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Review

Post-translational quantitation by SRM/MRM: applications in cardiology

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Abstract

Introduction: Post-translational modifications (PTMs) have an important role in the regulation of protein function, localization and interaction with other molecules. PTMs apply a dynamic control of proteins both in physiological and pathological conditions. The study of disease-specific PTMs allows identifying potential biomarkers and developing effective drugs. Enrichment techniques combined with high-resolution MS/MS analysis provide attractive results on PTMs characterization. Selected reaction monitoring/multiple reaction monitoring (SRM/MRM) is a powerful targeted assay for the quantitation and validation of PTMs in complex biological samples.

Areas covered: The most frequent PTMs are described in terms of biological role and analytical methods commonly used to detect them. The applications of SRM/MRM for the absolute quantitation of PTMs are reported and a specific section is focused on PTMs detection in proteins that are involved in cardiovascular system and heart diseases.

Expert commentary: PTMs characterization in relation to disease pathology is still in progress, but targeted proteomics by LC-MS/MS has significantly upgraded the knowledge in the last years. Advances in enrichment strategies and software tools will facilitate the interpretation of high PTMs complexity. Promising studies confirm the great potentiality of SRM/MRM to study PTMs in cardiovascular field and PTMomics could be very useful in a clinical perspective.

Keywords: Biomarkers; cardiovascular proteomics; mass spectrometry; multiple reaction monitoring; post-translational modifications; protein function; selected reaction monitoring; targeted proteomics.

1. Introduction

Post-translational modifications (PTMs) of proteins are very important, because they affect several cellular processes, among which protein localization in cells, regulation of protein function and protein-protein interactions (Figure 1). They modulate intra- and intermolecular interactions, introducing a dynamic control of protein activity and new protein functions [1]. PTMs have a crucial role in both physiological and pathological conditions. They control the fold and physiological function of the majority of proteins, but in some cases the presence of a modification can cause a disease development. The PTM alteration on a protein or the introduction of a new one can induce a change in the protein biological functions and, consequently, lead to pathological symptoms [2]. Otherwise, some proteins can be modified in a different way than under physiological state due to the disease. For example, phosphorylation is frequently involved in diseases, because it regulates a huge number of protein kinase/phosphatase signaling networks. To facilitate the reader, the PTMs are addressed in two sections, enzymatic and non-enzymatic modifications. Enzymatic PTMs are catalyzed in the presence of specific enzymes and are involved in protein function regulation, such as phosphorylation, glycosylation, ubiquitination, acetylation and methylation. On the other hand, non-enzymatic PTMs occur in the presence of compounds chemically reactive with proteins (e.g., reactive oxygen and nitrogen species) [3]. Enzymatic PTMs are specific, reversible and dynamic in comparison to non-enzymatic modifications that are generally irreversible and damaging in different organs (e.g., dynamic O-GlcNAcylation and irreversible glycation have been highlighted in important cardiomyocyte proteins in the diabetic cardiomyopathy [4]). However, it is important to point out that some non-enzymatic PTMs can be also reversed and they occurs in physiological conditions, influencing several cellular processes [3]. In this review, we summarize their physical and chemical properties, the biological role and the analytical methods commonly used to detect and quantify them. Therefore, the study of disease-specific modifications allows identifying potential biomarkers for early diagnosis, as well as developing effective drugs targeted to PTMs. An accurate analysis and quantitation of PTMs provides the possibility to monitor the disease progression, the treatment efficacy and understand better the pathogenesis of a diseased state. In the last few years, particular interest has been focused on the crosstalk among the PTMs that regulates the physiological and pathological states by several positive or negative pathways. In 2015, Stastna et al. discussed the PTMs that occur on lysine residue, in particular neddylation (i.e., ubiquitin-like protein NEDD8 is conjugated to targeted proteins) and sumoylation (i.e., attachment of small ubiquitin-like modifier SUMO to targeted proteins), and the proteomic approaches available to study these modifications in cardiovascular research [5]. They pointed out that different PTMs can compete for the same amino acid or multiple residues can be modified by the same reaction, making the MS identification and characterization very challenging. The type and site location of PTMs can influence protein functions, cell signaling and determine acute and

chronic effects on the cardiovascular system. Thus, the authors highlighted the functions of neddylation and sumoylation in heart physiology and pathophysiology together with the role of ubiquitination. Some years before, PTMs of the ATP synthase subunit found in the heart have been discussed, suggesting the possibility of differential regulation by these modifications in several cardiac pathologies, such as heart failure and preconditioning [6]. Van Eyk also reported that proteomic analysis of cardiac myocytes and tissue is particularly complicated, due to the extended dynamic range that requires effective fractionation or enrichment procedures to facilitate low abundant proteins detection [7]. Moreover, the high number of cardiac isoforms and PTMs in myocyte proteome enhances the composition variability and this biological complexity can interfere with the analysis. They stressed the importance of an accurate quantitative characterization of each modified amino acid in order to explain the dynamic changes of proteins, the interactions between PTMs and their implications on cellular functions. Selected reaction monitoring/multiple reaction monitoring (SRM/MRM) quantitative analysis has been proposed by the author as a robust technique to increase the sensitivity and protein coverage [8].

1.1. Analytical challenges of PTMs: the role of mass spectrometry

The study of PTMs in proteomics is really challenging. One of the challenges in quantifying the level of PTMs at individual sites is the presence of multiple residues on the modified proteins and it is difficult to have specific antibodies that are able to distinguish between closely related epitopes using immunological approaches [9]. Mass spectrometry (MS) has emerged as a powerful method to successfully characterize PTMs in proteomic research. MS is a sensitive and powerful analytical technique used to identify and quantify compounds within a biological sample and clarify their structural and chemical properties [10]. The molecules are characterized based on their mass-to-charge (m/z) ratio and abundance. Multiple ions are generated from the sample of interest by different ionization methods in the ion source, eventually fragmented (tandem MS/MS) and then separated in the analyzer according to their m/z ratio. The ion detector system detects the ions and measures their abundances converting data in electrical signals, thus the obtained mass spectrum displays the ion abundance versus m/z ratio. This entire system works under an extreme vacuum (up to 10^{-8} torr) to avoid non-specific reactions of gas molecules and contaminating ions with the sample, interfering with the correct interpretation of the analytical data. Several ionization methods are available, but electrospray ionization (ESI) [11] and matrix assisted laser-desorption ionization (MALDI) [12,13] are two soft ionization techniques that are commonly used in the protein analysis by MS. MALDI source is usually combined with time of flight (TOF) mass spectrometer, where the ions are detected by measuring the time required for them to travel the length of the flight tube. MALDI-TOF MS technique uses a laser energy absorbing matrix to create ions (i.e., singly protonated ions) from large biological molecules like proteins, which are then accelerated at a fixed potential to the analyzer and separate from each other based on their m/z value [14]. Instead, ESI

source produces multiple-charged ions using an electrospray, in which a high voltage is applied to a liquid to create an aerosol. Coupled with liquid chromatography (LC) for sample fractionation prior to MS analysis, LC/ESI-MS allows the analysis of both small and large molecules in complex biological samples. Mass spectrometers operating in MS/MS mode allow the selection of a specific ion and its fragmentation, in order to obtain additional information about the sequence and structure. The standard fragmentation method is collision-induced dissociation (CID), which is based on the collision of selected ions with molecules of a neutral gas generating a series of b and y ions. In the study of PTMs, alternate fragmentation techniques have been developed to improve PTMs identification and localization on peptide sequences [15]: electron capture dissociation (ECD) [16], electron transfer dissociation (ETD) [17] and higher-energy collision dissociation (HCD) [18]. These complementary fragmentation methods can be also used with large peptides and proteins. They have an important role in the direct analysis of intact proteins by MS (i.e., top-down proteomics) [19], thus giving full sequence coverage and precise localization of PTMs [20].

Several important technical points should be considered in MS analysis of PTMs: the low abundance of the modified peptides, the stability of the modification during the analysis, the mass shift of the peptide molecular weight due to the modification and the ionization efficiency of the modified peptides [21]. Moreover, the determination of the PTMs sites location is often difficult, because not all the modified peptides are easily detectable in MS due to the peptide concentration and sequence. High quality samples with high purity, low degradation and reduced concentration of contaminants are needed in the PTMs analysis [10]. PTM peptides are often present in very low concentration levels compared to their unmodified analogs, which can suppress the signal and further increase the dynamic range of concentrations required in the analysis [2]. The high biological complexity of a wide variety of samples (e.g., biofluids, tissues, cells) makes difficult the quantitation of proteins and the identification of their PTMs [10]. Protein concentration range in biological samples can span about 12 orders of magnitude [22], so sample fractionation and protein/peptide enrichment methods are fundamental to reduce sample complexity and improve the MS detection and quantitation of PTMs (Figure 2). Simultaneous enrichment of PTMs is another important aspect that is emerging in recent years to measure multiple modifications in a single analysis and evaluate the PTMs cross talk [23]. However, this type of study is still rare due to the high difficulties in experimental design and instrument availability.

LC-MS/MS is the method of choice to investigate proteoforms with high sensitivity and accuracy [2]. Several strategies for PTMs study are possible and they can be divided into top-down, bottom-up and middle-down approaches.

Bottom-up approach is the commonly used proteomic method, in which fractionated samples are proteolytically digested and peptides analyzed by LC-MS/MS. The introduction of high-resolution mass spectrometers and peptides separation techniques gave to bottom-up approach great possibilities to investigate modification sites individually on signature peptides. In contrast, top-

down approach involves the direct analysis of intact proteins [24] and allows the study of different isoforms of a modified protein. Several technical limitations have been highlighted over the years, preventing its application and making the bottom-up approach more competitive; the inadequate fragmentation efficiency of high molecular weight molecules and the presence of multiple charges with large precursor ions often reduce the sensitivity of the top-down method compared to bottom-up. Moreover, high efficiency chromatographic separations for proteins are limited, and consequently the quantitative analysis of PTMs in complex biological samples is very difficult. However, with the development of high resolution MS instruments such as fourier transform ion cyclotron resonance (FT-ICR) and Orbitrap, top-down approach is now considered one of the most promising proteomic method for the PTMs investigation. Top-down strategy ensures accurate mass measurements that provide a complete sequence coverage and greatly facilitate the identification of small mass differences between isoforms [22]. Moreover, the combined use of ECD/ETD fragmentation techniques provides several advantages for PTMs localization, identification of new modifications and sequence variations. Top-down MS is very useful in disease mechanisms characterization and in discovery of novel biomarkers and therapeutic targets. Several PTMs studies have been performed in cardiovascular research applying this strategy [25]. As an example, Zhang et al. studied PTMs on cTnl by top-down quantitative proteomics methodology and identified phosphorylation of cTnl as a candidate biomarker for chronic heart failure. This study was the first clinical application of top-down MS for the quantitation of PTMs changes for biomarker discovery from human tissues [19]. In the same way, Dong et al. used high resolution top-down MS to quantify cTnl phosphorylation changes in a spontaneously hypertensive rat model of hypertensive heart disease and failure [16]. The authors demonstrated that protein kinase C phosphorylation of cTnl was potentially associated with a cardiac dysfunction. In addition, an O-glycosylated form of apolipoprotein C-III has been quantified by Mazur et al. in a study on patients with high and low HDL-cholesterol levels and associated with coronary artery disease pathogenesis [26]. High-resolution top-down MS was also applied by Chen et al. to characterize α -cardiac actin and α -skeletal actin isoforms and quantify their relative expression in donor hearts and failing hearts with dilated cardiomyopathy [27]. In this study, they were able to distinguish between isoforms that have high degree of sequence similarity and suggested their clinical involvement as potential biomarkers for cardiac dysfunction. Finally, Gregorich et al. used top-down proteomics to characterize cardiac heterogeneity [28].

Another important aspect that is increasingly drawing attention is the urgent need of panels of PTM-based biomarkers to recognize cardiovascular co-morbidities and steer on a personalized therapeutic regime. Borges et al. proposed a combination of potential biomarkers able to discriminate between CVD and type II diabetes mellitus co-morbidities using a top-down approach [29]. They observed an increase in protein oxidation and truncation that allowed selecting different groups of diabetic patients with or without a myocardial infarction and/or congestive heart failure

history. Hereby, high-throughput large-scale protein quantitation by top-down MS has a great potential in the future to be translated into clinical practice, even if important challenges need to be solved.

A variant of the approaches described above is the middle-down strategy, which combines some advantages of both bottom-up and top-down. Proteins are digested, usually using AspN or GluC enzymes, and larger peptide fragments (masses higher than 3 kDa, but lower than 10 kDa) are analyzed in MS [10]. Jin et al. applied this effective strategy combined with size-exclusion chromatography (SEC) to characterize myosin heavy chain from human heart tissue [30]. The authors identified β -myosin heavy chain as the most expressed isoform in human left ventricular tissue; they achieved a complete sequence coverage that allowed observing, for the first time in human heart tissue, three PTMs: acetylation, methylation and trimethylation. However, when the aim of the study is a well-defined subset of modified proteins, targeted proteomics represents the strategy of choice for a precise and high-throughput measurement [31]. Targeted LC-MS/MS analysis has been widely applied to study and validate novel modification sites, overcoming the traditional use of site-specific antibodies due to their limitations in specificity, availability and production costs. SRM/MRM technique has been recently established as a powerful targeted assay for PTMs quantitation and validation of new modification sites. The assignment of a PTM site is essential to understand its biological effect on protein function [2]. Here, our focus is on the potentialities of SRM/MRM technique in PTMs quantitation and its application in protein biomarkers discovery studies. The most frequent and naturally occurring PTMs are described (Figure 3).

2. SRM/MRM quantitation of post-translational protein modifications

2.1. MS-based targeted proteomics: SRM/MRM approach

MS-based targeted proteomics has steadily grown over the recent years. Key advantages of targeted protein quantitation assays are high specificity, sensitivity, accuracy and reproducibility [32]. Although prior knowledge of the analytes is required, targeted assays represent the most precise available MS-based quantitation tool [33]. One of the most powerful targeted MS approach for a high-throughput quantitation of proteins/peptides is SRM/MRM, whose application has shown great interest in clinical research. The technique is known as selected reaction monitoring (SRM) when a specific target ion is monitored, while multiple reaction monitoring (MRM) when a series of target ions are screened (usually 50–100) [31]. Indeed, MRM MS allows a multiplex quantitative analysis of selected peptides derived from proteins of interest, including variants and modified forms [34]. It is possible to quantify proteins in a wide range of sample types, selecting peptides unique to a particular protein (proteotypic peptides) and highly detectable by LC-MS. These peptides, obtained by a proteolytic digestion of the proteome of complex biological samples, are

also known as quantotypic peptides, because they should be representative of both the protein identity and quantitative content [31]. SRM/MRM is performed using an LC system interfaced to a triple quadrupole (TQ) mass spectrometer (Figure 4A). The first mass analyzer (Q1) is used to select the targeted precursor peptide ion, whose fragments are obtained by CID in Q2 (collision cell). Specific fragment ions are then filtered in the third quadrupole (Q3). Several transitions (precursor/fragment ion pairs) can be monitored over the time, providing information on the absolute peptides abundance that can be used to estimate the concentration of the parent protein in the sample. Many software are freely available for setting up SRM/MRM assay and processing the MS data [35,36]. It is very important to choose the optimal transitions for the SRM/MRM analysis and several software solutions, such as Skyline [37], MRMAid [38], ATAQS [39], MRMer [40] and TIQAM [41], can help to design the entire targeted proteomic workflow. In silico digestion data, MS/MS data, retention time (RT) calculator tools and collision energy (CE) equations are available for the selection of the best transitions and for the optimization of the analytical parameters to improve the MS data quality. As an example, Skyline assists the user to choose precursor and product ions for specific target proteins based on previous results or available publicly spectral library. The user can adjust settings for peptide and transition selection, specify the number of transitions per MS method, and export a transition list that could be directly imported into the mass spectrometer method set-up. Moreover, SRM/MRM data obtained in a preliminary analysis allow identifying the optimal masses, the appropriate RT windows for further scheduled monitoring of each selected peptide and the optimal CE for each of them. The high complexity of the human proteome and the wide dynamic range of protein concentrations require an enrichment step before the MRM analysis, in order to have more sensitivity in the quantitation of endogenous low-abundant proteins, such as PTM molecules that are usually present in very low concentration levels in biological samples. High-abundance protein depletion step and fractionation procedures can improve the limit of detection and quantitation of the analysis [34], overcoming the limitations due to the presence of co-eluting contaminants that may interfere with the targeted transitions (i.e., precursor/fragment ion pairs of interest that are monitored in the MRM analysis).

The workflow for a high-throughput SRM/MRM assay includes several steps (Figure 4B): choice of the proteins of interest considering the biological relevance or clinical involvement in a specific disease, selection of the quantotypic peptides and fragment ions, validation and quantitation of transitions. Peptides selection is very important and undergoes some criteria. Peptides from 7 to 25 amino acids in length are preferred [42] and sites of known PTMs may be avoided, except in case the analysis is specifically focused on the study of isoforms and modified proteins. Moreover, peptides with missed cleavages sites for the proteolytic enzyme (i.e., the enzyme failed to cut one or more sites on the protein sequence) and peptides with two basic amino acids close to the cleavage site at one or both ends (i.e., KK, RR, KR and RK are known as ragged-ends) should be avoided. Online proteomics repositories, such as PeptideAtlas [43], Global Proteome Machine

Database [44], PRoteomics IDentification database (PRIDE) [45] and SRMATlas [46], that contain sequences and MS/MS spectra of peptides that have been already detected in previous analysis, can be consulted in order to choose the best peptides. When the experimental data are not accessible, computational software tools (e.g., ESP predictor [47] and PeptideSieve [48]) can be used to predict the transitions with the best LC and MS behaviors for each protein. The performance of the MRM assay is further optimized selecting the most intense fragment ions for each peptide, in order to have an higher sensitivity and minimize the interferences. Transitions can be optimized and validated experimentally by using synthetic reference peptide standards. Usually, the best two-four transitions for doubly and triply charged peptides are selected and it is recommended to choose ions with m/z values higher than the precursor mass, avoiding ions with m/z values close to the precursor because of their noise. Moreover, it is essential to optimize collision energy and duty cycle for each transition.

A scheduled SRM/MRM analysis can be performed to increase the number of transitions analyzed in a single chromatographic run without a loss of analytical performance, thus improving the efficiency of the assay and maintaining the high sensitivity. The transitions are acquired only in a defined small time window that corresponds to the elution time of the peptide; for this reason, the retention time of the targeted peptides must be known previously by means of experimental runs or predictive tools, and chromatographically reproducible [34].

Quantitative analysis is possible using both label and label-free methods (Figure 4C). A scheme with the relative and absolute protein quantitation strategies used with SRM/MRM MS is provided in Table 1. A relative quantitation is performed using a stable isotope dilution (SID) approach, in which the signal area or intensities of the isotope-labeled peptides from the control sample are compared with their unlabeled analogs in the pathological state [42]. A less costly alternative to SID is the labeled reference peptide (LRP) method, in which a single isotopically labeled peptide is used as a normalization reference for all target peptides [49]. Anyway, the most commonly used approach considering the MRM potentialities is the absolute quantitation, which provides a solid and precise analysis. Isotope-labeled internal standards are added to the sample at a known concentration before any pre-processing. These labeled standards are chemically identical to their endogenous peptides/proteins, but with a mass difference due to the isotope labeling. They have identical MS/MS fragmentation pattern with their unlabeled counterpart and both compounds co-elute in the chromatogram. Co-elution guarantee the correct signal identification and quantitation. Frequently, the analysis involves the use of stable isotope-labeled internal peptides (absolute quantification [AQUA] peptides), thus the peaks areas or intensities comparison between the endogenous peptide and spiked labeled standard provides an estimation of the concentration of the protein. Additionally, other strategies are available to perform an absolute characterization: quantification concatamers (QconCATs) that are generated by concatenating isotopically labeled peptide sequences of the molecules of interest into artificial proteins [50]; protein standard for

absolute quantification (PSAQTM) that involves the design of full-length isotope labeled proteins analogs of the targeted endogenous [51]; and, finally, stable isotope standards and capture by anti-peptide antibodies (SISCAPA) that requires the use of anti-peptides antibodies for the enrichment of targeted proteins [52].

MRM-initiated detection and sequencing (MIDAS) technique involves a hybrid quadrupole linear ion trap mass spectrometer with both triple quadrupole and linear ion trap capabilities. This combination provides a great opportunity for the studies on PTMs.

2.2. Quantitative detection of PTMs by SRM/MRM MS

We now discuss the use of SRM/MRM MS approach in the detection and quantitation of PTMs and its application in the cardiovascular proteomics. In the recent years, SRM/MRM has been successfully used to investigate the main protein modifications, thanks to the multiplexing capability and high accuracy that allow the quantitation of a protein and its circulating disease-modified forms in the same analysis.

Quantitation of PTMs is a real analytical challenge and SRM/MRM method using stable isotope-labeled modified standards provides a specific, sensitive and unambiguous analysis of the modified proteins [34]. SRM/MRM MS has emerged as a useful quantitative method to overcome several limitations of immunoassays, mainly related to the specificity of antibodies and their capability to distinguish sequence variants. Frequently, some antibodies are not able to detect specific modification sites on proteins, while MRM assures site-specific quantitation of PTMs with higher analytical reproducibility and lower costs [34].

Analysis of PTMs is often complicated by the poor chromatographic separation of some modified peptides that are not easily detectable in MS due to low ionization efficiency [31]. Moreover, besides the enzymatically controlled modifications, chemically induced PTMs can be generated *in vivo* or during sample preparation before the MS analysis, compromising the quantitation of the peptides of interest.

Another important aspect to be taken into consideration is the enzyme used for the digestion of the proteins. Trypsin is the most commonly used enzyme, but in case of tryptic peptides not suitable for SRM/MRM analysis (i.e., too long or too short peptides sequence) it is recommended to employ other proteases, if needed also in combination, such as Lys-C, Asp-N, Glu-C, chymotrypsin and elastase [9,53-55]. Moreover, acetylated or methylated lysine and arginine residues are resistant to trypsin digestion, whereas glycans often inhibit trypsin activity.

PTMs are present sometimes in more than one site within the amino acid sequence, so it is important to evaluate all possible combinations of modified and unmodified peptides and the ratio between them within the same sample [56], and not only among several samples. SRM/MRM analysis allows the estimation of PTM site occupancy, in order to clarify the biological impact of a specific PTM especially when multiple PTMs are present at the same amino acid.

In the next paragraphs the most common PTMs and MS-based approaches to quantify them will be discussed.

2.3. Most common enzymatic PTMs

2.3.1. Ubiquitination

Protein ubiquitination is an enzymatic PTM in which a single ubiquitin molecule (monoubiquitination or multi- monoubiquitination if the proteins are modified at multiple Lys residues) or a chain of ubiquitin (polyubiquitination) is attached to a targeted protein [57-61]. Ubiquitin binding to substrate proteins determines the removal of the protein by the proteasome or the alteration of its activity, localization or affinity to other proteins [62]. Indeed, ubiquitination is an important PTM involved in protein degradation, regulation of intracellular protein trafficking and protein-protein interactions, modification of cellular localization, cellular signaling and DNA damage response [63,64].

Moreover, ubiquitination has a central role in cell division and migration, providing important signals used in cellular control of stability and function [65]. Specific domains (ubiquitin binding domains, UBDs) are able to recognize and non-covalently bind the ubiquitin, in order to ensure the distribution of the ubiquitin signal for different molecular processes.

Proteins modification can occur on eight possible linkage sites, seven Lys residues and the amino-terminal Met [66]. The linking residues are identifiable by a K or M and a number corresponding to the position in the ubiquitin molecule (K6, K11, K27, K29, K33, K48, K63 and M1). The C-terminal carboxylate group of the ubiquitin molecule (glycine) most commonly binds covalently a specific lysine, but can also bind other sites called “non-canonical ubiquitination”, such as cysteine, serine, threonine or N-terminus of the target protein.

Ubiquitin can form several linkage-specific homotypic and heterotypic polymers on substrate proteins, generating a biological diversity of cellular responses. Moreover, the heterotypic ubiquitin chains can also be mixed, branched or modified by other PTMs, such as acetylation [67] and phosphorylation [68,69]. This assortment of the ubiquitin conjugates structures significantly increases the complexity of functions making the understanding of the ubiquitin control on cellular pathways not easy [62].

Alterations in the ubiquitination process give rise to cellular dysfunctions that cause multiple human diseases (e.g., neurodegenerative diseases, cardiovascular diseases and inflammatory diseases), cancer development and paediatric disorders [65]. Therefore, it is necessary to identify ubiquitinated proteins and their modification sites, in order to increase the knowledge of the ubiquitination mechanisms and improve the therapeutic strategy against several pathologies. During years, high-resolution MS analysis has demonstrated the highly heterogenous nature of polyubiquitin chains conjugates and the continuous improvements in MS technologies have increased the knowledge of the molecular structures and the multiple related functions. The

increase of the molecular weight of the ubiquitinated proteins depends on the number of conjugated ubiquitin molecules, so different protein forms need to be analyzed by MS. After digestion of ubiquitinated proteins with different proteases, it is possible to obtain unique peptides with a specific tag, which are used to recognize the ubiquitination sites through MS and, consequently, the corresponding ubiquitinated proteins. As an example, the trypsin cleavage of the ubiquitin-protein complex leaves two glycine (Gly) residues (monoisotopic mass is 114.04 Da) on the modified Lys of the target protein, producing specific peptides that are readily detected by MS. Occasionally, trypsin misses to cleave the peptide bond between Arg and Gly on the conjugated ubiquitin and a short adduct (Leu-Arg-Gly-Gly) remains on the substrates with a monoisotopic mass of 383.23 Da [70]. MALDI-TOF MS technique has been used for the detection of the ubiquitination sites in combination with immunoprecipitation of the ubiquitinated protein and its separation by gel electrophoresis [71,72]. However, this approach has many shortcomings, such as the need of gel separation before the MS analysis, the difficulty in the identification if there are other PTMs on the peptide and the relative low signal of the ubiquitinated peptides in the MS spectra [70]. To overcome these limitations, LC-MS/MS has been introduced to study ubiquitination sites, in combination with enrichment techniques of the protein sample before the MS/MS analysis [73-75]. LC enables an accurate separation of the components within a proteins/peptides mixture, which are then introduced into a mass spectrometer for the MS/MS sequencing. MS/MS analysis can provide greater structural information and facilitate the identification of PTMs site localization. However, the endogenous proteins are often ubiquitinated at low levels under physiological conditions and rapidly degraded by the proteasome or regulated in several cell signaling pathways [70]; thus, it is not easy to identify the ubiquitination sites using data-dependent experiments. Moreover, the ubiquitin has a larger size than other PTMs. For all these reasons, direct MS detection of the ubiquitination sites is very challenging. Therefore, as mentioned above, an enrichment of the ubiquitinated proteins or peptides is very helpful before the MS analysis to reduce the initial complexity of the analyzed sample, for example using affinity tags engineered at the N-terminus of ubiquitin or specific monoclonal antibodies. The use of affinity-tagged ubiquitin allows for the easy identification of hundreds of ubiquitination sites [70]. It is possible to obtain a more significant enrichment also purifying fragments derived from the ubiquitinated portion of proteins using chemical methods, such as cyanogen bromide (CNBr) or 2-nitro-5-thiocyanobenzoic acid (NTCB) [76], before the affinity tags procedure [77]. In addition, another method to purify the ubiquitinated proteins is based on the interaction with ubiquitin binding domains (UBDs); indeed, several UBDs can be used to recognize multiple different ubiquitin chains with high affinity [78-80], but this approach has some difficulty in the identification of monoubiquitinated proteins. In combination with these enrichment strategies, high-resolution mass spectrometers are recommended for data-dependent analysis in order to reduce false positive identifications, and

also different MS/MS fragmentation methods can be useful to have a complete view of all ubiquitination sites [81].

Ubiquitination is one of the most difficult PTMs to be identified and, sometimes, more precise and highly sensitive quantitative proteomic techniques are necessary to elucidate the complexity of protein ubiquitination in cell signaling and disease states. For this aim, MRM technique allows performing a targeted analysis for a global mapping of ubiquitination sites, without the use of large quantities of sample material and multiple analysis to obtain a full coverage. Data-dependent experiments alone are not adequate to characterize all combinations of ubiquitination, something that is happening using the high selectivity and sensitivity of MRM method. The application of MRM has been described in several proteomic studies to facilitate the interpretation of the ubiquitin topology and biological roles [82-85]. Monoubiquitination usually controls protein interactions and trafficking, whereas polyubiquitination is associated to protein signaling and proteasomal or autophagic degradation [86]. However, the different topologies of polyubiquitin chains allow characterizing a more complex regulatory activity. In particular, K48-linked chains and K63-linked chains have been well characterized up to now [87,88], while the knowledge of the precise role of the other lysine chains, M1-linked chains, branched and mixed chains is still limited. Ordureau et al. observed that K6-linked ubiquitin chains are present in mitochondrial outer membrane (MOM) proteins upon depolarization of the organelle [69]. In Parkinson's disease patients, the PARKIN E3 ubiquitin ligase is mutated and is involved in mitochondrial damage; indeed, in damaged mitochondria PINK1 accumulates on the MOM and stimulates the PARKIN recruitment that promotes the ubiquitination of numerous MOM proteins. The authors used an absolute quantification (AQUA)-based proteomics approach to determine the total abundance of ubiquitin conjugated on mitochondria that showed an increase of ~6-fold upon depolarization [69]. In details, the phosphorylation of PARKIN led to the elevated synthesis of not only K6 linkages, but also other multiple ubiquitin chain types, i.e. K48, K63 and non-canonical K11. Anyway, further studies are necessary to better elucidate the exact function of K6 and K11 linkages in this context. Several studies, instead, have reported a potential role of K27 linkages in the DNA damage response and innate immunity. Using SRM analysis, Gatti et al. showed that RNF168 ubiquitin ligase promotes the K27 polyubiquitination of the histones H2A and H2A.X both *in vitro* and *in vivo* [89]. The authors expressed wild-type FLAG-ubiquitin alone or with RNF168 in human embryonic kidney (HEK) 293T cells, extracted chromatin fraction, and enriched the ubiquitinated proteins that were tryptic digested and analyzed by SRM for an absolute quantitation. Moreover, they transfected HEK293T cells with FLAG-tagged K27-only ubiquitin mutant with or without RNF168 and performed a shotgun MS analysis, evaluating all proteins subjected to K27 ubiquitination. MIDAS technique has been successfully applied for the detection and characterization of ubiquitination. Mollah et al. performed an interesting study on receptor interacting protein (RIP), a mediator of the proximal TNF receptor 1 signaling complex. MIDAS workflow followed by full

MS/MS analysis for sequence confirmation was implemented to simultaneously target in a single experiment over 100 potential ubiquitination peptides on recombinant RIP [85].

Recent advances in high-resolution MS instruments and software allows to further improve the quality of the MRM analysis detecting all fragment ions and not only a subset of transitions per peptide [90], using the methodology called parallel reaction monitoring (PRM). In PRM, all products are measured simultaneously and this provides higher resolution and precision, higher confidence sequence identification and the feasibility to perform large-scale experiments [91]. This methodology has been successfully applied to quantify polyubiquitin chains expressed in low-abundance in Alzheimer and Parkinson's patients [92]. Ordureau et al. [93] used PRM approach to study the phosphorylation of ubiquitin on damaged mitochondria. They demonstrated that PARKIN and PINK1 were involved in autophagy of damaged mitochondria through polyubiquitination of numerous MOM proteins; PINK1 promotes phosphorylation of residue Ser65 (S65) of ubiquitin itself and in the ubiquitin-like domain of PARKIN, which is essential for the activation of PARKIN ubiquitin ligase and for the enrollment of autophagy receptors to the damaged mitochondria. The authors used PRM approach to assess total ubiquitin, chain linkage types and to detect phosphorylated S65 sites (p-S65) in ubiquitin in total purified mitochondria from nondepolarized cells expressing PARKIN^{WT} and depolarized cells expressing either PARKIN^{WT} or PARKIN^{C431S} (catalytically defective PARKIN).

2.3.2. Phosphorylation

Phosphorylation of a protein is the attachment of a phosphate group. Reversible phosphorylation of proteins is an important regulatory mechanism and can occur on several amino acids. The identification of phosphoamino acids in proteins is crucial, because phosphorylation influences protein biological functions regulating signal transduction, differentiation, cell division and metabolism [94]. Phosphorylation study is still a challenge in the proteomic research area, because protein phosphorylation is a highly dynamic PTM; some protein kinases and phosphatases modulate the function of target proteins, defining their phosphorylation state. Moreover, the alteration of this phosphorylation process can be responsible for some metabolic diseases and cancer progression. Therefore, sensitive methods are essential to evaluate the role of phosphorylation in normal or disease events, and offer a better knowledge for the development of new therapeutics. MS-based phosphoproteomics has been extensively employed over recent years, due to the advancement of MS techniques and the development of more efficient enrichment methods for phosphorylated proteins and peptides [95]. SRM/MRM assay offers the most specific, sensitive, and precise quantitative analyses of phosphopeptides. Phosphopeptides have complex intrinsic biological and chemical properties and they are usually present in low abundance; for these reasons, their MS detection is quite difficult because of the adherence to surfaces during LC-MS/MS and a poor ionization. Phosphopeptides enrichment by immobilized

metal ion affinity chromatography (IMAC) or other strategies (e.g., titanium or zirconium dioxide) can improve MS ionization and increase the sensitivity of MRM analysis further [96]. Titanium dioxide (TiO₂) provides high selective phosphopeptides enrichment and it is the most used method with complex biological samples; this approach is complementary to IMAC, because different phosphopeptide subpopulations are selected, and it is perfect for large-scale phosphoproteomics [97]. Before the application of these methods, a protein enrichment using phosphoamino acid-specific antibodies or phosphorylated consensus sequence-specific antibodies can be applied to ameliorate the analysis. Four types of phosphoamino acid residues are actually known: O-phosphates, obtained by the phosphorylation of hydroxyamino acids (serine, threonine or tyrosine); N-phosphates, formed by the phosphorylation of the amino groups in arginine, lysine or histidine; acyl phosphates, generated by the phosphorylation of aspartic or glutamic acid; finally, S-phosphates, produced by the phosphorylation of cysteine. It is established that the MS provides unambiguous detection of the sequence position of the modified residues and MS/MS analysis can differentiate sequence isomers [34]. Different MS scan modes can be used to identify unknown phosphoamino acid sequences; neutral loss scanning is useful to monitor peptides that lose a phosphate group (e.g., -98 Da, corresponding to H₃PO₄, -49 Da for doubly and -32.7 Da for triply charged peptides) from the parent ion when fragmented. Precursor ion scanning, instead, is applied to detect in negative ion mode the ion at *m/z* 79 (corresponding to PO₃⁻) for serine/threonine phosphorylation; in particular, this type of scan can be also performed in positive ion mode to identify the immonium ion at *m/z* 216.043 for tyrosine phosphorylation [31]. Although these MS methods are very useful for identifying unknown phosphopeptides from a complex mixture, targeted MRM approach allows the study of specific modified peptides, taking advantage of prior information about the protein of interest, and it does not require high amount of protein sample for the analysis. The knowledge of the amino acid sequence, the potential type of phosphorylation and phospho-consensus sites are information very helpful to design MRM transitions for the identification and quantitation of all possible phosphopeptides [98]. MIDAS approach allowed in several studies an efficient analysis of low-level protein phosphorylation and the evaluation of different fragmentation pathways with high sensitivity and specificity [96,99]. A list of the MRM transitions of the candidate phosphopeptides is created considering an addition of 80 Da (HPO₃) to the calculated mass of a peptide for every phosphorylated residue in the sequence; this calculation is usually performed on both doubly and triply charged forms of the peptide. However, because of the instability of the phosphoryl group, some modified peptides can lose their phosphoryl group during the ionization phase. Therefore, it is possible to observe a loss of 80 Da or 98 Da (if the phosphate group is lost as H₃PO₄) for each putative phosphorylated serine or threonine amino acids, whereas for phosphopeptides that contain tyrosine there can be only the loss of 80 Da. Of course, the loss of the phosphoryl group generates additional challenges to MS and MS/MS-based quantitation [100]. The linear ion trap scans combined with MRM analysis can

be helpful, because they offer a reliable confirmation of the phosphopeptides sequence detected in MRM and the exact position of the phosphorylation sites [96].

In 2012, Narumi et al. performed a phosphoproteomic study using Fe-IMAC enrichment coupled with the isobaric tags for relative and absolute quantification (iTRAQ) technique and SRM method for the identification of candidate prognostic biomarkers in human breast cancer tissues of high- and low-risk recurrence patients [101].

Some years later, Carr et al. successfully applied MRM technique to quantify the calcineurin (CN) phosphatase activity by measuring the dephosphorylation of a synthetic phosphopeptide substrate in peripheral blood mononuclear cells (PBMCs) isolated from calcineurin inhibitors (CNIs)-treated kidney transplant patients and healthy subjects [102], with the aim to monitor CNIs dose in a large-scale of transplant patients in clinics.

In a similar way, Yang et al. employed a cell-penetrating peptide biosensor and MRM technique to detect phosphorylated peptides resulting from Bcr-Abl activation and inhibition by imatinib in the human chronic myelogenous leukemia (CML) cell line K562, providing a potential model for monitoring drug sensitivity [103].

Recently, Whiteaker et al. reported a detailed method to multiplexed measure phosphopeptides. The protocol is focused on the ATM kinase phosphopeptides and their unmodified analogs in cellular lysates from cultured HeLa cell lines [32]. The authors pointed out the need of a peptide immunoaffinity enrichment before the MRM analysis in order to have appropriate sensitivity for the quantification of low-level analytes in complex biological matrix. Therefore, this quantitative immuno-MRM assay provides a view of the phospho-signaling and it could be adapted to other phosphopeptides in different samples.

Few studies have been performed on human plasma to investigate the variations in plasma phosphoproteins, which are very important to understand disease pathophysiology. As an example, Zawadzka et al. developed and optimized a strategy to identify phosphopeptides in plasma and measure the variability in plasma protein phosphorylation in healthy individuals using MRM MS [104]. In addition, the authors achieved SWATH (sequential window acquisition of all theoretical fragment ion spectra) MS2 data-independent acquisitions to compare the sensitivity and quantitative performance of this technique with the MRM assay. SWATH-MS2 analysis was performed with the aim to reduce sample volume, optimize the experimental time and increase the numbers of quantifiable phosphopeptides. Similar and complementary results were obtained with the two methods, corroborating their accurate and reproducible quantitation of PTMs. The quantified phosphopeptides derived from numerous proteins that are involved in homeostasis, immune response, cell surface interactions, lipid metabolism and signaling pathways. Several plasma protein phosphorylation sites were discovered, but there is little known about the biological variability in these sites among healthy human population. Therefore, this study was a good

reference for future analysis that target human phosphoproteins as potential biomarkers for disease diagnosis or monitoring progression of drug treatment.

2.3.3. Glycosylation

Glycosylation is a very complex PTM, which provides great proteomic diversity. The glycosylation process involves the linkage of monosaccharides together, the transfer of sugars from one molecule to another and the removal of sugars from the glycan structure. Many enzymes are responsible of the glycan synthesis and structure, and their activity is different based on the type of cell or intracellular compartment. Glycosyltransferases are enzymes that control the glycan length, transferring mono- or oligosaccharides with different specificity. Instead, glycosidases catalyze the hydrolysis of glycosidic linkages, removing sugars that are linked to a given protein [105].

Glycosylation involves several glycosidic linkages, including N-, O-, C- and S-linked glycosylation, glypiation (GPI anchor attachment), and phosphoglycosylation. Proteins are often glycosylated at multiple sites with different glycosidic linkages. N- and O-glycosylation are the most commonly detected types of glycosylation. In N-glycosylation (e.g., N-GlcNAc or N-acetylglucosamine addition) a glycan binds to the nitrogen of asparagine residue, while in O-glycosylation (e.g., GalNAc or mucin-type O-glycosylation) the glycans are mostly attached to the hydroxyl oxygen of serine, threonine or tyrosine. Both types of glycosylation occur in secreted and membrane proteins [106]. N-glycosylation begins in endoplasmic reticulum and N-glycans are involved in protein folding, then the folded glycoprotein is transferred to the Golgi apparatus where is further modified to obtain mature N-glycans with different biological functions and conjugated to secreted or membrane proteins. O-glycosylation occurs in Golgi apparatus by different GalNAc transferases. Sometimes both modifications can be observed in the same protein, increasing the complexity of the glycosylated species.

Glycosylation is important in many biological processes, including cell attachment to the extracellular matrix and protein-ligand interactions in the cell. O-linked glycans assist the protein localization and trafficking, protein solubility, antigenicity and cell-cell adhesion. N-linked glycans control protein stability and cell signaling, but they play an important role also in the immune system, by regulating the migration pattern of the immune cells. Therefore, mutations in genes involved in glycosylation result in several diseases, such as disorders that affect the nervous system, cancer progression and autoimmune diseases. Glycoproteins are often used as pharmaceuticals and alterations in glycosylation can influence bioactivity, pharmacokinetics, immunogenicity, and allergenicity [107].

Glycoproteins can be purified and analyzed by different strategies, including glycan staining, glycan crosslinking to agarose or magnetic resins, and MS analysis. A common large-scale characterization of glycoproteins is performed by separate analysis of detached glycans (glycomics) and peptides obtained by an enzymatic or chemical deglycosylation (i.e., indirect MS

analysis) [106,108]. The analysis of detached glycans often involves permethylation, which is the addition of methyl groups (CH₃) to all of the hydroxyl and N-acetyl groups. Permethylation improves and enhances the ionization efficiency of glycans on MS when compared to non-derivatized oligosaccharides. It stabilizes glycans and prevents gas phase rearrangements, allowing the detection of both neutral and acidic glycans using MALDI-TOF MS or the relative and absolute quantitation by LC-MS analysis. In addition, derivatization of the reducing end or terminal sialic acid is a useful strategy for structural and quantitation HPLC-MS analysis of glycans. N-glycans are analyzed after labelling the reducing end of the sugars with a fluorescent compound, such as 2-aminobenzamide, aminopyridin or 2-aminoacridone, while the study of O-glycans is carried out without any tags.

The most common technique for the analysis of protein glycosylation is HPLC-MS/MS, but several important aspects need to be considered in the detection and quantitative studies. Firstly, the proteolysis efficiency of glycans is critical; O-glycoforms tend to cluster and are resistant to proteolysis, that sometimes can be inhibited also by N-glycosylation. For example, proteolysis has been facilitated and improved by the used of immobilized proteases and denaturation under pressure cycling to 25 kpsi [109]. Moreover, glycopeptides have a lower ionization efficiency and they are in low abundance with multiple glycoforms per peptide. Therefore, many successful strategies for glycopeptides enrichment were developed over the years, including lectin affinity enrichment, covalent interactions, and chromatographic separations or solid-phase extraction (SPE) [107]. Lectins recognize glycan structures attached to glycoproteins and they have been used in different affinity formats. A limitation of lectin affinity chromatography is that a given lectin binds a specific glycan, so only a subgroup of glycopeptides is captured; mixtures of lectins or other fractionation steps, such as hydrophilic interaction liquid chromatography (HILIC), may be used to have a wider coverage of the glycoproteome. Another method employed in combination with lectins is PNGase F under H₂¹⁸O for deglycosylation of the peptides [110]. Methods based on covalent interaction between the glycopeptides and functionalized solid supports can be performed using a hydrazide solid support [111,112] or boronic acid interactions [113,114]. The enrichment and separation of glycopeptides based on their physico-chemical properties is an alternative approach and different stationary phases are available; for example, detached glycans can be successfully analyzed by porous graphitic carbon (PGC) chromatography with LC-MS/MS detection [115,116].

Deglycosylation facilitate the analysis separating the core protein from the glycan, but it does not provide a direct comprehension of the interaction between them [117]. For this reason, the combined analysis of the glycan motif and the protein, known as glycoproteomics, has seen a rapid evolution in the last few years in order to perform a direct assessment of glycosylation. Protein sample is enzymatically digested and the obtained glycopeptides are enriched using zwitterionic hydrophilic interaction (ZIC-HILIC) LC or other similar approaches. The MS/MS analysis is

performed using the combination of two fragmentation methods, HCD and ETD, which allow the direct characterization of the intact glycopeptides. Glycosidic bonds are broken by HCD fragmentation, while ETD maintains the glycan attachment and fragments only peptide backbone [118]. As an example, Yin et al. provided the most comprehensive list of human glycosylated endothelial proteins to date applying three proteomic strategies, including HCD-ETD workflow [119]. In 2016, Mayr and colleagues defined the glycosylation profile of human cardiac extracellular matrix (ECM) proteins and evaluate ECM remodelling during persistent atrial fibrillation [120]. The efficient glycopeptides enrichment, the MS-based analysis of detached glycans and glycosylated peptides and ever more updated informatic solutions allowed an important glycoproteomic discovery study, which still needs a targeted verification workflow in order to quantify the differences in abundance, especially in different physiological or diseased contexts. Multiplex MRM MS approach is suited for glycan biomarker research and allows quantifying a large numbers of glycopeptides in a single analysis. As an example, Ahn et al. applied a lectin-coupled MRM MS platform to identify multiple biomarkers from human plasma according to liver cancer progression [121]. The authors enriched with a specific lectin the aberrantly glycosylated proteins and quantified them by MRM as potential biomarkers for hepatocellular carcinoma (HCC). MRM analysis of site-specific glycoforms of proteins provides a more specific information than the quantitation of detached glycans or deglycosylated peptides. The efficiency of ionization depends on the peptide sequence and also on the attached glycan. Moreover, the MRM design is strongly dependent to the fragmentation of glycopeptides. CID fragmentation of glycopeptides in positive mode generates dominant Y- and B- ions (glycosidic bond fragments) [106]. In particular, intense glycan fragments (B-type oxonium ions) and glycosylated peptide Y-ions characterize MS/MS spectra of N-glycopeptides. The fragmentation of the peptide backbone is limited and the amount of b- and y-ion fragments observed depends on the peptide sequence. Instead, the major fragment of the O-glycosidic bond is typically the Y0 fragment.

Hong et al. used the high intensity oxonium ions (i.e., typical glycan fragment ions under CID) to quantify IgG, IgA and IgM glycopeptides directly in digests of plasma without protein enrichment [122], and they were able to quantify immunoglobulins and their site-specific glycans simultaneously using MRM. Specific and non-specific proteases, trypsin and pronase E, were applied to obtain a complete site-specific N-glycan map of the abundant serum glycoproteins and LC-MS/MS analysis was performed to characterize the fragmentation profile of these glycopeptides. Then, only the tryptic peptides were used to design the MRM analysis, because the specificity of trypsin provides well-defined peptides suitable for quantitation. Glycosite coverage is better with non-specific proteases, but they usually produce variable peptide sequences that complicate data analysis and quantitation. The authors demonstrated that it is possible to study glycosylation of immunoglobulins by MRM directly from serum without any sample cleanup. This

method could be applied to other glycosylated proteins with a protein enrichment to increase the signal of low abundant molecules and the specificity of quantitation.

An increase in sensitivity and specificity for particular glycopeptides can be achieved by multiple reaction monitoring cubed (MRM³) quantitation. Fortin et al. used MRM³ to quantify O-glycoforms of hemopexin, demonstrating the power of this technique [123]. Fragmentation of O-glycopeptides produced dominant Y0 ions (MS2), which were then selected for the ion-trap fragmentation. Monitoring of linear ion trap peptide fragments allowed obtaining an increased sensitivity of detection of a low-abundant glycoform of hemopexin compared with the MRM approach. In 2016, Kumar et al. applied MRM and MRM³ to detect and quantify, in undepleted healthy human plasma, apolipoprotein F (Apo-F), a novel and promising low abundant liver fibrosis biomarker [124]. MRM³ allowed removing interferences, obtaining improved quantitation data compared to MRM. The authors also demonstrated the possibility to detect and confirm a glycopeptide using MRM-enhanced product ion (EPI) scanning on a QTRAP instrument.

Recently, Lin et al. applied a targeted high resolution MRM strategy to analyze purified glycoproteins and SWATH for an accurate investigation of highly complex biological samples [125]. Both approaches allowed high quality data for the interpretation of glycopeptide identity and structure as well as a reliable quantitation. Data-independent acquisition (DIA) did not require the use of a spectral library with already known glycan compositions, but it allowed detecting and quantifying unexpected glycan structures. The power of this workflow was demonstrated by the identification of 59 N-glycosylation sites from 41 glycoproteins from a HILIC enriched human plasma digest.

In the same way, Sanda et al. used a DIA workflow of N-glycopeptides with soft CID fragmentation to study fucosylated complex glycoforms of ten abundant plasma glycoproteins in liver cirrhosis patients [126]. In contrast with the previous study by Lin et al., the authors established a library of N-glycopeptides and their glycoforms by data dependent LC-MS/MS and scheduled PRM analysis of a tryptic digest of 14 human proteins retained on a multiple affinity removal column.

Finally, Darebna et al. used MRM to quantify changes in N- and O-glycopeptides in patients with colorectal cancer and hepatocellular carcinoma and they also proposed full scan high resolution FT-ICR strategy as a reliable alternative for the detection of glycan differences between different types of cancers or diseases [127].

2.3.4. Acetylation

Protein acetylation is one of the major PTMs in the cell, in which an acetyl group from acetyl coenzyme A is transferred to a specific site on a polypeptide chain [128]. The acetyl group can be co- or post-translationally attached on the N-terminus of proteins and on the side chain of lysine residues of several proteins, among which non-histone proteins and histones/transcription factors, thus affecting chromatin functionality [129]. N-terminal and lysine acetyltransferases (NATs and

KATs, respectively) are responsible for the acetylation process. N-terminal acetylation is an irreversible modification and it is generally involved in the synthesis, stability and localization of proteins. Moreover, protein N-terminal acetylation plays an important role in cell cycle regulation and apoptosis, and they are implicated in tumorigenesis and neurodegenerative diseases [128]. Instead, lysine acetylation is enzymatically reversible and it is regulated by the activity of lysine deacetylases or histone deacetylases. Therefore, a good balance of acetylation and deacetylation is fundamental for important cellular processes. However, lysine acetylation is not only limited to histone proteins, but it significantly affects other proteins involved in signal transduction and regulation of the kinase activity [130,131].

In recent years, with new techniques emerging in MS field, protein acetylation has been significantly studied. Many protein acetylation sites have been identified, but not all the acetylation events are explained because of their dynamic property and low abundance. Several studies also described important links between acetylation and other PTMs (e.g., phosphorylation and methylation) [132]. Immunoaffinity purification strategies are necessary to enrich lysine-acetylated proteins and peptides before the MS/MS analysis. After a protein digestion, isoelectric focusing, strong cation-exchange (SCX) chromatography or other purification methods allow the peptides enrichment by specific antibodies. High-resolution MS instruments and computational data analysis are finally used to identify and quantify the extent of acetylation. Some years ago, Zhao et al. separated human liver tissues into nuclear, mitochondrial, and cytosolic fractions in order to study non-nuclear protein acetylation, leading to the identification of more than 1300 acetylated peptides [133].

Concerning the analysis of histone acetylation, some sample preparation steps are essential to facilitate the MS investigation; for example, the histones are extracted in presence of acids or high concentration salts and further purified using different techniques (e.g., reverse-phase HPLC, SDS-PAGE, ion-exchange chromatography, HILIC and capillary electrophoresis). In particular, HILIC can simultaneously separate acetylated and methylated histones [134]. The MS identification can be performed based on a bottom-up approach or a top-down approach. The derivatization of histones with propionic anhydride is an effective method to reduce the complexity of the peptide mixture, generating specific peptides with a mass shift of +56 Da. In addition, this strategy can be combined with an esterification reaction in order to apply a quantitative analysis of different histone samples. On the other hand, the analysis of the intact histones directly at the protein level allows a complete sequence coverage, but the need to use expensive and not easy high-resolution MS instruments (e.g., FT-ICR) as well as an high-efficiency prefractionation step reduces its application with respect to the bottom-up approach. For the first time in 2013, Evertt et al. studied the dynamics of histone modifications and quantified the rate at which histones are acetylated and deacetylated on a residue-specific basis [135]. Over the years, the epigenetic remodeling of chromatin histone proteins by acetylation has been also studied for the identification of biomarkers

indicative of late onset cognitive loss. Zhang et al. applied SRM-based targeted proteomics to quantify the histone acetylation in human brain tissue from AD patients with different degrees of vascular amyloid deposition and neurologic controls [136].

Recently, Thomas et al. applied a LC-MS/MS analysis to study the lysine-directed PTMs of protein tau, which is associated with neurofibrillary lesion formation in the pathogenesis of AD [137]. Lysine-directed PTMs, such as acetylation, ubiquitination and methylation, control tau turnover and aggregation. Therefore, deeper detection and quantitation of these modifications are important for drug target discovery and validation [138]. In general, the MS data processing should consider an addition of 42.01 Da (under CID fragmentation) for each acetylated lysine residue. It is important to consider that lysine trimethylation has a similar mass of 42.04 Da that usually requires a high-resolution instrument to distinguish both PTMs. Beside CID fragmentation, also ETD has been recently applied to study acetylation. Sweredoski et al. described a PRM method, using ETD fragmentation, to quantify the effects of histone modifying enzyme drugs, such as histone deacetylases inhibitors, on the histone PTMs [54]. In particular, the authors focused the attention on the acetylation and methylation events on the histone H3 after treatment with butyric acid.

2.3.5. Methylation

Methylation is another important PTM that, like acetylation, controls the function of several proteins together with histones. Protein methylation involves the addition of methyl groups on arginine or lysine amino acids in the protein sequence. Enzymes known as protein arginine methyltransferases (PRMTs) and lysine methyltransferases (KMTs), respectively, catalyze the transfer of methyl groups from S-adenosylmethionine to the nitrogens of Arg and Lys residues [129]. Methylation occurs on single or multiple sites of a protein sequence and can regulate protein-protein interactions and other important protein functions, such as enzymatic activation of receptor tyrosine kinases.

Methyl-specific antibodies are available for affinity purification and have been successfully used to study human methylome in combination with high-throughput MS analysis [139].

Protein methylation has been mainly studied in the histones, because it is known that methylated histones play an important role in either activation or repression of gene transcription, based on the site and state of the modification. Moreover, some studies have described a cross talk between methylation and other PTMs, such as phosphorylation pathways [140], even if the knowledge is still incomplete.

Liu et al. developed an integral strategy that combines peptide array, bioinformatics and MS to identify lysine-methylated proteins and the corresponding methylation sites [141], and they applied it to study heterochromatin protein 1 β (HP1 β) methylated associated proteins. Moreover, the use of heavy methyl SILAC (stable isotope labeling by amino acids in cell culture) combined with the MRM approach allowed to quantify changes in lysine methylation and show that proteins with

different function in the HP1 β interactome had specific Lys methylation dynamics in response to DNA damage. The MRM method has been used also in another study for the quantitation of histone H3 and H4 acetylation and methylation simultaneously [142]. The identification was directly performed from tryptic digests of core histones that were separated on a special capillary column without derivatization. The authors used HCD fragmentation and all modified ions were measured using PRM. Neutral loss ions and immonium ions of modified and unmodified residues, obtained with HCD fragmentation, allow distinguishing multiple modifications on lysine residues in histones.

2.4. Non-enzymatic PTMs: oxidative post-translational modifications (oxPTMs)

Protein oxidative modifications involve the reaction between an amino acid residue and reactive oxygen species (ROS) or reactive nitrogen species (RNS). Oxidative stress causes an alteration of the cell biological functions, while oxidation-reduction (redox) reactions are usually controlled and are important in several physiological roles [143]. Reactivity and specificity with several cellular components is different for each ROS and, generally, the cysteine thiol group is one of the most reactive. Cysteine residues can undergo several PTMs, such as oxidation, glutathionylation, nitrosation and prenylation. Other amino acid residues that are usually oxidized are methionine, histidine, proline, lysine and arginine [33]. The oxidation of amino acid side chains can be classified into irreversible and reversible oxPTMs. Low concentration of ROS/RNS causes a reversible oxidation of cysteine residues and formation of S-nitrosylation, sulfenic acids, S-glutathionylation or disulfide bridges, which are responsible of redox signaling events. On the other hand, irreversible oxidation occurs with high ROS/RNS levels and modifications of cysteines, nitration of tryptophane/tyrosine, hydroxylation of histidine, advanced glycation end products (AGEs) formation or carbonylation of proteins are associated with protein dysfunction, protein misfolding, protein accumulation or degradation [144]. Therefore, an impaired redox homeostasis can lead to a cellular damage or death, and consequently to a pathological condition.

High throughput technologies are essential for the characterization and quantitation of specific oxPTMs, in order to clarify diseases mechanisms and define novel therapeutic targets. Redox proteomics is focused on the identification of redox-sensitive proteins involved in both physiological processes and disease onset, the nature of the oxPTMs in proteins and the identification of multiple oxidation sites within target proteins. The identification and quantitation of oxidized cysteine residues is not easy due to low abundance of cysteines, different types of redox modifications and labile and dynamic condition of their oxidation. Cysteine oxidation is affected by the concentration and specificity of various oxidants and antioxidant enzymes, thiol pKa, solvent accessibility, and subcellular compartment [145]. For these reasons, it is necessary to have accurate and high sensitive enrichment strategies and MS detection. Several approaches were developed to maintain the endogenous oxidative state of protein cysteines during the sample preparation before the MS analysis, such as trichloroacetic acid (TCA) during cell or tissue lysis

and thiol-quenching reagents (e.g., N-ethylmaleimide (NEM) and iodoacetamide). It is important to avoid oxidation artefacts and limit a general increase in the cysteine oxidation process. Moreover, differential alkylation procedure allows labeling cysteine-thiols before and after reduction with coded cysteine-specific reagents (e.g., dithiothreitol (DTT) and tris(2-carboxyethyl)phosphine (TCEP)), in order to measure the reversibly alkylated level of cysteine residues [146]. Affinity and activity-based methods or capture compound probes are very useful to enrich oxidized and nitrosylated cysteine residues, allowing a multiplex MS analysis. Immunoenrichment with antibodies against oxPTMs has been widely used, although this approach can introduce a high background of unselected proteins, due to the low specificity of any antibodies. MS techniques are divided in gel-based (e.g., Redox-difference gel electrophoresis (DIGE)) and nongel-based approaches. The quantitation using two-dimensional gel electrophoresis (2DE)-based technique is limited and not accurate enough, because it is very difficult to analyze proteins with high (> 120 kDa) or very low (< 10 kDa) molecular weight and highly hydrophobic or membrane proteins [144]. To overcome these limitations, several nongel-based approaches are available for a more accurate label-free or label dependent quantitation of redox proteins and peptides in complex biological samples. Examples of methods for the enrichment and identification of irreversibly oxidized cysteine residues are long column ultra-high pressure liquid chromatography-pseudo SRM (UPLC-pSRM), SCX-HILIC and poly-arginine-coated nanodiamonds (PA-NDs). In contrast, several methods are currently applied to study reversible redox modifications such as oxICAT method [147], cysteine-reactive tandem mass tag (CysTMT) [148], iodoacetyl tandem mass tag (iodoTMT) [149], CysTMTRAQ [150], q-oxPTPome for the study of class I protein tyrosine phosphatases [151] and oxidative MRM (oxMRM) [145]. In particular, oxMRM allows the identification and quantitation of specific oxidized cysteine residues, both reversible and irreversible, in targeted low abundance proteins with high sensitivity. The only limitation of oxMRM is that the use of a single protease often does not produce peptides with appropriate LC-MS/MS characteristics and able to cover the entire protein sequence. Therefore, if the aim of the study is the detection of all cysteines in a protein of interest, it is necessary to employ a combination of several proteolytic enzymes to improve the sequence coverage. This technology includes different steps: irreversible alkylation of free thiols with NEM, reduction of oxidized cysteines and tagging with a stable isotope labeled NEM, affinity purification of the targeted proteins, proteolytic digestion and MRM analysis. In 2010, Held et al. described for the first time oxMRM, in order to quantify site-specific cysteine oxidation of endogenous tumor suppressor protein p53 and the oxidation levels of protein tyrosine phosphatase-1B (PTP1B) in primary fibroblasts and a human breast cancer cell line [145]. The abundance and nature of oxPTMs are clinical sample type dependent [33]. Body fluids and tissue extracts are the main sources for proteomics analysis of oxPTMs. Urine can be obtained in a non-invasively way, but oxPTMs concentration is too low for routine MS detection. Therefore, plasma is a preferred source, even if it is the most complex biological fluid and it reflects the

general systemic status rather than a disease or organ-specific condition. Other alternative biological fluids have been used to study protein oxidation, such as cerebrospinal fluid [152], amniotic fluid [153] and saliva [154]. In addition, oxidized proteins have been analyzed in tissue biopsies of tumors and surgical biopsies of diseased human tissues such as heart [155].

2.4.1. S-nitrosylation

S-nitrosylation is a dynamic redox-based PTM of a protein and it involves the covalent attachment of nitric oxide (NO) to a reactive thiol group on a cysteine residue to form an S-nitrosothiol (SNO). Cysteine residues with low pKa and exposed thiols are more susceptible to NO. Moreover, thiols in a hydrophobic environment, such as membrane proteins and hydrophobic protein domains, are more prone to stable S-nitrosylation. This modification regulates several biological processes, among which the localization and activity of proteins responsible for cellular growth and regulation [156]. Aberrant S-nitrosylation and abnormal NO production have been associated with multiple diseases including stroke, cancer, neurological disorders and respiratory disorders. S-nitrosylation is a reversible and targeted modification. The reverse of S-nitrosylation is denitrosylation and can be done either by nitric oxide release or by direct transfer to other cellular thiols [156]. Multiple enzymes are involved in denitrosylation of proteins, such as S-nitrosoglutathione reductase (GSNOR) that mediates the decomposition of S-nitrosoglutathione (GSNO) and SNO-proteins. GSNOR regulates the reactive nitric oxide availability in the cell and a dysregulation of GSNOR can significantly induce a modification of the cellular homeostasis, causing several diseases [157]. Several proteomics studies are focused on the identification and quantitation of endogenous sites of S-nitrosylation, even if the lability of this modification and the low abundance of S-nitrosylation proteins *in vivo* make the analysis more challenging. SNOs are preferably detected by ESI MS, because they are unstable when analyzed by MALDI MS. The increase of 29 Da (NO attachment) on the unmodified peptide mass is indicative of an S-nitrosylated cysteine on the peptide sequence. The labile nature of this modification sometimes prevents the direct MS analysis. Indeed, in MS/MS analysis the S-NO bonds are fragmented easier than the peptide backbone and this often causes a neutral loss of 29 Da in y and b ions with S-nitrosylated cysteine. For this reason, the identification of nitrosylation sites when more cysteine residues are present on a single peptide is very difficult [158]. Therefore, several methods have been developed for an indirect identification of S-nitrosocysteines and, of course, in MS/MS spectrum different mass shifts are present on modified peptides based on the type of enrichment used. These methods break the S-NO bonds and capture both sulfur and nitrogen parts for the MS detection [159]. Initially, chemiluminescent, colorimetric or electrochemical methods allowed detecting total nitric oxide from nitrosylated thiols, but these techniques were not able to provide information about the nitrosylation sites and dynamics on targeted proteins [156]. Actually, the biotin-switch technique (BST) is widely used in the characterization of S-nitrosylated proteins in complex biological systems and it consists

following biochemical steps: blocking of all free cysteine thiols, trans-nitrosation with ascorbate of the S-nitrosothiols, and finally capture of these new thiols by S-biotinylation. The enriched S-nitrosylated proteins are then proteolytic digested before the LC-MS/MS analysis. Alternative approaches include resin assisted-capture of SNO-proteins (SNO-RAC) [160], phosphine-based chemical probes and organo-mercury enrichment. These labeling methods simplify the proteomic analysis of S-nitrosylation in cells and tissue. Their combination and high-resolution MS enables a global profiling of protein nitrosylation and a quantitative analysis. Isotopic labeling (e.g., SILAC, ICAT, iTRAQ and cystTMT) and label-free approaches are both applicable to the study of S-nitrosylation [161]. Anyway, MRM technique allows a great reduction of false-positive identifications and an increase of sensitivity, providing important quantitative information of S-nitrosylation in diverse biological samples. Indeed, MRM has the advantage to allow a quantification of site-specific changes if multiple sites are present on a single protein [162]. Held et al. have shown that oxMRM can be applied to evaluate S-nitrosylation oxidation of proteins [145]. They verified the stability of the S-nitrosylation during their analytical procedure, spiking the cell samples with nitrosylated recombinant p53 at different phases of the protocol.

2.4.2. Glycation

Glycation is the non-enzymatic covalent attachment of a sugar molecule (e.g., glucose or fructose) to a protein. Protein glycation occurs mainly on lysine, arginine and N-terminal residues of proteins. Glycation products are classified in two groups: early glycation adducts (Schiff's base and Amadori products or fructosamine residues formed at early stages of glycation processes) and AGEs [163]. AGEs are heterogeneous and irreversible molecules, which can accumulate becoming biomarkers of age and age-related chronic diseases (e.g., cardiovascular diseases, Alzheimer's disease, diabetes and cancer) [144]. Only few glycation products are chemically stable, indeed most of them are repaired or the glycated proteins are degraded and new molecules are synthesized. Therefore, the analysis of glycated proteins is actually an estimation of dynamic equilibrium of the glycation, repair process and de-glycation or clearance.

Quantitative analysis of glycan-modified proteins allows understanding better the protein damage due to the modification in ageing and various disease conditions, encouraging a future clinical use in health screening, disease diagnosis and drug development/therapeutic monitoring [164]. It is very important to preserve the glycation status unchanged during the sample preprocessing before the MS analysis. The enrichment step can be performed at protein or peptide level and it can be global or targeted; the latter can be used to isolate a specific subset of glycated peptides or proteins based on specific glycan structures. Boronate affinity chromatography (BAC) is an example of enrichment technique that involves the reaction of boronic acids with cis-diol groups present in many glycated proteins or peptides [144,165]. An alternative technique can be surface-enhanced laser desorption/ionization (SELDI) approach, which offers the boronate affinity chip for

the selection of glycosylated proteins in combination with MALDI-TOF MS [166]. However, LC-MS/MS analysis is necessary to obtain more information about the peptide sequence of a glycosylated protein and identify the residues susceptible to glycosylation. Thereby, the protein extract of a sample of interest is proteolytically digested using trypsin or other enzymes and the peptides are analyzed by high-resolution MS (as an example, please see [164]). Multiple fragmentation strategies can be used in the study of protein glycosylation. ETD offers an extensive fragmentation of the peptide, allowing the study of glycosylation sites localization, while CID and HCD are very useful for the characterization of glycan composition [167]. Zhang et al. used data dependent LC-ETD-MS/MS and neutral loss triggered ETD methods for the fragmentation of glycosylated proteins in control and diabetic human plasma and erythrocytes [113]. LC-MS^E is another technique used to quantify even the low abundant label-free AGE modified peptides [168]. Bioinformatic software for protein glycosylation research are poorly developed, and new sequence search engines to predict glycosylated lysine and arginine sites in proteins would be very useful.

Quantitative high-resolution MS using stable isotope labeled proteins/peptides (isotopic labeling with ¹³C⁶ glucose [169] and isotopic exchange of ¹⁸O [170]) provides a more robust analysis of the glycosylation extent.

MRM technique is the best available targeted method to absolute quantify glycosylation with higher sensitivity and specificity. It allows the identification and quantitation of the glycosylated sites and the multiple glycosylated forms of a protein of interest. Brede et al. applied the MRM technique to measure the glycosylated albumin in serum and plasma of diabetic patients with end stage renal disease (ESRD) [171]. The authors demonstrated that glycosylated albumin measurement by detecting a proteotypic glycosylation peptide with MRM could be a useful support to the analysis of glycosylated hemoglobin levels for the control of increased blood glucose in these patients. Recently, Spiller et al. performed another interesting analysis using MRM of the glycosylation sites in human serum albumin as potential diagnostic biomarkers for type 2 diabetes mellitus [172]. They used a quantitative strategy based on BAC to enrich glycosylated peptides, obtained after digestion with trypsin of plasma samples, and MRM analysis with stable isotope-labeled standards. The same authors evaluated also the glycosylation degree of 27 sites in nine plasma proteins, in type 2 diabetic patients and non-diabetic controls, in order to find novel biomarkers for an early diagnosis and prognosis [173]. Quantitation was achieved by MRM using isotope-labeled peptides as internal standard.

2.4.3. Carbonylation

Protein carbonyl formation is an irreversible PTM that can occur on several residues, among which lysine, arginine, serine, threonine and proline, following metal-catalyzed oxidation [174] or attack by hypochlorous acid [33]. Aldehydes, ketones or lactams can be obtained in the amino acid side chains of proteins or by the oxidative protein backbone cleavage. Several mechanisms lead to

carbonyl-proteins: reaction of hydroxyl radicals attacking the side chains of specific amino acids or peroxidation of lipids by ROS with the production of reactive carbonyl species (RCS), which can react with amino acid side chains of cysteine, histidine, and lysine. In addition, also glycation can contribute to form reactive carbonyl groups because of the reaction of the amino group of lysine residues with AGEs [144]. Cellular enzymes cannot repair carbonylated proteins, which aggregate and induce cell death [175]. Therefore, a removal of carbonylated proteins in the organism is essential to the cell homeostasis.

Protein carbonylation is one of the most studied oxPTMs aimed to identify potential biomarkers of oxidative stress-related human diseases. In particular, this modification is associated with numerous diseases among which atherosclerosis, diabetes, neurodegenerative diseases and cancers. The identification and quantitation of carbonylated proteins can be performed with several biochemical and analytical methods: immunoblotting and ELISA assays, spectrophotometric and chromatographic methods, and mass spectrometry. MS analysis allows not only the identification of the modified proteins, but also the characterization of the specific carbonyl sites. Carbonyl-modified proteins are commonly low abundant in biological samples, so derivatization and enrichment steps are very useful prior to MS analysis. The use of carbonyl-reactive probes as tags to label modified peptides or proteins can improve MS detection. Carbonyl groups react with DNPH (2,4-dinitrophenylhydrazine), a frequently used derivatization reagent that forms a stable hydrazone, which is detectable by specific antibodies [176]. Alternatively, biotinylated probes, such as biotin hydrazide (BHZ) and N'-amino-oxymethylcarbonylhydrazino-D-biotin, can be employed and combined with an enrichment by affinity chromatography using avidin- or streptavidin-antibodies [177,178]. Moreover, many fluorescent compounds attached to the carbonyl reactive derivatization agents allow the detection of carbonyl proteins after a 2DE separation. Few years ago, Hollins et al. developed another interesting enrichment method where an oxalylidihydrazide acts as a crosslinker on a microfluidic chip for the capture of carbonylated proteins [179]. Girard P reagent and hydrazide-functionalized resins are also suitable to select and release oxidized compounds.

With regard to the gel-based methodologies for the analysis of carbonylated proteins, "OxyBlot" is often applied; it includes the immunoblotting of carbonyl groups, previously derivatized with DNPH, by specific anti-DNPH antibodies and 2DE separation. Since, this analytical procedure is time consuming and less accurate, the use of fluorophores has been introduced to overcome these limitations. Baraibar et al. presented Oxi-DIGE technology that significantly improves the specificity and reproducibility of the proteomic analysis of carbonylated proteins [180].

Certainly, a more accurate identification and quantitation of carbonyl proteins is reached by LC-MS/MS analysis [176]. Carbonyl proteins are enriched using affinity methods, proteolytically digested into peptides and identified in MS using different fragmentation techniques. For a quantitative approach, proteins are labeled with heavy isotopologues of C, H, N, and O or it is possible to label

enriched biotin hydrazide-labeled protein digests prior to LC-MS/MS analysis with isobaric reagents (i.e., HICAT and iTRAQ). To avoid the potential loss of target peptides during the enrichment process, the iTRAQ reagent can be paired with a hydrazide moiety in a technique called iTRAQ hydrazide (iTRAQH), which is very useful for quantitative large-scale proteomic profiling of carbonylation [181]. Tag-specific ions and neutral losses significantly complicate MS data interpretation. The bioinformatics software can partially help in the interpretation of these complications and the development of further tools is necessary to resolve these challenges [175]. Carbonylation research is still in progress and requires new high-throughput MS approaches, even though several proteomics workflows are already described. Highly sensitive techniques are needed for a deeper study of carbonylation and isotopically labeled internal standards should be used in the mixtures of carbonylated proteins or peptides, in order to perform an absolute quantification. In this context, MRM technique allows a multiplex analysis of different carbonyl modifications in a single run and the absolute quantification of protein carbonylation sites. MRM has been already applied to quantify carbonylation sites in carbonylated proteins isolated from plasma samples of diabetic rats [182]. The modified proteins were derivatized with biotin hydrazide and enriched with avidin affinity chromatography before the MS analysis. First, the authors performed a non-targeted analysis of the carbonylated peptides by tandem mass spectrometry, then for the first time the quantitation of the peptides bearing carbonyl modification was obtained by MRM approach. Studies on carbonylated proteome in human samples using MRM technique have not been described to date.

3. SRM/MRM analysis of PTMs in cardiovascular proteomics

High specificity, sensitivity and multiplexing capability of the SRM/MRM technology allow the discovery and quantitation of potential biomarkers that can be used to clarify cardiovascular disease (CVD) development and progression. The importance to study disease-related protein PTMs is crucial to evaluate functional alteration of proteins that are involved in CVDs pathophysiology and novel therapeutic targets for the treatment of patients. SRM/MRM is an emerging approach in the field of CVDs and, therefore, limited studies have been performed till now using this technique to study PTMs in cardiovascular field. In this review, we reported the published studies on the application of SRM/MRM for the PTMs characterization in cardiovascular diseases (Table 2).

A recent study by Barallobre-Barreiro et al. paid particular attention to the application of proteomics in the study of cardiac extracellular matrix (ECM), whose remodeling is an important feature of CVD [108]. They proposed a sequential extraction and enrichment strategy for vascular ECM proteins before the LC-MS/MS analysis. In particular, the authors emphasized the capabilities of SRM/MRM to obtain an accurate protein quantitation and validation of potential

diagnostic/prognostic CVD biomarkers, as well as targets for drug discovery [117]. Also reviews by Lindsey's lab contributed to the discussion on the technological advancements in proteomics for the study of cardiac ECM and the comprehension of the mechanisms underlying remodeling in myocardial infarction [183]. Moreover, Lindsey et al. proposed an important scientific statement in which there is a summary of the proteomic advances for basic research laboratory and for future clinical applications in cardiovascular field [184].

3.1. Phosphorylation

Several studies demonstrated the accuracy of SRM/MRM approach to identify and quantify phosphopeptides and their specific phosphorylation sites in cardiovascular proteomic research. Phosphorylation is the best described PTM in cardiac proteome.

Few years ago, Lam et al. proposed an MRM-based study to monitor and quantify mitochondrial protein phosphorylation in human (heart) and mouse (heart, liver) tissues [185]. Mitochondria play an important role in the cardiovascular system and the characterization of their proteome modifications is therefore mandatory. The authors developed an MRM method that included transitions of several proteins from the tricarboxylic acid cycle, pyruvate dehydrogenase complex and branched-chain α -keto acid dehydrogenase complex, and found significant phosphorylation differences between human and mouse cardiac mitochondria and between heart and liver in mouse. Hence, these data showed the possibility to successfully apply MRM as a quantitative approach for the study of changes in cardiac mitochondrial phosphorylation signaling in a specific disease state.

Zhang et al., in another important study, employed an MRM technique to quantify for the first time the phosphorylation levels of 14 sites on human cardiac troponin I (cTnI), including 6 new residues, in human explanted hearts from patients with ischemic heart disease or idiopathic dilated cardiomyopathy in comparison with donor hearts from healthy subjects [186]. Cardiac myofilament protein phosphorylation is physiologically and clinically important for heart function [187]. Indeed, cTnI is a cardiac regulatory protein that controls the myocardial contractility, and it is highly phosphorylated at multiple sites by several kinases. The balance of site-specific phosphorylation stoichiometry of cTnI is a critical factor for a good cardiac functionality. In a previous study, the same authors demonstrated that heart failure may be associated with a reduced phosphorylation of cTnI at cAMP-dependent protein kinase sites [19] and now they confirmed this evidence.

Therefore, phosphorylation has a significant role in the functional status of cTnI and, consequently, the phosphorylated forms of this circulating protein could be potential diagnostic markers for myocardial infarction. Kooij et al. also investigated the effects of protein kinase C α (PKC α) phosphorylation of cTn on myofilament function in human failing cardiomyocytes and explored the potential targets involved [188]. The MRM analysis showed target-specificity in the in vitro PKC α -mediated phosphorylation of several sites on human recombinant cTnI and the extent of

phosphorylation was quantified before and after treatment with PKC α . Particular attention should be paid to these data when considering the *in vivo* situation; however, the phosphorylation extent measured in recombinant cTn complex by Kooij et al. has proved comparable to phosphorylation levels of the different phosphorylation sites observed in human cardiac tissue by Zhang et al. [186]. In the same years, Wijeratne et al. used SRM approach as a verification tool for a site-specific quantitation of another important protein, the phosphoSer-282 on cardiac myosin binding protein C (cMyBPC) [189]. The authors presented for the first time a MS-based strategy that included an acetone-based peptide labeling technique (reductive alkylation by acetone, RABA) to perform a quantitative phosphoproteomics of cardiac tissue extracts from mouse hearts not expressing FGF2 (fibroblast growth factor-2) and hearts expressing low molecular weight FGF2 (LMW FGF2) subjected to ischemia and reperfusion. For the SRM analysis, cardiac tissue was proteolytic digested with trypsin, desalted using Oligo R3 and RABA-labeling was performed on the peptides. A TiO₂-chromatography was applied to enrich phosphopeptides before the SRM analysis. A relative quantitation was obtained using isotopically-labeled acetone (+ 48 Da of mass) and non-labeled acetone (+ 42 Da of mass) for heavy and light labeling of peptides, respectively. Data showed significant differences in the phosphorylation of many proteins, among which cMyBPC that has been never identified related to the LMW FGF2-mediated cardioprotective signaling. This study provided a better knowledge about the LMW FGF2-mediated phosphorylation changes in proteins that are important for the cardioprotection induced by ischemia/reperfusion injury of hearts. Moreover, these observations could contribute to identify potential targets useful for therapeutics against cardiac ischemia and reperfusion injury. Li et al. also studied myocardial ischemia reperfusion injury (MIRI) in a rat MIRI model and in cultured human cardiac microvascular endothelial cells (HCMECs) after treatment with Scutellarin, which is a flavonoid glycoside compound used in China in clinic against ischemic diseases [190]. MIR injury causes the vascular endothelium dysfunction (ED) that is responsible for the onset of several cardiovascular diseases. Also the vascular smooth muscle cell dysfunction is involved in the ED, where a reduced endothelium-dependent vasorelaxant response to acetylcholine is present. cGMP dependent protein kinase (PKG) is involved in the endothelium-dependent relaxation. The authors evaluated the role of PKG and the protective effects of Scutellarin on endothelial cells of coronary artery in a rat MIRI injury model and *in vitro* model of HCMECs, in which it was simulated an hypoxia reoxygenation injury. A MIDAS method was developed to detect and quantify PKG-I α phosphopeptides in a specific amino acid sequence of autophosphorylation domain. This study demonstrated that increased activation of PKG-I α by Scutellarin treatment has a main role in the protective effects of Scutellarin on ED.

Even before the studies reported above, Cox et al. evaluated the phosphorylation levels of myocyte enhancer factor 2A (MEF2A) using MRM MS in a mammalian cell line [98]. MEF2A is a member of the MEF2 family. This study is not focused on a specific cardiovascular disease, but it

is well known that MEF2 is a transcriptional regulator involved in the development of cardiac and skeletal muscle cells and whose activity is controlled by phosphorylation. Moreover, it regulates the stress-response during cardiac hypertrophy and tissue remodeling. The phosphorylation of this protein causes an increase or reduction of its activity based on the type of modified amino acids. Despite of this study focused on mammalian MEF2A, the authors wanted to demonstrate that targeted MRM-information dependent acquisition (IDA) successfully identify low fmol levels of phosphopeptides and it is a powerful technique for a future quantitation of phosphorylated proteins also in human complex biological matrix.

3.2. Glycosylation

Hülsmeier et al. developed a quantitative method of the glycosylation sites on transferrin and α 1-antitrypsin, using MRM MS with isotopically labeled standard peptides [55]. The authors reported a reduced N-glycosylation site occupancy in serum from congenital disorder of glycosylation type 1 (CDG type 1) patients that is significantly correlated with the severity of the disease. Moreover, they observed a selective underglycosylation of N-glycosylation sites in transferrin and α 1-antitrypsin. Congenital disorders of glycosylation are frequently associated with endocrine and coagulation dysfunctions. The abnormal glycosylation results in multisystem clinical manifestations, among which cardiovascular symptoms, and an accurate measurement of the glycosylation site occupancy in healthy subjects and in CDG patients is essential.

Recently, Kailemia et al. applied a targeted dynamic MRM method to measure glycoprotein levels and site-specific glycoisoforms of the proteins in human plasma in comparison with high-density lipoprotein (HDL) particles isolated from the same plasma samples [191]. The authors for the first time were able to profile the O- and N-linked glycosylation of HDL associated-proteins simultaneously. In several conditions, such as heart diseases, oxidative stress and diabetes, HDLs may become pro-atherogenic due an alteration of their composition. Therefore, an accurate analysis of the glycosylation in HDL can be helpful to clarify the loss of functionality associated with altered HDL and provide potential marker for diseases. Among the glycosylated proteins of HDL, α 1-antitrypsin, fetuin A and apolipoprotein CIII are implicated in many cardiovascular and other disease conditions. Plasma proteins were differentially glycosylated when compared with glycoproteins isolated in HDL, so these data showed a glycan specificity of proteins recruited into HDL particles and provided key information about their function.

3.3. Oxidation

The impact of the oxidative modifications in cardiac proteome was also studied using SRM/MRM. In particular, Yassine et al. developed a MRM assay to measure the Apolipoprotein A-I (Apo A-I) methionine oxidation in HDL of healthy subjects and type 2 diabetic patients with and without prior

CVD [192]. Oxidative modification of M148 in Apo A-I is associated with a HDL dysfunction *in vitro* [193], and several studies have suggested Apo A-I methionine oxidation as a potential indicator of diabetic complications and, therefore, stable isotope-labeled peptides for M148 oxidation were synthesized and used as internal standards to perform an accurate quantitation. Moreover, the authors obtained an estimation of the total HDL Apo A-I level, monitoring another Apo A-I peptide without any methionine residue in the sequence. HDL Apo A-I concentrations were significantly reduced in patients with diabetes and CVD in comparison with diabetic patients without CVD. Instead, the ratio of the oxidized M148 peptide to the native sequence was increased in HDL from patients with diabetes and CVD compared to diabetic group without CVD and controls. Some years before, also Shao et al. applied SRM technology to quantify the regiospecific oxidation of Apo A-I *in vivo* [194]. HDL normally protects against atherosclerosis, but the oxidative damage changes HDL composition and structure blocking its cardioprotective effects. The enzyme myeloperoxidase (MPO), which is expressed at high levels by macrophages in the human artery wall, has been associated to the nitration and chlorination of specific tyrosine residues of Apo A-I in *in vitro* studies. These oxidative modifications cause dysfunctional HDL in the artery wall. The authors employed SRM to quantify chlorinated and nitrated tyrosine residues in Apo A-I isolated from HDL, from human atherosclerotic tissue and plasma. They used an isotope-labeled Apo A-I protein as an internal standard, in order to quantify the site-specific oxidation. In this study, the authors suggested that Apo A-I chlorination and nitration quantitation might contribute to the diagnosis and treatment of human cardiovascular diseases. Recombinant Apo A-I could be a potential therapeutic agent and mutant oxidation-resistant forms of this protein could be cardioprotective in humans in the prevention of cardiovascular diseases.

4. Conclusion

PTMs have a pivotal role in the diversification and regulation of the cellular proteome [195]. PTMs influence biological processes of normal and pathological cells, thus making essential a better characterization of human proteome in the study of diseases at the molecular level. Indeed, PTMs may give additional information for the pathological condition and could be used as potential biomarkers in the detection and progression of a specific disease, as well as targets for effective drugs [31]. To date, selective enrichment techniques combined with high-resolution MS/MS quantitative analysis have been applied to describe several PTMs in proteins, including determination of the PTMs sites location and different isoforms of a modified protein. Enrichment methods are essential to improve the MS analysis of PTMs and LC-MS/MS has been resulted the best method to detect and quantify proteoforms with high sensitivity and accuracy. In particular, SRM/MRM is a powerful targeted assay for the quantitation of PTMs and validation of new modifications sites. This approach has been successfully applied in the PTMs study of several enzymatic and non-enzymatic PTMs, contributing in the comprehension of their biological effect on

protein function and potential implications in pathophysiology. Some interesting studies have been performed in cardiac proteomic research using SRM/MRM to study PTMs, establishing its capability to absolute quantify in complex biological samples changes in proteins that are relevant in cardiovascular diseases. Phosphorylation, glycosylation and oxidation have been characterized until now in proteins involved in cardiovascular system and these studies showed the possibility to apply SRM/MRM also for other important modifications, extending the PTMs investigation in cardiovascular field.

5. Expert commentary

Compared to human genome, proteome is more complex due to many factors, including splicing, mutations and a great variety of PTMs [2]. The number of proteoforms significantly exceeds the number of genes. PTMs of proteins affect several cellular processes and modulate intra- and intermolecular interactions, regulating protein activity and new protein functions. Indeed, the human proteome is highly dynamic and changes in response to several stimuli and PTMs. PTMs are very important in both physiological and pathological conditions; they control the physiological function of the majority of proteins, but sometimes their presence can cause a disease development. Today, more than 450 PTMs are described, but only few of them are currently investigated in proteomic research. The most extensively studied PTMs are phosphorylation and glycosylation, due to their role in a great variety of biological pathways; the other PTMs, such as acetylation, methylation and ubiquitination, are now receiving more attention thanks to the improvements in mass spectrometry strategies. Huge efforts have been done in PTMomics, but less frequent PTMs are still complex and difficult to be analyzed with actual available analytical strategies. Moreover, the future studies should be focused mostly on the characterization of PTMs in pathophysiological conditions.

The characterization of PTMs in relation to the disease pathology is still in progress and the majority of studies have been performed using shotgun approaches that provide a wider view of the protein modifications in a relative short time. Shotgun proteomics allows the identification of new modified proteins and PTM-sites easily. However, targeted strategies have a higher dynamic range and reproducibility as well as lower limits of detection in comparison with shotgun proteomics [196]. Targeted proteomics can detect with high specificity and sensitivity site-specific modification events that could be important for disease diagnostics and prognostics [2]. The methods and instruments employed for studying PTMs by LC-MS/MS have significantly upgraded in the last years, but they still need of improvements in enrichment strategies to obtain higher sensitivity and identification of low-abundant peptides from low amount of sample. Moreover, the development of sophisticated and automated software tools for data processing will be helpful in the interpretation of high complexity of PTMs. These advances would allow the application of PTMomics in a clinical perspective. PTM cross talk is having a strong relevance in PTMomics field

and the analysis of multiple PTMs simultaneously could be useful to obtain detailed information about the biological mechanisms in pathological conditions. However, this type of study is very complex and still present challenges concerning the optimization of experimental design and MS acquisition. Recently, label-free approaches, such as data-independent acquisition, have showed the possibility to use SWATH MS also for the PTMs analysis [2]. SWATH MS combines the traditional shotgun proteomics with the high reproducibility and sensitivity of SRM, allowing the detection of all fragment ions of the PTM peptide precursors that are present in a given sample. In this way, SWATH MS provides highly multiplexed fragment ions maps that can be defined using spectral libraries generated by previous shotgun experiments. This method is highly reproducible and sensitive in the detection of low-abundance peptides.

6. Five-year view

The standardization of sample preparation and analysis on a great number of samples is very important in the application of LC-MS/MS for the study of PTMs in a clinical field. In clinical research, the investigation of PTMs requires simplicity and robustness of the analytical method and the possibility to use small amount of sample material. The quantitation methods should be accurate and reproducible and SRM/MRM analysis is a valid solution for this aim. More sophisticated data analysis software are currently being developed, because the creation of complete spectral libraries of all modified peptides for a specific tissue/biofluid sample would be very useful in the discovery of potential disease markers and targets for a personalized treatment of patients. Indeed, several PTMs control the interaction between drug and protein targets and the therapeutic effects.

Advancement of knowledge of CVDs have created a fundamental shift in the paradigm of biomarker research, in which the major challenges will not be the discovery of new markers but rather the selection and validation of a subgroup of clinically useful markers from the large pool of candidates useful for the screening and general assessment of the disease. The value of these new markers will need to be evaluated in the context of readily available clinical information, including existing markers and other relevant demographic and clinical variables [197]. Finally, considering that CVDs, such as chronic heart failure, are characterized by maladaptive signaling within intertwined molecular pathways, leading to a complex syndrome in which the diverse pathways and pathological processes can manifest in circulation as biomarkers [198], use of combined biomarkers can improve risk stratification. Indeed, due to the complexity of CVDs pathophysiology, it is unrealistic that a single marker is able to reflect all of the features of this syndrome, whereas the combined use of more parameters would certainly give more comprehensive insight into an individual patient (Figure 5). To this purpose, according to the criteria established in a recent roadmap [199] an MRM approach has the potential to improve CVDs care, diagnosis and prognosis, and hopefully, to provide a better pharmacological treatment.

Key issues

- PTMs play an important role in the control of protein functions, localization and interaction with other molecules in the cell.
- PTMs modulate protein activity both in physiological and pathological conditions. Sometimes, the presence of a modification can cause a specific disease onset.
- PTMomics is really challenging and MS has become a powerful method to define PTMs and identify new modification sites on proteins.
- SRM/MRM approach provides a high-throughput targeted assay for the quantitation of PTMs in complex biological samples.
- SRM/MRM has been successfully applied in the PTMs characterization of several enzymatic and non-enzymatic PTMs, extending the knowledge of their biological effect on protein function and consequent implications in pathophysiology.
- Interesting studies on PTMs have been performed in cardiac proteomic research using SRM/MRM, pointing out its capability to absolute quantify changes in proteins that are relevant in cardiovascular system. These data confirmed the possibility to apply SRM/MRM for the investigation of new modifications, extending the PTMs knowledge in cardiovascular field.
- More selective enrichment methods, robust quantitative techniques and sophisticated software tools for data processing are strongly necessary in the near future, in order to increase the PTMs characterization and explain pathological processes, such as those underlying cardiovascular diseases.

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Papers of particular interest have been highlighted as:

* = of interest

** = of considerable interest

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Figure legends

Figure 1. Schematic summary of the most frequent PTMs of proteins that can be divided in enzymatic and non-enzymatic alterations.

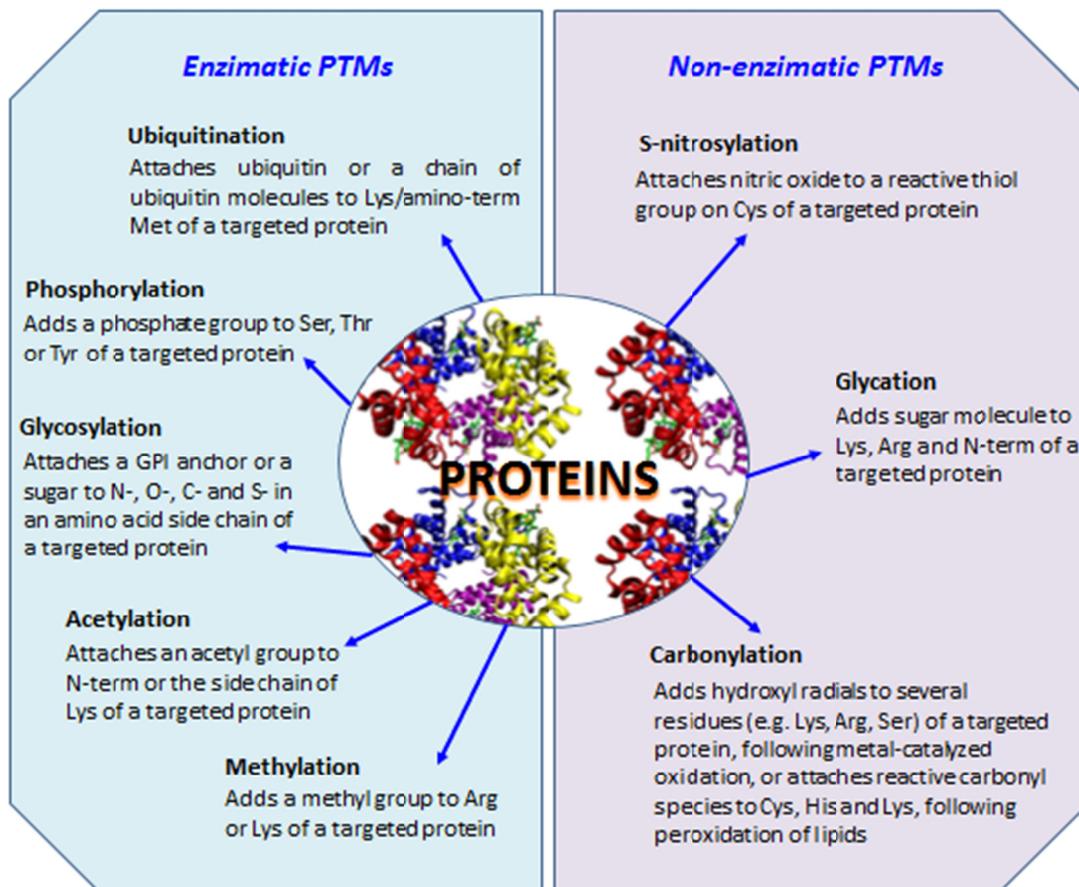
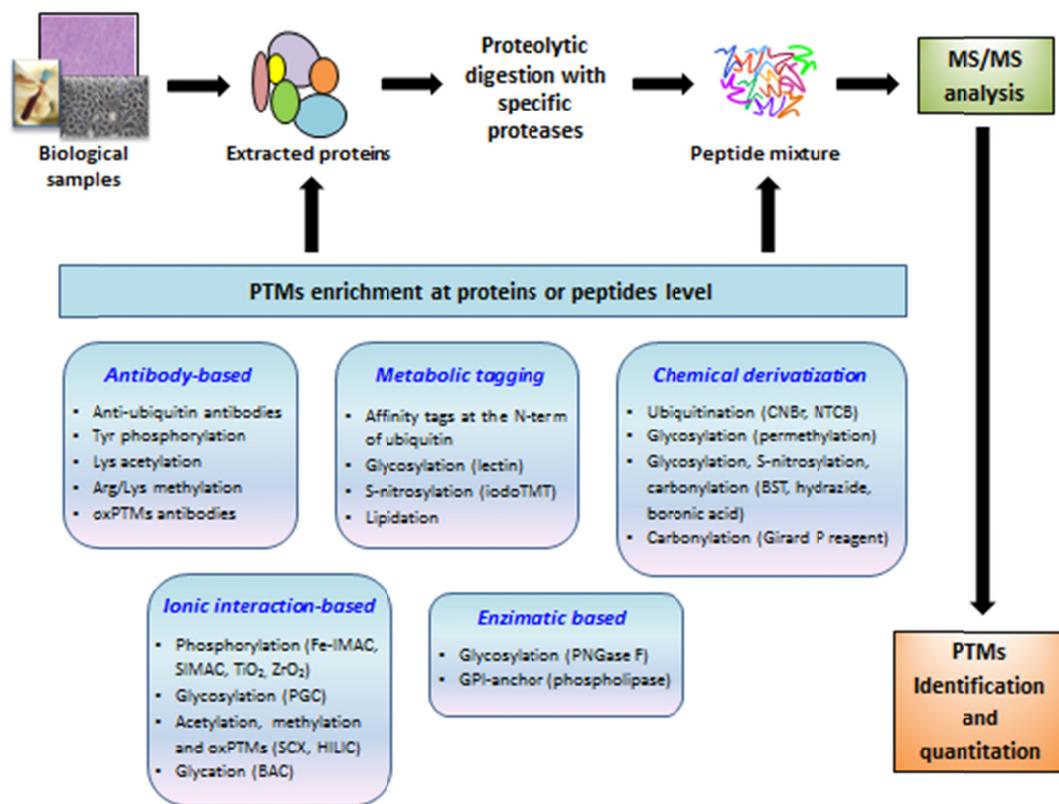


Figure 2. Overview of the proteomic analysis for PTMs detection and quantitation. The most commonly used PTMs enrichment methods are reported based on the type of studied modification.



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Figure 3. Schematic summary of the most frequent enzymatic and non-enzymatic PTM reactions.

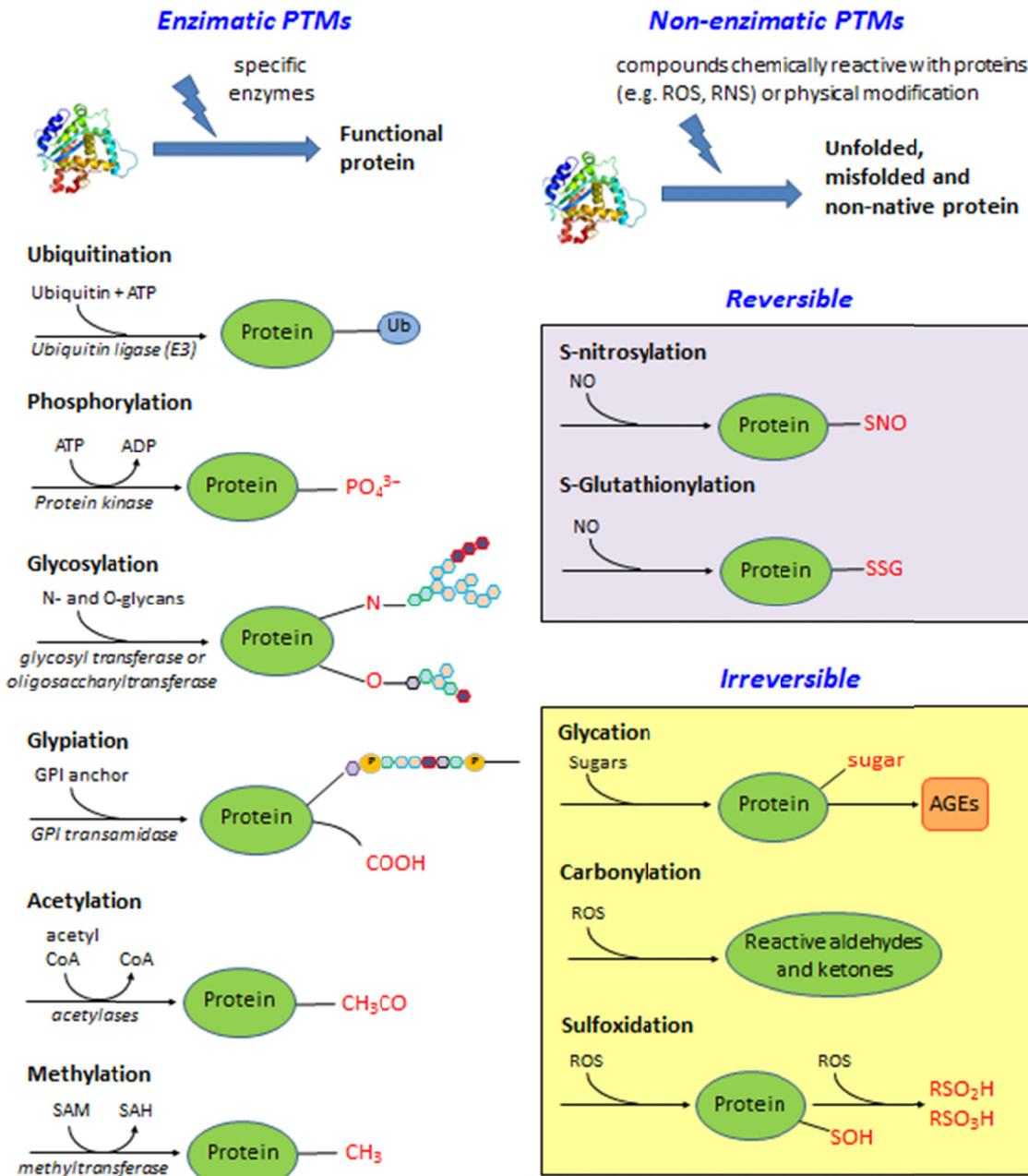


Figure 4. A. Selected reaction monitoring (SRM)/multiple reaction monitoring (MRM) on a triple quadrupole mass spectrometer. Targeted precursor peptide ion is selected in the first mass analyzer (Q1), fragmented in the collision cell and fragment ions (transitions) are filtered in Q3. A chromatogram for each monitored transition is obtained over the time. LC-ESI, liquid chromatography-electrospray ionization; CE, collision energy. **B.** The workflow for a high-throughput SRM/MRM assay. Selection of the proteins of interest based on their biological and clinical significance, definition of the proteotypic peptides and appropriate fragment ions, optimization and validation of each transition and, finally, quantitation by SRM/MRM. **C.** Overview of targeted PTMs protein quantitation by SRM/MRM. Proteins are extracted from biological samples and proteolytic digested. Fractionation strategies and PTMs enrichment are performed at

protein or peptide level for improving sensitivity and accuracy by reducing sample complexity. SRM/MRM allows the monitoring of specific peptides in a multiplex quantitative analysis using label or label-free method. For the absolute quantitation, isotope-labeled peptides matching the endogenous analogs sequence are added as internal standards before sample processing.

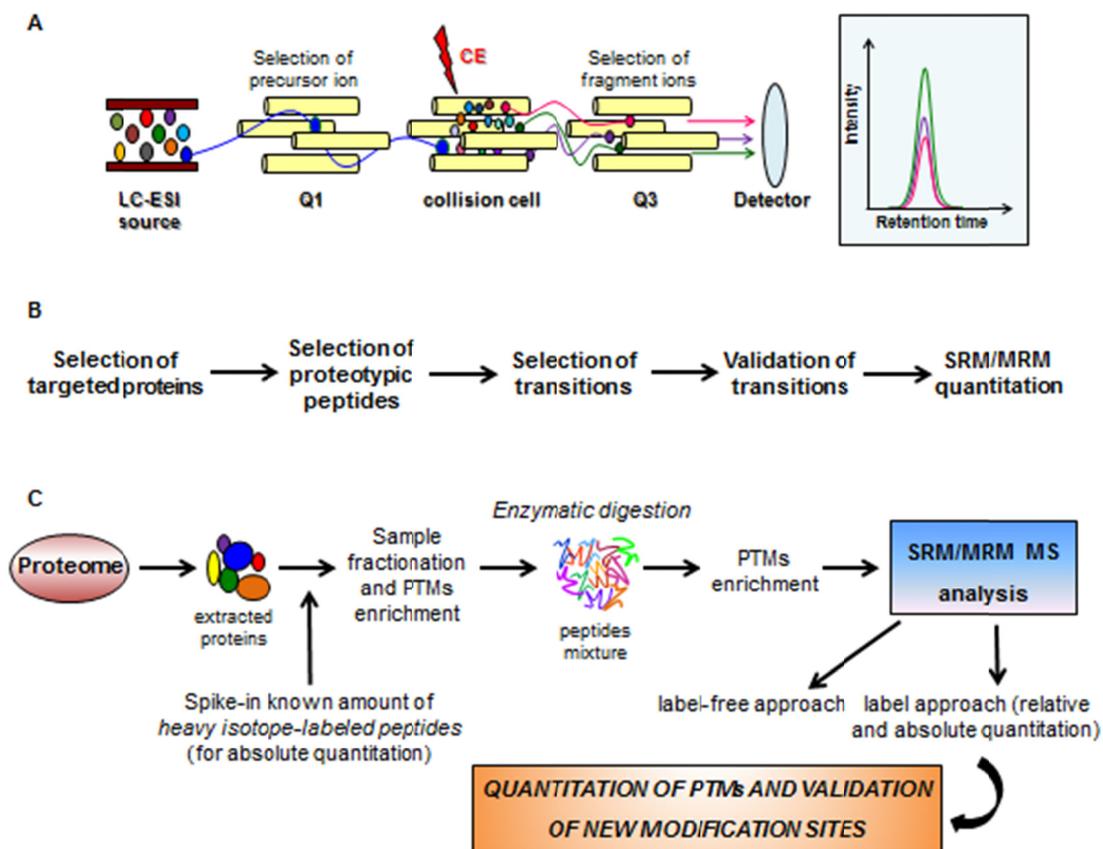
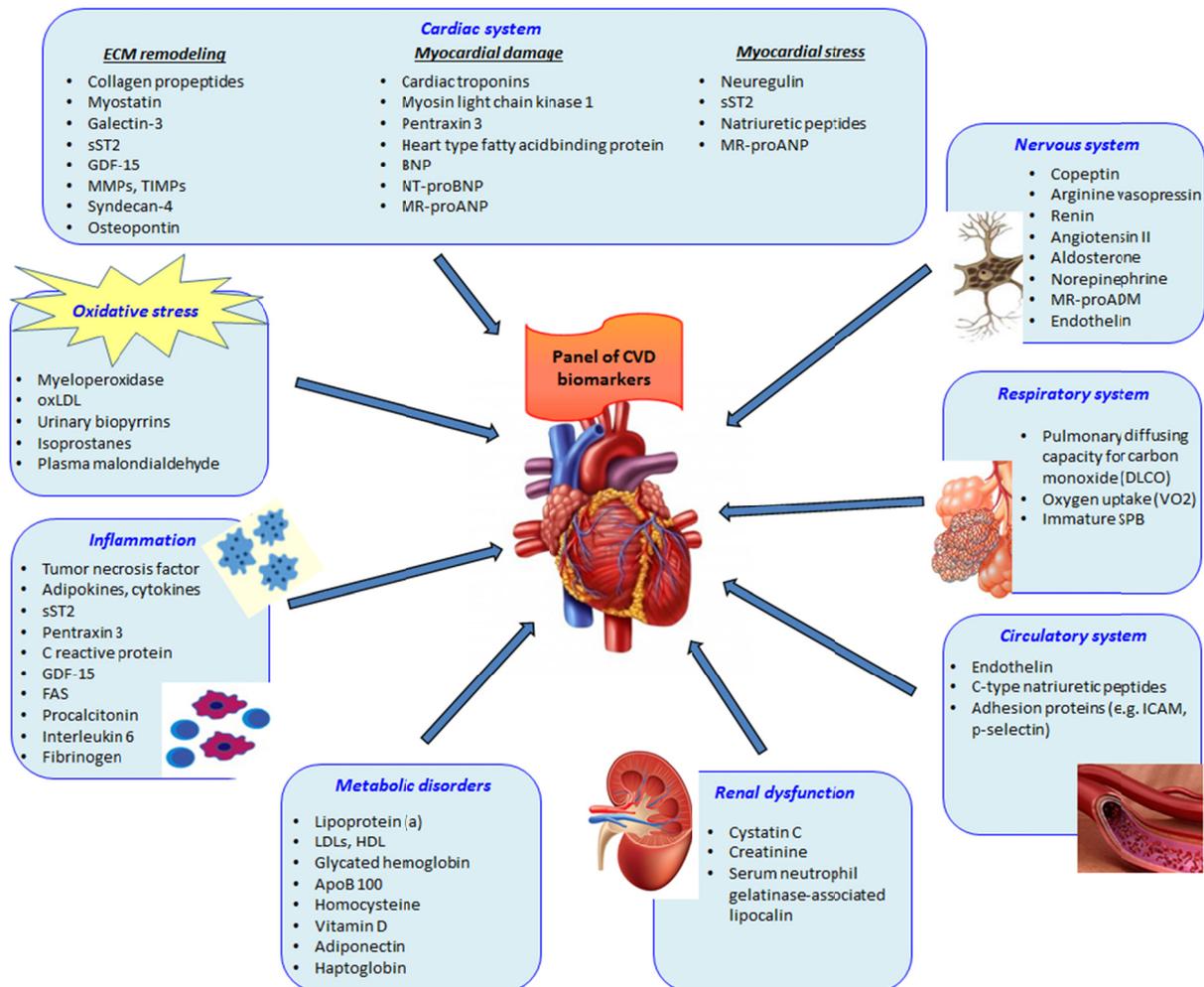


Figure 5. Classification of cardiovascular disease (CVD) biomarkers according to the pathologic process they represent. OxLDL, oxidative low-density lipoprotein; sST2, soluble ST2; GDF-15, growth differentiation factor 15; FAS, tumor necrosis factor receptor superfamily member 6; HDL, high-density lipoprotein; ApoB 100, apolipoprotein B 100; ICAM, intercellular cell adhesion molecule; MR-proADM, mid regional-pro adrenomedullin; MR-proANP, mid regional-pro atrial natriuretic peptide; BNP, brain natriuretic peptide; NT-proBNP, N-terminal-pro brain natriuretic peptide; MMPs, matrix metalloproteinases; TIMPs, tissue inhibitor of metalloproteinases.



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Table 1. Relative and absolute protein quantitation strategies used with SRM/MRM MS. MS, mass spectrometry; QconCATs, quantification concatamers.

Quantitation type	Method name	Description
Relative	Label-free approach	<ul style="list-style-type: none"> Relative measurement based on the MS signals intensity of defined transitions Lower accuracy compared to label-based methods Endogenous reference proteins (i.e. housekeeping proteins) are quantified together with the targets of interest, allowing the calculation of relative changes in protein expression without the addition of labeled standards
Relative	Stable isotope dilution (SID) approach	<ul style="list-style-type: none"> Signal intensities comparison of the stable isotope-labeled peptides from the control sample and their unlabeled counterparts in the pathological sample
Relative	Labeled reference peptide (LRP) method	<ul style="list-style-type: none"> Single isotopically labeled peptide used as a normalization reference for all the endogenous analytes Less costly alternative to SID approach
Absolute	Stable isotope-labeled internal standards (AQUA peptides)	<ul style="list-style-type: none"> Chemical stable isotope labeling Synthesis of AQUA peptides chemically identical to the corresponding native peptides with the incorporation of stable isotopes (¹⁵N, ¹³C, ¹⁸O, etc.) Known amounts of AQUA peptides spiked into biological sample and used as internal standards
Absolute	QconCAT internal standards	<ul style="list-style-type: none"> Metabolic stable isotope labeling Synthesis of QconCATs by concatenating isotopically labeled peptide sequences of the target proteins into artificial proteins Known amounts of QconCAT proteins spiked into biological sample and used as internal standards
Absolute	Protein Standard Absolute Quantification (PSAQ)	<ul style="list-style-type: none"> Metabolic stable isotope labeling Design of full-length isotope labeled proteins as internal standards analog of the target proteins Known amounts of recombinant labeled proteins spiked into biological sample before any pre-processing

		<ul style="list-style-type: none">• Immunoaffinity enrichment of intact target proteins
Absolute	Stable isotope standards and capture by anti-peptide antibodies (SISCAPA)	<ul style="list-style-type: none">• Metabolic stable isotope labeling• Design and purification of anti-peptide antibodies• Known amounts of stable isotope standards spiked into biological sample• Immunoaffinity enrichment of the peptides

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Table 2. Applications of SRM/MRM MS in cardiovascular proteomics for the characterization of PTMs. The reported studies are focused on the PTMs analysis of proteins that are important in different pathophysiological conditions in cardiovascular field. HDL, high-density lipoprotein.

Reference	Condition relevant to cardiovascular field	Sample	PTMs	Quantitation strategy	Proteins quantified	Study
Cox <i>et al.</i> 2005 [98]	Development of cardiac muscle cells and stress-response during cardiac hypertrophy	Mammalian cell line	Phosphorylation	No quantitation	1	Phosphorylation of myocyte enhancer factor 2A
Schröter <i>et al.</i> 2010 [200]	Hypertensive cardiac hypertrophy and heart failure	Human embryonic kidney cell line (HEK293) and cultured murine cardiac microvascular endothelial cells	Phosphorylation	Relative	1	Phosphorylation of guanylyl cyclase A receptor before and after atrial natriuretic peptide (ANP)-induced desensitization
Zhang <i>et al.</i> 2012 [186]	Ischemic heart disease or idiopathic dilated cardiomyopathy	Human tissue (left ventricular free wall transmural tissue from explanted end-stage failing hearts)	Phosphorylation	Absolute	1	Site-specific phosphorylation of human cardiac troponin I
Kooij <i>et al.</i> 2013 [188]	End-stage idiopathic dilated cardiomyopathy	Human tissue (left ventricular samples from end-stage failing idiopathic dilated myocardium)	Phosphorylation	Absolute	1	Protein kinase C α (PKC α) phosphorylation of human cardiac troponin

Lam <i>et al.</i> 2013 [185]	Heart failure	Human tissue (anterior left ventricular wall from heart transplantation) and mouse tissues (heart and liver)	Phosphorylation	Relative	11	Mitochondrial proteins phosphorylation
Wijeratne <i>et al.</i> 2013 [189]	Ischemia/reperfusion injury	Mouse tissue (hearts)	Phosphorylation	Relative	1	Site-specific quantitation of the pSer282 on cardiac myosin binding protein C
Li <i>et al.</i> 2015 [190]	Ischemia/reperfusion injury	Human cardiac microvascular endothelial cells (HCMECs) and endothelial cells of coronary artery in a rat model	Phosphorylation	Relative	1	Phosphorylation of cGMP dependent protein kinase-I α
Hülsmeier <i>et al.</i> 2007 [55]	Congenital disorder of glycosylation type 1 (CDG type 1) associated with coagulation disorders	Human serum	Glycosylation	Absolute	2	Site-specific glycosylation of transferrin and α 1-antitrypsin
Kailemia <i>et al.</i> 2018 [191]	Pro-atherogenic function	Human plasma and HDL	Glycosylation	Absolute	3	O- and N-linked glycosylation of HDL associated-proteins (α 1-antitrypsin, fetuin A and apolipoprotein CIII) simultaneously
Shao <i>et al.</i> 2012 [194]	Atherosclerosis	Human HDL from atherosclerotic tissue and plasma	Oxidation (nitration and chlorination)	Relative	1	Regiospecific oxidation of Apolipoprotein A-I

Yassine <i>et al.</i> 2014 [192]	Type 2 diabetes with and without prior cardiovascular events	Human HDL	Oxidation	Relative	1	Apolipoprotein A-I M148 oxidation
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