faa, G., Messana, I., Castagnola, M., RP-HPLC-ESI-MS evidenced that salivary cystatin B is detectable in adult human saliva mostly as S-modified derivatives: S-glutathionyl, S-cysteinyl and S-S 2-mer. *J. Proteomics* 2012, *75*, 908–913.
116. Iavarone, F., Cabras, T., Pisano, E., Sanna, M. T., Nemolato, S., Vento, G., Tirone, C., Romagnoli, C., Cordaro, M., Fanos, V., Faa, G., Messana, I., Castagnola, M., Top-down HPLC-ESI-MS detection of S-glutathionylated and S-cysteinylated derivatives of cystatin B and its 1–53 and 54–98 fragments in whole saliva of human pre-term newborns. *J. Proteome Res.* 2013, *12*, 917–926.
117. Zaballa, M. E., van der Goot, F. G., The molecular era of protein S-acylation: spotlight on structure, mechanisms, and dynamics. *Crit. Rev. Biochem. Mol. Biol.* 2018, *53*, 420–451.
118. Doulias, P. T., Gould, N. S., Analysis of cysteine post-translational modifications using organic mercury resin. *Curr. Protoc. Protein Sci.* 2018, *94*, e69.
119. Gould, N. S., Site-specific proteomic mapping of modified cysteine residues. *Methods Mol. Biol.* 2019, *1967*, 183–195.
120. Yount, J. S, Zhang, M. M., Hang, H. C., Emerging roles for protein S-palmitoylation in immunity from chemical proteomics. *Curr. Opin. Chem. Biol.* 2013, *17*, 27–33.
121. Kang, R., Wan, J., Arstikaitis, P., Takahashi, H., Huang, K., Bailey, A. O., Thompson, J. X., Roth, A. F., Drisdel, R. C., Mastro, R., Green, W. N., Yates, J. R. 3rd, Davis, N. G., El-Husseini, A., Neural palmitoyl-proteomics reveals dynamic synaptic palmitoylation. *Nature* 2008, *456*, 904–909.
122. Wilson, J. P., Raghavan, A. S., Yang, Y. Y., Charron, G., Hang, H. C., Proteomic analysis of fatty-acylated proteins in mammalian

cells with chemical reporters reveals S-acylation of histone H3 variants. *Mol. Cell. Proteomics* 2011, *10*, M110.001198.
123. Cao, X. J., Garcia, B. A., Global proteomics analysis of protein lysine methylation. *Curr. Protoc. Protein Sci.* 2016, *86*, 24.8.1–24.8.19.
124. Evich, M., Stroeva, E., Zheng, Y. G., Germann, M. W., Effect of methylation on the sidechain pK a value of arginine. *Protein Sci. Publ. Protein Soc.* 2016, *25*, 479–486.
125. Paik, W. K., Kim, S., Enzymatic methylation of protein fractions from calf thymus nuclei. *Biochem. Biophys. Res. Commun.* 1967, *29*, 14–20.
126. Ambler, R. P., Rees, M. W., Epsilon-*N*-methyl-lysine in bacterial flagellar protein. *Nature* 1959, *184*, 56–57.
127. Murn, J., Shi, Y., The winding path of protein methylation research: milestones and new frontiers. *Nat. Rev. Mol. Cell Biol.* 2017, *18*, 517–527.
128. Dhar, S., Vemulapalli, V., Patananan, A. N., Huang, G. L., Di Lorenzo, A., Richard, S., Comb, M. J., Guo, A., Clarke, S. G., Bedford, M. T., Loss of the major Type I arginine methyltransferase PRMT1 causes substrate scavenging by other PRMTs. *Sci. Rep.* 2013, *3*, 1311.
129. Bedford, M. T., Richard, S., Arginine methylation an emerging regulator of protein function. *Mol. Cell* 2005, *18*, 263–272.
130. Biggar, K. K., Li, S. S., Non-histone protein methylation as a regulator of cellular signalling and function. *Nat. Rev. Mol. Cell Biol* 2015, *16*, 5–17.
131. Scoumanne, A., Zhang, J., Chen, X., PRMT5 is required for cell-cycle progression and p53 tumor suppressor function. *Nucleic Acids Res*. 2009, *37*, 4965–4976.
132. Sylvestersen, K. B., Horn, H., Jungmichel, S., Jensen, L. J., Nielsen, M. L., Proteomic analysis of arginine methylation sites in human cells reveals dynamic regulation during transcriptional arrest. *Mol. Cell. Proteomics* 2014, *13*, 2072–2088.
133. Krapivinsky, G., Krapivinsky, L., Renthal, N. E., Santa-Cruz, A., Manasian, Y., Clapham, D. E., Histone phosphorylation by TRPM6's cleaved kinase attenuates adjacent arginine methylation to regulate gene expression. *Proc. Natl. Acad. Sci. U.S.A.* 2017, *114*, E7092-E7100.
134. Wang, Q., Wang, K., Ye, M., Strategies for large-scale analysis of non-histone protein methylation by LC-MS/MS. *Analyst* 2017, *142*, 3536–3548.
135. Cao, X. J., Arnaudo, A. M., Garcia, B. A., Large-scale global identification of protein lysine methylation in vivo. *Epigenetics* 2013, *8*, 477–485.
136. Guo, A., Gu, H., Zhou, J., Mulhern, D., Wang, Y., Lee, K. A., Yang, V., Aguiar, M., Kornhauser, J., Jia, X., Ren, J., Beausoleil, S. A., Silva, J. C., Vemulapalli, V., Bedford, M. T., Comb, M. J., Immunoaffinity enrichment and mass spectrometry analysis of protein methylation. *Mol. Cell. Proteomics* 2014, *13*, 372–387.
137. Wu, Z., Cheng, Z., Sun, M., Wan, X., Liu, P., He, T., Tan, M., Zhao, Y., A chemical proteomics approach for global analysis of lysine monomethylome profiling. *Mol. Cell. Proteomics* 2015, *14*, 329–339.
138. Larsen, S. C., Sylvestersen, K. B., Mund, A., Lyon, D., Mullari, M., Madsen, M. V., Daniel, J. A., Jensen, L. J., Nielsen, M. L., Proteome-wide analysis of arginine monomethylation reveals widespread occurrence in human cells. *Sci. Signal.* 2016, *9*, rs9.
139. Ong, S. E., Mittler, G., Mann, M., Identifying and quantifying in vivo methylation sites by heavy methyl SILAC. *Nat. Methods* 2004, *1*, 119–126.
140. Bremang, M., Cuomo, A., Agresta, A. M., Stugiewicz, M., Spadotto, V., Bonaldi, T., Mass spectrometry-based identification and characterisation of lysine and arginine methylation in the human proteome. *Mol. Biosyst.* 2013, *9*, 2231–2247.
141. Uhlmann, T., Geoghegan, V. L., Thomas, B., Ridlova, G, Trudgian, D. C., Acuto, O., A method for large-scale identification of protein arginine methylation. *Mol. Cell. Proteomics* 2012, *11*, 1489–1499.
142. Wang, K., Dong, M., Mao, J., Wang, Y., Jin, Y., Ye, M., Zou, H., Antibody-free approach for the global analysis of protein methylation. *Anal. Chem.* 2016, *88*, 11319–11327.
143. Ma, M., Zhao, X., Chen, S., Zhao, Y., Yang, L, Feng, Y., Qin, W., Li, L, Jia, C., Strategy based on deglycosylation, multi-protease, and hydrophilic interaction chromatography for large-scale profiling of protein methylation. *Anal. Chem.* 2017, *89*, 12909–12917.
144. Zhang, M., Xu, J. Y., Hu, H., Ye, B. C., Tan, M., Systematic proteomic analysis of protein methylation in prokaryotes and eukaryotes revealed distinct substrate specificity. *Proteomics* 2018, *18*, 1700300.
145. Binda, O., Boyce, M., Rush, J. S., Palaniappan, K. K., Bertozzi, C. R, Gozani, O., A chemical method for labeling lysine methyltransferase substrates. *ChemBioChem*. 2011, *12*, 330–334.
146. Peng, C., Wong, C. C., The story of protein arginine methylation: characterization, regulation, and function. *Expert Rev. Proteomics* 2017, *14*, 157–170.
147. Carlson, S. M., Moore, K. E., Green E. M., Martín G. M., Gozani O. Proteome-wide enrichment of proteins modified by lysine methylation. *Nat. Protoc.* 2014, *9*, 37–50.
148. Liu, H., Galka, M., Mori, E., Liu, X., Lin, Y. F., Wei, R., Pittock, P., Voss, C., Dhami, G., Li, X., Miyaji, M., Lajoie, G., Chen, B., Li, S. S., A method for systematic mapping of protein lysine methylation identifies functions for HP1β in DNA damage response. *Mol. Cell* 2013, *50*, 723–735.
149. Bürger, M., Chory, J., Structural and chemical biology of deacetylases for carbohydrates, proteins, small molecules and histones. *Commun. Biol.* 2018, *1*, 217.
150. Aksnes, H., Van Damme, P., Goris, M., Starheim, K. K., Marie, M., Støve, S. I., Hoel, C., Kalvik, T. V., Hole, K., Glomnes, N., Furnes, C., Ljostveit, S., Ziegler, M., Niere, M., Gevaert, K., Arnesen, T., An organellar Nα-acetyltransferase, Naa60, acetylates cytosolic N termini of transmembrane proteins and maintains Golgi integrity. *Cell Rep*. 2015, *10*, 1362–1374.
151. Ree, R., Varland, S., Arnesen, T., Spotlight on protein *N*-terminal acetylation. *Exp. Mol. Med.* 2018, *50*, 90.
152. Nguyen, K. T., Mun, S. H., Lee, C. S., Hwang, C. S., Control of protein degradation by *N*-terminal acetylation and the *N*-end rule pathway. *Exp. Mol. Med.* 2018, *50*, 91.
153. Li, Y. T., Yi, C., Chen, C. C., Lan, H., Pan, M., Zhang, S. J., Huang, Y. C., Guan, C. J., Ly, Y. M., Yu, L., Liu L., A semisynthetic Atg3 reveals that acetylation promotes Atg3 membrane binding and Atg8 lipidation. *Nat. Commun*. 2017, *8*, 14846.
154. Drazic, A., Myklebust, L. M., Ree, R., Arnesen, T., The world of protein acetylation. *Biochim. Biophys. Acta* 2016, *1864*, 1372–1401.
155. James, A. M., Hoogewijs, K., Logan, A., Hall, A. R., Ding, S., Fearnley, I. M., Murphy, M. P., Non-enzymatic *N*-acetylation of lysine residues by acetyl-CoA often occurs via a proximal S-acetylated thiol intermediate sensitive to glyoxalase II. *Cell Rep*. 2017, *18*, 2105–2112.

156. Choudhary, C., Kumar, C., Gnad, F., Nielsen, M. L., Rehman, M., Walther, T. C., Olsen, J. V., Mann, M., Lysine acetylation targets protein complexes and co-regulates major cellular functions. *Science* 2009, *325*, 834–840.
157. Zhou, Z., Chen, Y., Jin, M., He, J., Guli, A., Yan, C., Ding, S., Comprehensive analysis of lysine acetylome reveals a site-specific pattern in rapamycin-induced autophagy. *J. Proteome Res.* 2019, *18*, 865–877.
158. Miyagi, M., Site-specific quantification of lysine acetylation using isotopic labeling. *Methods Enzymol*. 2017, *586*, 85–95.
159. Lundby, A., Lage, K., Weinert, B. T., Bekker-Jensen, D. B., Secher, A., Skovgaard, T., Kelstrup, C. D., Dmytriyev, A., Choudhary, C., Lundby, C., Olsen, J. V., Proteomic analysis of lysine acetylation sites in rat tissues reveals organ specificity and subcellular patterns. *Cell Rep*. 2012, *2*, 419–431.
160. Schilling, B., Meyer, J. G., Wei, L., Ott, M., Verdin, E., High-resolution mass spectrometry to identify and quantify acetylation protein targets. *Methods Mol. Biol.* 2019, *1983*, 3–16.
161. Baeza, J., Dowell, J. A., Smallegan, M. J., Fan, J., Amador-Noguez, D., Khan, Z., Denu, J. M., Stoichiometry of site-specific lysine acetylation in an entire proteome, *J. Biol. Chem.* 2014, *289*, 21326–21338.
162. Gil, J., Ramírez-Torres, A., Chiappe, D., Luna-Peñaloza, J., Fernandez-Reyes, F. C., Arcos-Encarnación, B., Contreras, S., Encarnación-Guevara, S., Lysine acetylation stoichiometry and proteomics analyses reveal pathways regulated by sirtuin 1 in human cells. *J. Biol. Chem.* 2017, *292*, 18129–18144.
163. Staes, A., Impens, F., Van Damme, P., Ruttens, B., Goethals, M., Demol, H., Timmerman, E., Vandekerckhove, J., Gevaert, K., Selecting protein N-terminal peptides by combined fractional diagonal chromatography. *Nat. Protoc.* 2011, *6*, 1130–1141.
164. Bienvenut, W. V., Giglione, C., Meinnel, T., SILProNAQ: a convenient approach for proteome-wide analysis of protein N-Termini and N-Terminal acetylation quantitation. *Methods Mol. Biol.* 2017, *1574*, 17–34.
165. Marino, G., Eckhard, U., Overall, C. M., Protein termini and their modifications revealed by positional proteomics. *ACS Chem. Biol.* 2015, *10*, 1754–1764.
166. Ciechanover, A., The unravelling of the ubiquitin system. *Nat. Rev. Mol. Cell Biol.* 2015, *16*, 322–324.
167. Komander, D., Rape, M., The ubiquitin code. *Annu. Rev. Biochem.* 2012, *81*, 203–229.
168. Behuliak, M., Celec, P., Gardlik, R., Palffy, R., Ubiquitin-the kiss of death goes nobel. Will you be quitting? *Bratisl. Lek. Listy* 2005, *106*, 93–100.
169. Welchman, R. L., Gordon, C., Mayer, R. J. Ubiquitin and ubiquitin-like proteins as multifunctional signals. *Nat. Rev. Mol. Cell Biol.* 2005, *6*, 599–609.
170. Hochstrasser, M., Origin and function of ubiquitin-like proteins. *Nature* 2009, *458*, 422–429.
171. Vertegaal, A. C. O., Uncovering ubiquitin and ubiquitin-like signaling networks. *Chem. Rev.* 2011, *111*, 7923–7940.
172. Takada, K., Nasu, H., Hibi, N., Tsukada, Y., Ohkawa, K., Fujimuro, M., Sawada, H., Yokosawa, H., Immunoassay for the quantification of intracellular multi-ubiquitin chains. *Eur. J. Biochem.* 1995, *233*, 42–47.
173. Matsumoto, M., Hatakeyama, S., Oyamada, K., Oda, Y., Nishimura, T., Nakayama, K. I., Large-scale analysis of the human ubiquitin-related proteome. *Proteomics* 2005, *5*, 4145–4151.
174. Spence, J., Gali, R. R., Dittmar, G., Sherman, F., Karin, M., Finley, D., Cell cycle-regulated modification of the ribosome by a variant multiubiquitin chain. *Cell* 2000, *102*, 67–76.
175. Peng, J., Schwartz, D., Elias, J. E., Thoreen, C. C., Cheng, D., Marsischky, G., Roelofs, J., Finley, D., Gygi, S. P., A proteomics approach to understanding protein ubiquitination. *Nat. Biotechnol.* 2003, *21*, 921–926.
176. Tagwerker, C., Flick, K., Cui, M., Guerrero, C., Dou, Y., Auer, B., Baldi, P., Huang, L., Kaiser, P., A tandem affinity tag for two-step purification under fully denaturing conditions: application in ubiquitin profiling and protein complex identification combined with in vivo cross-linking. *Mol. Cell. Proteomics* 2006, *5*, 737–748.
177. Aillet, F., Lopitz-Otsoa, F., Hjerpe, R., Torres-Ramos, M., Lang, V., Rodríguez, M. S., Isolation of ubiquitylated proteins using tandem ubiquitin-binding entities. *Methods Mol. Biol.* 2012, *832*, 173–183.
178. Beaudette, P., Popp, O., Dittmar, G., Proteomic techniques to probe the ubiquitin landscape. *Proteomics* 2016, *16*, 273–287.
179. Bustos, D., Bakalarski, C. E., Yang, Y., Peng, J., Kirkpatrick, D. S., Characterizing ubiquitination sites by peptide-based immunoaffinity enrichment. *Mol Cell Proteomics* 2012, *11*, 1529–1540.
180. Kim, W., Bennett, E. J., Huttlin, E. L., Guo, A., Li, J., Possemato, A., Sowa, M. E., Rad, R., Rush, J., Comb, M. J., Harper, J. W., Gygi, S. P., Systematic and quantitative assessment of the ubiquitin-modified proteome. *Mol. Cell* 2011, *44*, 325–340.
181. Na, C. H., Jones, D. R., Yang, Y., Wang, X., Xu, Y., Peng, J., Synaptic protein ubiquitination in rat brain revealed by antibody-based ubiquitome analysis. *J. Proteome Res.* 2012, *11*, 4722–4732.
182. Saitoh, H., Pu, R. T., Dasso, M., SUMO-1: wrestling with a new ubiquitin-related modifier. *Trends Biochem. Sci.* 1997, *22*, 374–376.
183. Saitoh, H., Hinchey, J., Functional heterogeneity of small ubiquitin-related protein modifiers SUMO-1 versus SUMO-2/3. *J. Biol. Chem.* 2000, *275*, 6252–6258.
184. Ulrich, H. D., The fast-growing business of SUMO chains. *Mol. Cell* 2008, *32*, 301–305.
185. Owerbach, D., McKay, E. M., Yeh, E. T., Gabbay, K. H., Bohren, K. M., A proline-90 residue unique to SUMO-4 prevents maturation and sumoylation. *Biochem. Biophys. Res. Commun.* 2005, *337*, 517–520.
186. Galisson, F., Mahrouche, L., Courcelles, M., Bonneil, E., Meloche, S., Chelbi-Alix, M. K., Thibault, P., A novel proteomics approach to identify SUMOylated proteins and their modification sites in human cells. *Mol. Cell. Proteomics* 2011, *10*, M110 004796.
187. Li, T., Evdokimov, E., Shen, R. F., Chao, C. C., Tekle, E., Wang, T., Stadtman, E. R., Yang, D. C., Chock, P. B., Sumoylation of heterogeneous nuclear ribonucleoproteins, zinc finger proteins, and nuclear pore complex proteins: a proteomic analysis. *Proc. Natl. Acad. Sci. U. S. A.* 2004, *101*, 8551–8556.
188. Panse, V. G., Hardeland, U., Werner, T., Kuster, B., Hurt, E., A proteome-wide approach identifies sumoylated substrate proteins in yeast. *J. Biol. Chem.* 2004, *279*, 41346–41351.
189. Becker, J., Barysch, S. V., Karaca, S., Dittner, C., Hsiao, H. H., Berriel Diaz, M., Herzig, S., Urlaub, H., Melchior, F., Detecting endogenous SUMO targets in mammalian cells and tissues. *Nat. Struct. Mol. Biol.* 2013, *20*, 525–531.

190. Zhan, Y., Li Y., Tang B., Zhang C.-Y., The strategies for identification and quantification of SUMOylation. *Chem. Commun.* 2017, *53*, 6989–6998.
191. Okanishi, H., Kim, K., Masui, R., Kuramitsu, S., Lysine propionylation is a prevalent post-translational modification in *Thermus thermophilus*. *Mol. Cell. Proteomics* 2014, *13*, 2382–2398.
192. Peng, C., Lu, Z., Xie, Z., Cheng, Z., Chen, Y., Tan, M., Luo, H., Zhang, Y., He, W., Yang, K., Zwaans, B. M., Tishkoff, D., Ho, L., Lombard D., He, T. C., Dai, J., Verdin, E., Ye, Y., Zhao, Y., The first identification of lysine malonylation substrates and its regulatory enzyme. *Mol. Cell. Proteomics* 2011, *10*, M111.012658.
193. Xie, L., Liu, W., Li, Q., Chen, S., Xu, M., Huang, Q., Zeng, J., Zhou, M., Xie, J., First succinylproteome profiling of extensively drug-resistant *Mycobacterium tuberculosis* revealed involvement of succinylation in cellular physiology. *J. Proteome Res.* 2015, *14*, 107–119.
194. Yang, M., Wang, Y., Chen, Y., Cheng, Z., Gu, J., Deng, J., Bi, L., Chen, C., Mo, R., Wang, X., Ge, F., Succinylome analysis reveals the involvement of lysine succinylation in metabolism in pathogenic *Mycobacterium tuberculosis*. *Mol. Cell. Proteomics* 2015, *14*, 796–811.
195. Tan, M., Peng, C., Anderson, K. A., Chhoy, P., Xie, Z., Dai, L., Park, J., Chen, Y., Huang, H., Zhang, Y., Ro, J., Wagner, G. R., Green, M. F., Madsen, A. S., Schmiesing, J., Peterson, B. S., Xu, G., Ilkayeva, O. R., Muehlbauer, M. J., Braulke, T., Muhlhausen, C., Backos, D. S., Olsen, C. A., McGuire, P. J., Pletcher, S. D., Lombard, D. B., Hirschey, M. D., Zhao, Y., Lysine glutarylation is a protein posttranslational modification regulated by SIRT5. *Cell Metab*. 2014, *19*, 605–617.
196. Zhang, Z., Tan, M., Xie, Z., Dai, L., Chen, Y., Zhao, Y., Identification of lysine succinylation as a new post-translational modification. *Nat. Chem. Biol.* 2011, *7*, 58–63.
197. Park, J., Chen, Y., Tishkoff, D. X., Peng, C., Tan, M., Dai, L., Xie, Z., Zhang, Y., Zwaans, B. M., Skinner, M. E., Lombard, D. B., Zhao, Y., SIRT5-mediated lysine desuccinylation impacts diverse metabolic pathways. *Mol. Cell* 2013, *50*, 919–930.
198. Weinert, B. T., Scholz, C., Wagner, S. A., Iesmantavicius, V., Su, D., Daniel, J. A., Choudhary, C., Lysine succinylation is a frequently occurring modification in prokaryotes and eukaryotes and extensively overlaps with acetylation. *Cell Rep*. 2013, *4*, 842–851.
199. Colak, G., Xie, Z., Zhu, A. Y., Dai, L., Lu, Z., Zhang, Y., Wan, X., Chen, Y., Cha, Y. H., Lin, H., Zhao, Y., Tan, M., Identification of lysine succinylation substrates and the succinylation regulatory enzyme CobB in *Escherichia coli*. *Mol. Cell. Proteomics* 2013, *12*, 3509–3520.
200. Kosono, S., Tamura, M., Suzuki, S., Kawamura, Y., Yoshida, A., Nishiyama, M., Yoshida, M., Changes in the acetylome and succinylome of *Bacillus subtilis* in response to carbon source. *PLoS One* 2015, *10*, e0131169.
201. Pan, J., Chen, R., Li, C., Li, W., Ye, Z., Global analysis of protein lysine succinylation profiles and their overlap with lysine acetylation in the marine bacterium *Vibrio parahemolyticus*. *J. Proteome Res.* 2015, *14*, 4309–4318.
202. Li, X., Hu, X., Wan, Y., Xie, G., Li, X., Chen, D., Cheng, Z., Yi, X., Liang, S., Tan, F., Systematic identification of the lysine succinylation in the protozoan parasite *Toxoplasma gondii*. *J. Proteome Res.* 2014, *13*, 6087–6095.
203. Longxiang, X., Juan, L., Wanyan, D., Zhaoxiao, Y., Wenjie, F., Min, C., Wanqing, L., Jianping, X., Weihua, P., Proteomic analysis of lysine succinylation of the human pathogen *Histoplasma capsulatum*. *J. Proteomics* 2017, *154*, 109–117.
204. Okanishi, H., Kim, K., Fukui, K., Yano, T., Kuramitsu, S., Masui, R., Proteome-wide identification of lysine succinylation in thermophilic and mesophilic bacteria. *Biochim. Biophys. Acta* 2017, *1865*, 232–242.
205. Wu, L., Gong, T., Zhou, X., Zeng, J., Huang, R., Wu, Y., Li, Y., Global analysis of lysine succinylome in the periodontal pathogen *Porphyromonas gingivalis*. *Mol. Oral Microbiol*. 2019, *34*, 74–83.
206. Vyas, S., Matic, I., Uchima, L., Rood, J., Zaja, R., Hay, R.T., Ahel, I., Chang, P., Family-wide analysis of poly(ADP-ribose) polymerase activity. *Nat. Commun.* 2014, *5*, 4426.
207. Hottiger, M. O., Nuclear ADP-ribosylation and its role in chromatin plasticity, cell differentiation, and epigenetics. *Annu. Rev. Biochem.* 2015, *84*, 227–263.
208. Daniels, C. M., Ong, S. E., Leung, A. K., The promise of proteomics for the study of ADP-ribosylation. *Mol. Cell* 2015, *58*, 911–924.
209. Martello, R., Leutert, M., Jungmichel, S., Bilan, V., Larsen, S. C., Young, C., Hottiger, M. O., Nielsen, M. L., Proteome-wide identification of the endogenous ADP-ribosylome of mammalian cells and tissue. *Nat. Commun.* 2016, *7*, 12917.
210. Zhang, Y., Wang, J., Ding, M., Yu, Y., Site-specific characterization of the Asp- and Glu-ADP-ribosylated proteome. *Nat. Methods* 2013, *10*, 981–984.
211. Daniels, C. M., Ong, S. E., Leung, A. K. L., ADP-ribosylated peptide enrichment and site identification: the phosphodiesterase-based method. *Methods Mol. Biol.* 2017, *1608*, 79–93.
212. Gibson, B. A., Zhang, Y., Jiang, H., Hussey, K. M., Shrimp, J. H., Lin, H., Schwede, F., Yu, Y., Kraus, W. L., Chemical genetic discovery of PARP targets reveals a role for PARP-1 in transcription elongation. *Science* 2016, *353*, 45–50.
213. Kalesh, K., Lukauskas, S., Borg, A. J., Snijders, A. P., Ayyappan, V., Leung, A. K. L., Haskard, D. O., Di Maggio P. A., An integrated chemical proteomics approach for quantitative profiling of intracellular ADP-ribosylation. *Sci. Rep.* 2019, *9*, 6655.
214. Hendriks, I. A., Larsen, S. C., Nielsen, M. L., An advanced strategy for comprehensive profiling of ADP-ribosylation sites using mass spectrometry-based proteomics. *Mol. Cell. Proteomics* 2019, *18*, 1010–1026.
215. Udenwobele, D. I., Su, R. C., Good, S. V., Ball, T. B., Varma Shrivastav, S., Shrivastav, A., Myristoylation: an important protein modification in the immune response. *Front. Immunol.* 2017, *8*, 751.
216. Goya Grocin, A., Serwa, R. A., Morales Sanfrutos, J., Ritzefeld, M., Tate, E. W., Whole proteome profiling of *N*-myristoyltransferase activity and inhibition using sortase A. *Mol. Cell. Proteomics* 2019, *18*, 115–126.
217. Nguyen, K. T., Lee, C. S., Mun, S. H., Truong, N. T., Park, S. K., Hwang, C. S., N-terminal acetylation and the N-end rule pathway control degradation of the lipid droplet protein PLIN2. *J. Biol. Chem.* 2019, *294*, 379–388.

**How to cite this article:** Pieroni L, Iavarone F, Olianas A, et al. Enrichments of post-translational modifications in proteomic studies. *J Sep Sci* 2019;1–23. https://doi.org/10.1002/jssc.201900804