Protein Post-Translational Modification Crosstalk in Acute Myeloid Leukemia Calls for Action

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Running title: AML post-translational modification crosstalk

Abstract

Background: Post-translational modification (PTM) crosstalk is a young research field. However, there is now evidence of the extraordinary characterization of the different proteoforms and their interactions in a biological environment that PTM crosstalk studies can describe. Besides gene expression and phosphorylation profiling of acute myeloid leukemia (AML) samples, the functional combination of several PTMs that might contribute to a better understanding of the complexity of the AML proteome remains to be discovered.

Objective: By reviewing current workflows for the simultaneous enrichment of several PTMs and bioinformatics tools to analyze mass spectrometry (MS)-based data, our major objective is to introduce the PTM crosstalk field to the AML research community.

Results: After introduction to PTMs and to PTM crosstalk, this review introduces several protocols for the simultaneous enrichment of PTMs. Two of them allow a simultaneous enrichment of at least three PTMs when using 0.5-2 mg of cell lysate. We have reviewed many of the bioinformatics tools used for PTM crosstalk discovery as its complex data analysis, mainly generated from MS, becomes challenging for most AML researchers. We have presented several non-AML PTM crosstalk studies throughout the review in order to show how important the characterization of PTM crosstalk becomes for the selection of disease biomarkers and therapeutic targets.

Conclusion: Herein, we have reviewed the advances and pitfalls of the emerging PTM crosstalk field and its potential contribution to unravel the heterogeneity of AML. The complexity of sample preparation and bioinformatics workflows demands a good interaction between experts of several areas.

Keywords: Acute myeloid leukemia, post-translational modifications, crosstalk, proteome, phosphoproteome, acetylproteome, methylproteome, glycoproteome, ubiquitinome, mass spectrometry, biomarkers, proteoform

1. INTRODUCTION

Acute myeloid leukemia (AML) is a complex hematopoietic malignancy, characterized by differentiation arrest and rapid proliferation of abnormal myeloid precursor cells. Accumulation of these abnormal cells in the bone marrow will interfere with the production of normal blood cells and eventually lead to morbidity if left untreated [1]. AML is associated with a relatively poor outcome and the long-term outcomes have not significantly improved over the last three decades. For old adults (≥ 67 years of age) the five-year survival rate is 5% compared to 40% in young patients [2]. Recent advances in the understanding and identification of the new genomic landscape of AML have increased the use of individualized and targeted treatment strategies. The therapeutic and prognostic indicators of AML are chromosomal rearrangements or mutations involving tumor suppressor genes, oncogenes, transcription factors and epigenetic modifiers [3]. The tumor suppressor gene *TP53*, a guardian of genomic integrity by activating responses to DNA damage and a central driver of apoptosis, is often mutated or lost in human cancer, but is reported to be mutant in only 7% of AML cases [4]. Oncogene activating mutations in genes encoding epigenetic modifiers (*DNMT3A*, *ASXL1*, *TET2*, *IDH1* and *IDH2*), involved in DNA methylation and histone post-translational modifications (PTMs), are found at early stages of AML, while mutations in ribosome biogenesis and signaling genes (*NPM1*, *FLT3* and *RAS*) occur later in leukemogenesis [5]. Epigenetic mutations in AML are not sufficient to initiate the disease. However, they will co-occur with classical mutations in transcription factors and signaling effectors. Mutations on *DNMT3A* often co-occur with *FLT3* internal tandem duplication (ITD) and *NPM1* mutations, conferring adverse risk [6]. Thus, the analysis of epigenetic modifications during leukemogenesis becomes relevant to understand disease emergence.

In the past four decades, the AML treatment has remained relatively unchanged with a general poor outcome, especially for the elderly patients unfit for induction therapy. Consolidative treatments such as hematopoietic stem cell transplantation (SCT) are usually recommended to younger and fit patients [7]. However, 2017 was a pivotal year for AML researchers, clinicians and patients, with four new AML drug approvals. Midostaurin is a multitargeted kinase inhibitor, described to significantly improve the median overall survival (OS) of patients with *FLT3* mutation, when added to induction chemotherapy [8]. Gemtuzumab ozogamicin is a humanized immunoglobulin antibody, improving OS when added to induction chemotherapy. CPX-351 is a liposomal formulation of the DNA interactors daunorubicin and cytarabine in a 1:5 molar ratio for fit older patients, newly diagnosed with secondary AML or therapy-related AML. Enasidenib is a non-cytotoxic selective inhibitor of *IDH2*-mutated patients [9].

The biological research on the AML heterogeneity has experienced many innovations in the past years. Research technologies with suffix "-omics" such as genomics, transcriptomics, proteomics, metabolomics and lipidomics, and the integration of some of them are widely used in current cancer research (Fig. **1**). Proteomics studies are a valuable complement of the genome translation by analyzing the entire set of proteins and their modifications in an organism or system [10]. Proteomics technologies are based on global and high throughput analytical methods, such as two-dimensional (2D)-gel electrophoresis, microarray and liquid chromatography tandem mass spectrometry (LC-MS/MS), producing large-scale datasets [11]. Additional approaches for proteomic profiling related to AML have been described somewhere else [12]. LC-MS/MS allows the identification and quantification of several thousands of proteins, including PTMs, and has been used in all phases of AML drug development, from the identification of protein targets to the bioanalytical studies of pharmacokinetics and pharmacodynamics during therapeutic drug monitoring and early phase clinical trials [13].

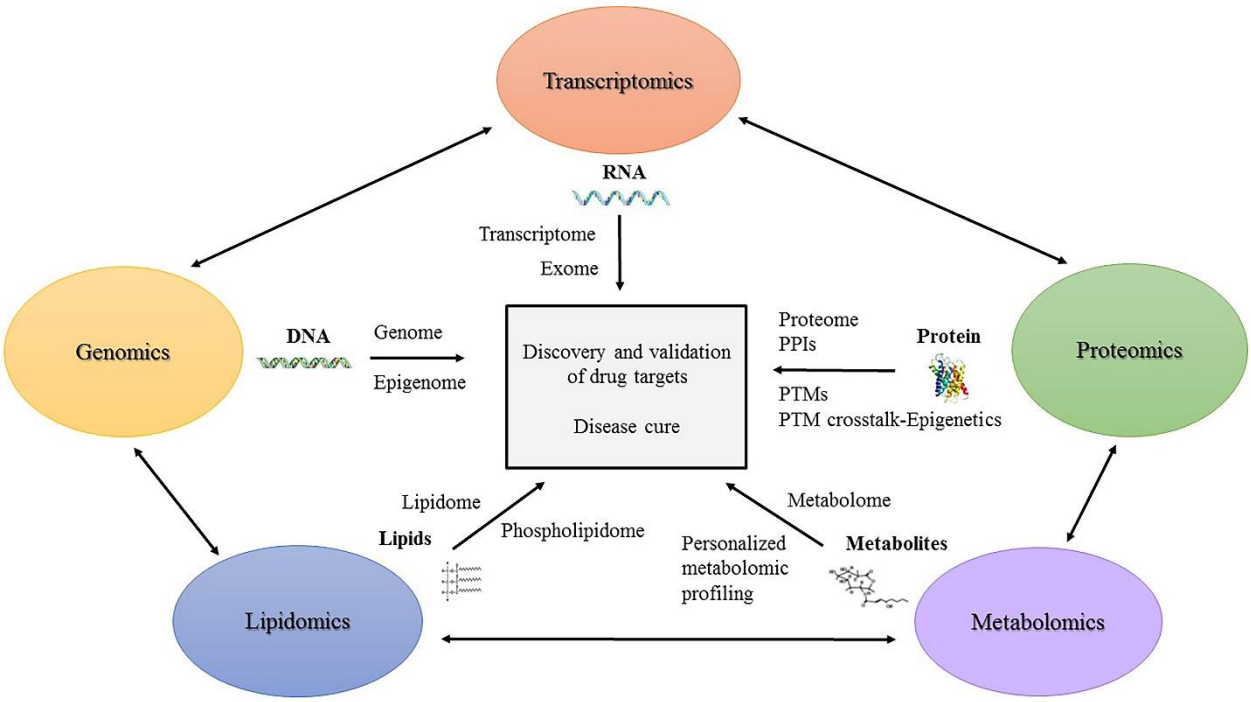

Fig. (1). Some of the –omics technologies used in cancer research, aims, outputs and connections.

(PTMs: post-translational modifications; PPIs: protein-protein interactions)

LC-MS/MS-based proteomics has emerged as a powerful tool for gene expression and epigenetic research, allowing the unbiased and comprehensive analysis of histone PTMs and the characterization of chromatin and its associated proteins [14]. Moreover, LC-MS/MS has gained an increasingly important role in deconvolution of epigenetic drug targets. However, analysis of histones and non-histone proteins and their PTMs has been challenging due to the high complexity of the numerous modifications and the large amount of cellular material required to process samples for LC-MS/MS analysis [15].

The aim of this review is to introduce PTMs and PTM crosstalk to the AML community by describing most occurring PTMs on proteins and suggesting sample preparation workflows and biocomputational tools that might encourage future AML PTM crosstalk studies. Major achievements on PTM crosstalk research will also be described. Current limitations of starting material amounts for some of the sample preparation methods and data analysis workflows are still major challenges in this research field. However, the review we present here of some of the most employable approaches that could work in AML research might promote upcoming investigations to elucidate the unknown PTM crosstalk in AML.

2. RELEVANT POST-TRANSLATIONAL MODIFICATIONS (PTMs) IN AML

PTMs are enzymatic and non-enzymatic modifications that occur on proteins during or after translation. More than 200 PTMs are known now, including small chemical modifications (e.g. phosphorylation) and the addition of whole proteins (ubiquitination) [16]. The same protein sequence can have multiple biological functions as a result of its different PTMs. Moreover, PTMs can contribute to abnormal cellular proliferation, differentiation, adhesion and morphology in disease [17]. Thus, PTM discovery appears to contribute to the understanding of cellular signaling pathways and might be used to find new clinical biomarkers. Some protein modular domains can recognize, transfer or remove PTMs. In the PTM field, these proteins are termed “readers”, “writers” or “erasers”, respectively. Covalently attached PTMs require the activity of specific “writers” enzymes, e.g. kinases in phosphorylation, methylases in methylation, acetyltransferases in acetylation, ubiquitinases in ubiquitination and glycosyltransferases in glycosylation events.

Non-enzymatic PTMs might occur randomly, depending on the tertiary structure of proteins. The nature, site and extent of such modifications on a specific protein might alter its structure and affect its protein functionality ranging from a fully active to a totally inactive molecule [18]. A well-known example of non-enzymatic PTM is protein oxidation

originated by covalent modifications directly induced by reactive oxygen species or indirectly with secondary by-products of oxidative stress [19]. Revising all the PTMs that might be relevant in AML is beyond the scope of this review. However, we will describe herein some enzymatic PTMs of recognized importance in cancer biology, i.e., phosphorylation, acetylation, glycosylation, methylation and ubiquitination. Excellent reviews of other PTMs of clinical relevance can be found elsewhere [20].

2.1 Protein phosphorylation

Phosphorylation events mostly occur at serine, threonine and tyrosine residues by kinase enzymes ("writers"). These residues can also be dephosphorylated by phosphatases ("erasers"). Phosphorylation can affect protein activity, conformation, localization, interaction and degradation. It is involved in cell growth, survival, apoptosis and extracellular signaling [21]. Phosphotyrosine signaling plays a central role in cell-cell and cell-environment interactions [22]. It is estimated that one third of the human proteome is substrate for nearly 500 kinases at any time point [23]. Protein phosphorylation is considered the most abundant PTM in eukaryotes and represents the most studied PTM with nearly 265,547 publications in PubMed (as of October 2018 using protein phosphorylation as search words). Protein phosphorylation is widely studied in cancer research. Phosphorylation data has the potential to unveil significant insights into kinase signaling networks.

In AML cases, it was early discovered that constitutive transcription factor STAT5 phosphorylation is caused by phosphorylation on mutated FLT3 [24]. Such investigations have encouraged the use of FLT3-ITD inhibitors such as sorafenib and quizartinib in clinics and in Phase III trials, respectively [25]. Recently, phosphorylation of transcription activator MEF2C has been identified in most of the studied cases of chemotherapy resistance in AML [26].

2.2 Protein acetylation

This modification occurs at the N-terminus during protein translation to preserve protein stability, interactions or subcellular localization [27]. More than 80% of human proteins carry an acetyl group on the first amino acid. After N-terminus acetylation, further acetylation on lysine residues can occur enzymatically by acetyltransferases, which transfer an acetyl group from acetyl-coenzyme A to the primary amine in the ε-position of the lysine side chain [28]. Lysine residues are deacetylated by deacetylases. Acetylation produces the neutralization of the positive electrostatic charge of the lysine residues regulating protein function, association with other proteins or, in the case of histones, with DNA [21]. Histone acetylation results in an open and transcriptionally active structure of chromatin that allows the binding of transcription factors and transcription co-activators necessary for gene expression. Protein acetylation

is affected by the metabolism. In the absence of nutrients, levels of NAD^+ rise producing an increase of deacetylase activity of SIRT [29].

Histone acetylation and other chromatin-modifying enzymes that are often altered in AML have quickly become of major interest in AML. Recently, it was found that ANP32A, a nuclear phosphoprotein that participates in the INHAT (inhibitor of histone acetyltransferases) complex, promotes leukemogenesis [30]. The expression of histone acetyltransferases such as MYST2 has also been found to be suppressed in AML [31]. Thus, either histone acetylation or deacetylation inhibition processes might represent effective therapeutic approaches in AML. In fact, histone deacetylation inhibitors such as valproic acid alone or in combination with all-trans retinoic acid (ATRA) are used in chemotherapies for unfit patients [32].

2.3 Protein glycosylation

Glycosylation events affect mostly cell surface and secreted proteins. Specific glycosyltransferases of cell compartments can attach multiple oligosaccharide chains (glycans) to a single protein facilitating a proper conformational folding [21]. There are several types of protein glycosylations, with the most studied being attachment of glycans to the amino group of asparagine (*N*-glycosylation) and to the hydroxyl group of serine or threonine residues (*O*-glycosylation). Protein glycosylation processes, involving changes in glycoprotein abundance, glycosylation site occupancy or glycan heterogeneity at different sites on a glycoprotein, are dysregulated in several diseases such as cancer, inflammation, Alzheimer's disease, multiple sclerosis and cystic fibrosis [33].

The dynamic of plasma membrane glycoproteins has been studied during differentiation of the monocytic suspension AML cell line THP1 into macrophage-like adherent cells by selective biotinylation [34].

2.4 Protein ubiquitination

Ubiquitination affects all proteins at some time point in their life cycle by tagging lysine residues with ubiquitin, a small 8.5-kDa protein, for degradation via the 26S proteasome. The ubiquitination process involves the action of three proteins: ubiquitin-activating enzymes (E1), ubiquitin-conjugating enzymes (E2) and ubiquitin ligases (E3) [35]. Ubiquitination is a reversible process. The E3 ligases tasks are opposed by deubiquitinating enzymes (DUB), which cleave ubiquitin off its protein conjugate targets [36]. While protein monoubiquitination appears to be involved in the regulation of the target protein binding and activity, polyubiquitation however seems to call for polypeptide degradation [21]. Additional phosphorylation, phosphoribosylation, acetylation or glutamine deamination on the ubiquitin chain can promote, among many diverse functionalities, the covalent attachment of ubiquitin moieties onto

substrate proteins or inhibit polyubiquitin chain elongation [37-39]. E3 ligases, and therefore the stability and activity of E3 substrates, are deregulated in cancer, and this might lead to downregulation of tumor suppressor activities and upregulation of oncogenic events [40].

Recently, the expression of E3 ligase WWP1 was found significantly increased in primary AML cells and in several AML cell lines. The WWP1 inactivation remarkably disturbed the growth of both primary AML and line-derived cells *in vitro* while *in vivo* mouse models bearing WWP1-depleted AML cells showed a reduced leukaemogenic development [41]. Thus, E3 ligases such as WWP1 might serve as potential AML biomarkers and drug-targeted molecules in future AML therapies.

2.5 Protein methylation

Methylation is one of the most unknown and least studied PTMs. It involves the attachment of a methyl group to proteins on arginine or lysine residues, usually on glycine- and arginine-rich regions known as GAR motifs [42]. Methylation and demethylation processes are catalyzed by methyltransferases and demethylases, respectively. Arginine methylation usually affects proteins with RNA binding activities. Recently, methylarginine sites have been recognized as binding motifs for members of the Tudor protein family, which are now known to be part of PIWI complexes regulating the PIWI-interacting RNA (piRNA) pathway in the germ line [43]. Arginine methyltransferases have recently been linked to cancer, as well as metabolic, neurodegenerative and muscular disorders using transgenic mouse models [44].

Up to three methyl groups can be attached to lysine residues forming mono-, di- and trimethylated derivatives. Di- and trimethylation of lysine residues are believed to prevent the addition of other PTMs to the same residue [21]. This might occur in the case of methylated histones, as a mechanism to prevent acetylation on lysine residues. Lysine methylation, mostly studied in histones because of its relevance on chromatin biology and gene regulation, appears to also be involved in growth signaling and DNA damage responses [45-46]. Furthermore, lysine methylation has been found to regulate chaperone proteins. SETD1 methylation of HSP70 on lysine residue 561 promoted Aurora kinase B activation and stimulated the proliferation of cancer cells [47]. TP53 is methylated at several residues, and demethylation at lysine 370 by LSD1 removed the recognition site for TP53BP1, thus hindering TP53 in reacting against DNA damage [48].

To our knowledge, protein methylation has not been studied in AML yet.

3. MULTIPLE PTM TYPES ON A SINGLE PROTEIN SEQUENCE: THE PTM CROSSTALK

The co-ocurrence of several PTMs on the same protein sequence has been well documented in the past decades. The direct competitive crosstalk between *O*-phosphorylation and *O*-linked *N*-acetylglucosamine (O-GlcNAc) was described more than 20 years ago [49]. The effect of several PTMs working together, sequentially or in combination, was characterized on histone proteins and it was termed the histone code [50]. The "PTM code" concept has recently been introduced and describes the combinations of different PTMs on the same protein sequence that might determine its activation/inhibition state, affect its interaction with other proteins and induce allosteric conformational changes in complex with other proteins, therefore modulating downstream signaling [51]. PTM crosstalk is now recognized to regulate proteins' functions like TP53, tau or tubulin as well as being one of the major cell regulatory mechanisms [51-55]. PTM crosstalk can occur within the same protein (intra) or between different proteins (inter). Examples of both are observed in the histone PTM crosstalk: histone PTM intra-crosstalk occurs when the phosphorylation on serine 10 on histone H3 increases acetylation of lysine 14 on the same histone protein [56-57]; histone PTM inter-crosstalk occurs when ubiquitination on histone H2B promotes di- and trimethylation of lysine 4 on histone H3 [58].

In general, PTM crosstalk can be divided into two types: (*i*) in positive crosstalk, the addition of a first PTM can serve as a signal for the addition or removal of a second PTM, or it can serve as a recognition site for other "writers" that carries out a second modification (e.g. phosphorylation-dependent ubiquitination and SUMOylation); (*ii*) in negative crosstalk, there is either a direct competition of several PTMs for the same residue in a protein, or an indirect competition by masking the recognition site for additional PTMs [51,59-61]. Thus, taking into account single PTM types and combinations of PTM types, it is estimated that the number of unique proteoforms can dramatically increase up to millions [62].

Although specific crosstalk between O-GlcNAc and other PTMs such as phosphorylation, ubiquitination, acetylation and methylation has been well studied in numerous proteins [63], large-scale PTM crosstalk investigations started to emerge only few years ago. The study of single PTM types and PTM crosstalk highly depends on the availability of sample material for the enrichment protocols on one hand, and on fast and sensitive instrumentation to achieve the identification and quantification of a high number of modifications on the other. Because of their importance, enrichment methodologies and MS evolution have become key players in the PTMs discovery field. Therefore, the most popular enrichment strategies and MS features for the study of PTMs and PTM crosstalk will be described next.

We will also review the most relevant bioinformatics tools for the analysis of PTM crosstalk, highlighting advances in data analysis of simultaneous PTMs as well as the challenges and shortfalls of current bioinformatics workflows.

4. STRATEGIES FOR PTM AND PTM CROSSTALK DISCOVERY

4.1 Enrichment methodologies for single PTM types

Protein expression and PTM changes have been widely studied with immunoblot analysis, ELISA microarray and immunohistochemistry in the past [17,64-66]. Although these antibody-based techniques are still widely used for the study of individual protein expression and phosphorylation, the limited commercial availability of highly specific anti-PTM antibodies and the challenges associated with antibody cross-reactivity represent the major restraints of those techniques. Nonetheless, we should not underestimate antibody-based methodologies and remark that one of them, immunoaffinity purification (IAP), is the most effective approach to isolate modified peptides such as acetyl-, ubiquitin- and methylpeptides from complex biological samples.

Most strategies for PTMs analysis begin by digestion of the cell lysate into peptides, followed by enrichment for the modified peptides. Phosphopeptide enrichment can be carried out using agarose-Fe(III) beads in the immobilized metal ion affinity chromatography (IMAC) or titanium dioxide spheres in the metal oxide chromatography affinity (MOAC) [67-69]. It is also possible to use both reagents sequentially in the sequential elution from IMAC (SIMAC) procedure, as well as in combination with hydrophilic interaction liquid chromatography (HILIC) in the TiSH strategy [70-71].

Both IMAC and MOAC approaches have been successfully used in the study of the AML phosphoproteome from primary cells [72-74]. However, our research group have selected IMAC as it was found to give the highest number of quantified phosphosites at high specificity (86%) [75]. We were able to quantify 3,036 phosphosites from 320 μg of lysate from primary blasts with the IMAC methodology [76]. The number of phosphosites can increase approximately 1.5x using the same enrichment approach and amount of lysate when the samples are analyzed on more sensitive MS equipment such as the Q Exactive HF (unpublished results). The enrichment of tyrosine-phosphorylated (pY) peptides is effectively performed by IAP with P-Tyr-1000 antibody, which requires large amounts of lysate (approx. 5 mg as recommended by manufacturers) [77]. Nonetheless, more than 200 pY peptides have been identified using 1 mg of biopsy samples [78]. Using the standard IAP protocol with 5 mg of lysate, the pY proteome of 12 AML patient samples has recently described 219 pY sites from 159 proteins showing activation of both receptor and nonreceptor protein tyrosine kinases [79].

The enrichment of lysine-acetylated peptides can also be performed by IAP using an anti-acetyl-lysine antibody. Although manufacturers recommend a high amount of protein lysate (10-15 mg), effective enrichment can be carried out with 2 mg of starting material [80].

For several years, effective enrichment of arginine-methylated peptides has been performed with antibodies recognizing the modification in the context of an GAR motif [81]. The use of methylation antibodies generated using the peptide library approach has proven efficient in the isolation of approximately 1000 arginine monomethylated sites and between 300 and 400 arginine asymmetric dimethylated sites in HCT116 cells [82]. The enrichment of lysine-methylated peptides was notably improved with the use of commercially available pan-specific anti-mono/di/ trimethyl-lysine antibodies [83]. Recently, IAP of lysine-methylated peptides with a commercially available or homemade antisera-derived pan anti-monomethyl-lysine antibody have routinely identified more than 1000 monomethylation sites in a single experiment. By using high quality of antibodies and large protein amounts as starting material, 1246, 59 and 53 mono-, di- and trimethylated sites, respectively, were identified [84]. The large recommended amounts of starting material that are required for the IAP enrichment of arginine- or lysine-methylated sites, 10-30 mg, makes the characterization of the methylproteome of AML patients difficult. Alternatively, enrichment of mono- and dimethylated sites using the triple malignant brain tumor domains of L3MBTL1 can perform well with few mg of starting material [85].

There is a large variety of strategies to isolate glycopeptides, either intact or derivatized. In this review, we will describe some of the most widely used protocols to enrich only for intact *N*- or *O*-glycosylated peptides and sialic acid (SA)-containing glycopeptides. *N*-glycosylation enrichment is widely carried out with HILIC solid phase extraction (SPE) as a result of the hydrophilic property of polar hydroxyl groups found in glycopeptides [33]. The HILIC SPE enrichment carried out with high concentrations of detergents (SDS or Triton X-100) identified 811 *N*-glycosylation sites from 567 membrane proteins using 200 μg of protein digest [86]. Zwitter-ionic-HILIC (ZIC-HILIC) SPE-based methods have also been proved to enrich *N*-glycopeptides efficiently [87-88]. A ZIC-HILIC tip format used in the study of the *N*-glycosecretome of human hepatocellular carcinoma metastatic cell lines found 1637 unique *N*-glycosites from 635 proteins [88]. HILIC has also been employed in the characterization of *O*-glycosites from pooled blood plasma samples [89]. However the sequential use of lectin weak affinity chromatography (LWAC) identified 1123 unique *O*-glycosites from 649 glycoproteins in human plasma, platelets and endothelial cells [90].

SA-containing glycopeptides have been identified from 100-500 μg of HeLa cell lysate using MOAC enrichment of sialylated glycopeptides followed by HILIC separation of deglycosylated peptides. This method identified 1,632 unique sialylated glycopeptides belonging to 817 sialylated proteins [91]. A combination of MOAC and IMAC beads has been used in the filter-assisted sample preparation (FASP) format for the enrichment of SA-containing *N*-glycopeptides in human serum identifying 194 sites from around 50 μg of sample [92].

The enrichment of lysine-ubiquitinated peptides is carried out by IAP using highly specific antibodies against the K-ε-diglycine remnant motif that is originated upon digestion with trypsin of ubiquitinated proteins [93]. A variant of this standard workflow, which includes high pH reverse-phase fractionation prior to IAP and peptide cleaning with pipette tip-based filter plugs before MS, identified more than 23,244 ubiquitination sites in the HeLa cells ubiquitinome [94]. FASP has also been shown suitable for analyzing the ubiquitinome when used with large ultrafiltration units [95]. However, both in solution- and FASP-based sample preparation methods require more than 5 mg of starting material. An overview of some of the PTMs protocols with high modification coverage is shown in Table **1**.

Table 1. Overview of high coverage protocols for enrichment of single PTMs types

PTM	Enrichment	Starting protein material (species)	Number of quantified sites	Protocol ref. (year of publication)
STY Phosphorylation	IMAC	~300 μg (human/mouse)	>37,000	[96] (2018)
Y Phosphorylation	P-Tyr-1000 IAP	1 mg (human)	150-217	[78] (2017)
STY Phosphorylation in AML	MOAC	250 μg (human)	9,500	[74] (2018)
	IMAC	72-1720 μg (human)	7,831	[73] (2014)
Lysine Acetylation	Acetylated lysine IAP	5 mg (human)	973	[97] (2015)
N-Glycosylation (intact)	HILIC-SPE	1 mg (human)	453	[98] (2014)
Arginine Methylation	Monomethylated arginine IAP	10 mg* (human)	5,700	[99] (2016)
Lysine Ubiquitination	Lysine-ε-GG IAP	4 mg (human)	1746	[94] (2018)

*The amount of peptides, not of proteins, is described in this paper

(S: serine; T: threonine; Y: tyrosine)

4.2 Enrichment workflows for multiple PTMs types

Taken into account the different enrichment protocols for single PTMs types, several combinations of them have been suggested for the study of various PTMs from the same sample. Herein, we will describe those that showed a high coverage of protein PTMs using amounts of starting material that are compatible with the amounts normally obtained from typical AML cell samples. As for the enrichment of single PTMs types, the number of sites from multiple PTMs types simultaneously identified and quantified depends on the amount of starting material, protocol used and the biological event under study.

Simultaneous enrichment of phosphopeptides and *N*-linked sialylated glycopeptides using MOAC, followed by deglycosylation and HILIC, from 200 μg peptides obtained after digestion of a HeLa enriched membrane fraction identified and quantified 3588 phosphosites and 1809 *N*-glycosylation sites [100]. A modified protocol of the TiSH strategy that included the latter methodologies together with SIMAC and acetylation enrichment has recently been described to identify 10,192 phosphopeptides, 964 sialylated *N*-glycopeptides and 81 acetylated peptides from 500 μg of HeLa cell lysate (Fig. **2A**) [101]. The low number of isolated acetylated peptides obtained after this workflow might suggest that a larger amount of starting material could be more efficient for the IAP step. A better enrichment of acetylated peptides (2,898 quantified peptides) was carried out simultaneously with phosphorylation isolation when 200 μg of rat heart tissue per Tandem Mass Tags (TMT) reagent, 1200 μg in total, were first immunoprecipitated with the anti-lysine-acetylation antibody, before the supernatant was used for the enrichment of phosphorylation according to MOAC and HILIC methodologies [102].

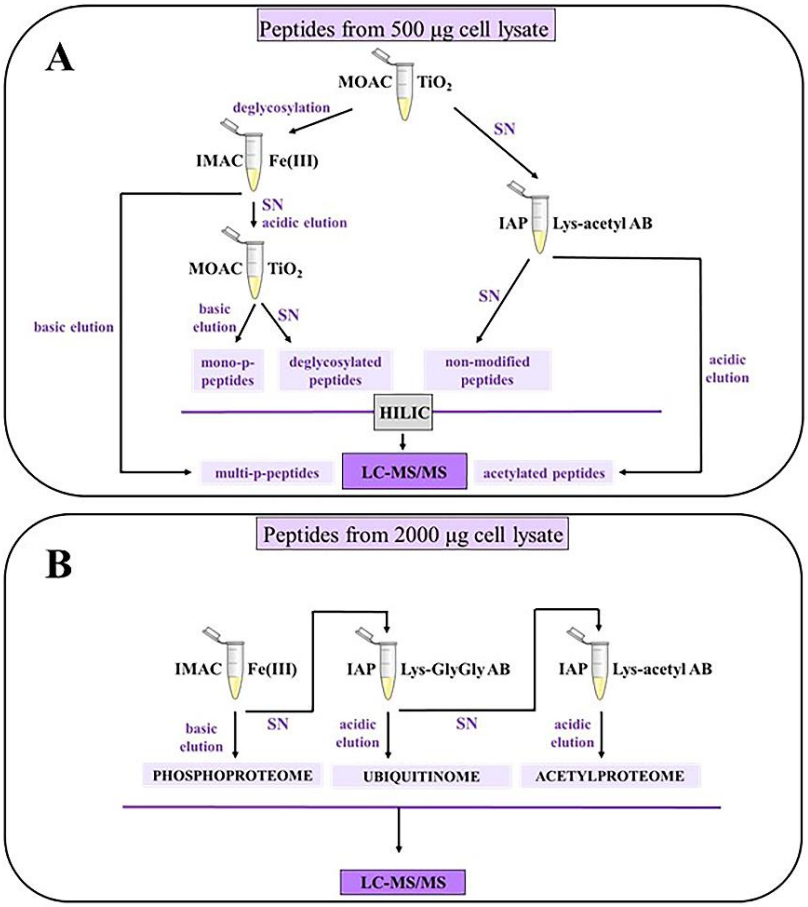

Fig. (2). Sample preparation workflows for the enrichment of multiple PTMs types **A)** Simultaneous phosphorylation, lysine acetylation and *N*-linked sialylated glycosylation in a branched experimental design using MOAC, SIMAC, lysine-acetyl IAP and HILIC techniques; **B)** Simultaneous phosphorylation, ubiquitination and lysine acetylation based on a sequential IAP in the SEPTM (serial enrichments of different post-translational modifications) strategy.

(SN stands for supernatant; AB for antibody; p for phospho)

The SEPTM (serial enrichments of different post-translational modifications) has proved to be an efficient strategy for the quantification of 5,466 phosphorylation sites, 2,078 ubiquitination sites and 317 acetylation sites using 2 mg of metabolically labeled Jurkat cell lysates (Fig. **2B**) [80]. A large-scale SEPTM that involves enrichment for pY, serine/threonine-phosphorylated, lysine-acetylated, lysine-methylated and arginine-methylated sites has recently been described to outline signaling networks in lung cancer cell lines [103]. However, the large amount of starting material that this approach required (approx. 6 mg) might not be feasible to obtain from AML patient samples.

4.3 MS identification and PTMs quantification

MS-based technologies such as Matrix-Assisted Laser Desorption/Ionization (MALDI)-Time Of Flight (TOF), Surface-Enhanced Laser Desorption/Ionization (SELDI)-TOF and electrospray ionization (ESI) tandem MS have been used for the identification and quantification of PTMs for nearly two decades. However, the field of MS-based proteomics is continuously evolving as a result of the availability of faster and more sensitive instruments. Specially advances in the Orbitrap technology over the past and current decades have provided MS equipment with optimized resolution and fast MS/MS scan rates in both the linear ion trap (low resolution) and Orbitrap (high resolution) to maximize protein and PTM identifications and measurement accuracy [104]. In addition, peptide fragmentation techniques that improve PTM identification and localization have remarkably contributed to high coverage studies of PTMs in complex biological samples [105]. Among them, electron transfer dissociation (ETD) and electron capture dissociation (ECD) fragmentation are widely used in the analysis of PTMs. Furthermore, the ETD fragmentation technique using supplemental activation with either collision-induced dissociation (CID) or higher-energy collision dissociation (HCD), i.e. ETciD or EThcD methods, with improved sequence coverage is now available in Orbitrap Fusion Lumos mass spectrometers [106]. Besides discovery/global MS-based approaches, targeted MS can measure specifically annotated modified peptides using their isotope-labeled versions as an internal standard [107].

PTMs can be quantified using label-free (LF) approaches, by metabolic labeling (e.g. stable isotope labeling by amino acids in cell culture, SILAC), or chemically, e.g. by isobaric tags (isobaric tags for relative and absolute quantification, iTRAQ, and TMT). To our knowledge, there are no comparative studies on the error measurement of the different quantification methods for most of the PTMs described in this review and for the simultaneous enrichment for multiple PTM types. A recent study has compared some of these quantification techniques for global phosphoproteomics [108]. LF and SILAC were found to be the most accurate methods whereas MS^2-based TMT yielded the most precise quantification. We recommend the use of labeled strategies for the quantification of simultaneous PTMs due to the complexity of current workflows for sample preparation.

4.4 Bioinformatic tools

MS-based proteomics experiments produce increasingly larger and more complex datasets. Therefore, sophisticated bioinformatics tools are becoming a major bottleneck in many projects. Modern mass spectrometers acquire spectra of high quality, allowing the identification and quantification of proteins across a high dynamic range. However, a large fraction of the acquired spectra remains unidentified, partially due to post-translationally modified peptides [109-110].

A large number of search engines are available, most of which allow for several PTMs as variable modifications during the database search [111]. A search engine can only identify peptides from proteins that are in the selected database and it will not be able to identify a modified peptide correctly unless the specific modification(s) are added to the search [112]. Failure to add the proper modifications in a search can lead to both poor identification rates due to many unmatched spectra, as well as false positives, as the search engine will try to match spectra to something else in the database. The proper addition of variable PTMs is therefore a crucial step in setting up a database search when analyzing MS data. However, adding many variable modifications to a search is also problematic because the search space will increase dramatically for each modification added. Increasing the search space will result in longer search time, more computation power required, higher chances of false identifications and potentially reduced identification rates [113]. The above may explain why identification of spectra from peptides with PTMs is currently a major challenge in the field, and why few software offer reliable pipelines for data analysis of multiple PTMs and PTM crosstalk experiments. Incomplete PTM profiles in MS-based experiments are problematic because PTMs often represent important biological functions [109]. It is therefore important to work towards solving the challenges associated with PTM data analysis. The complexity of PTM crosstalk data analysis will be outlined below. We will review three types of bioinformatics tools relevant for the investigation of PTM crosstalk: PTM prediction tools, databases containing PTM data and software/pipelines for analysis of MS-derived PTM data. Some relevant papers will also be highlighted.

4.4.1 PTMs prediction tools

Several *in-silico* prediction and molecular modeling tools are available to predict PTMs on proteins/peptides, as recently reviewed [114]. Most of the tools are online and focused on single PTM types. Some are based on neural network algorithms (YingOYang and NetPhos 2.0) and some can predict modified sites based on machine learning algorithms (GlycoMine) [115-117]. Few tools are optimized for prediction of PMT crosstalk or PTM hotspots (highly enriched protein regions for PTMs). ModPred is a sequence-based predictor of 23 types of PTM sites and it was the first unified tool for a simultaneous prediction of PTM sites [118]. PTM-X is a webserver for prediction and characterization of intra- and inter-protein PTM crosstalk between two sites [119]. The developers used 193 PTM crosstalk pairs from 77 human proteins and tested residue co-evolution, motif co-evolution and co-modification across species and different conditions. They used a naïve Bayes classifier with kernel density estimation to predict PTM crosstalk and found evidence of crosstalk events preferably occurring among nearby PTM sites, and that crosstalk pairs

tended to co-evolve. Structural analysis of PTM hotspots (SAPH-ire) is a PTM hotspot ranking method of its potential to impact biological functions, integrating experimental PTM observations, sequence conservation, protein structure and protein-protein interaction (PPI) data [120]. SAPH-ire has been used to study PTMs in diverse G protein families and it showed to be predictive for PTMs biological function. In another study, SAPH-ire was applied to eukaryotic protein families containing PTM and 3D structure data (50,839 unique PTM sites organized into 31,747 modified alignment positions) [121]. PTMscape is a recent open source tool available as an R package to predict PTM sites and map modification crosstalk in protein domains and biological processes [122]. This tool predicts sites based on a unified set of descriptors of the physico-chemical microenvironment of the sites. Downstream modules test enrichment of PTMs (individual or pairs) in protein domains. The authors discovered key protein domain crosstalk in histones, protein kinases and RNA recognition motifs, across biological processes such as RNA processing, DNA damage response, signal transduction and regulation of cell cycle.

4.4.2 Databases and resources of PTM data

Scientific data sharing through online databases and repositories is becoming a standard practice, and several journals are now demanding or strongly recommending sharing of underlying raw data [123]. In the PTM field, this is also of utmost importance and several online databases/repositories specialized on PTM data now exist. The dbPTM is an integrated resource for PTMs that provides both experimentally verified PTM data and serves as a platform for accessing many available databases and tools for PTM analysis, including site prediction tools, 3D structure viewers and network investigators [124]. The dbPTM platform was recently updated with more than 30 external PTM resources and now includes more experimentally validated PTMs from available databases, manual literature curation and disease association based on non-synonymous single nucleotide polymorphisms (nsSNPs). Phosphosite Plus (PSP) is a popular online systems biology repository providing comprehensive information and tools for the study of PTMs [125]. This resource is dedicated to mammalian PTMs including phosphorylation, ubiquitination, acetylation and methylation data. PSP provides information about PTMs involvement in disease [126]. PTMfunc is a repository of functional predictions for protein PTMs [127]. From nearly 200,000 phosphorylation, acetylation and ubiquitination sites, the functional relevance of PTMs were prioritized by predicting those that likely participate in cross-regulatory events, regulate domain activity or mediate PPIs. PTMCode is another database containing known and predicted functional associations between PTMs within and between protein [128]. PTMCode aims to provide a framework presented as an interactive web interface to enable hypothesis-driven experimental or computational analysis. PTMs

functional associations are based on five evidence channels: a literature survey, residue co-evolution, structural proximity, PTMs at the same residue and location within hotspots. An updated version (PTMCode v2) was recently published presenting a new strategy to propagate PTMs from validated modified sites through orthologous proteins [129]. This version is focused on the regulatory role of PTMs in PPIs and reports 8 million associations of PTMs regulating single proteins and over 9.4 million interplays tuning protein-protein interactions. The database CrosstalkDB focuses on PTM crosstalk in histones and was developed to collect and organize MS data of multiply modified histones or histone tails [130]. HIstome is a manually curated histone-focused database and relates PTMs to their modifying proteins [131]. The SysPTM was developed as a systematic resource of proteomics PTM data and a suite of web tools for annotation of PTMs [132]. It has been updated recently (SysPTM 2.0) and currently contains a knowledge base of manually curated multi-type modification data and four fully developed, in-depth data mining tools [133].

4.4.3 Software and pipelines for the analysis of MS-derived PTMs data

Most proteomics software are capable of handling PTM data, however, few software tools or pipelines are optimized for the analysis of experimental PTM MS-derived data. Recently, the performance of eight search engines (pFind, Mascot, SEQUEST, ProteinPilot, PEAKS, OMSSA in COMPASS, X!Tandem in TPP and Andromeda in MaxQuant) was compared in terms of their ability to identify histone PTMs, and it was found that pFind and Mascot gave the best performance [109]. The PTMeta computational pipeline gives increased identification rates by using a pre-scanning to find the most abundant modifications [134]. Extensive database search follows, together with a statistical framework to combine the results from database search runs with different modification settings. The ISPTM is another and iterative search algorithm for systematic identification of PTMs from complex proteome mixtures [135]. An example of a commercially available software package that has a special focus on PTM analysis is Byonic by Protein Metrics [136]. Byonic is a promising tool for PTM crosstalk studies because it (*i*) allows an essentially unlimited number of variable modification types, (*ii*) allows for a separate number of occurrences for each modification type, (*iii*) offers the wildcard search for unknown PTMs alongside known ones, and (*iv*) is also one of few software that are able to identify intact glycopeptides. The popular MaxQuant tool has also been used in several multi PTMs and PTM crosstalk studies [137-138]. However, many research groups prefer to use in-house developed tools and pipelines, or rely on manual interpretation of mass spectra, often resulting in highly complex analysis workflows [16,139]. Some PTM crosstalk studies used both commercial and free proteomics software in the same study, e.g. crosstalk between phosphorylation

and O-GlcNAc was investigated using both Byonic and MaxQuant, as well as manual spectrum interpretation for site annotation [140]. In the SEPTM study mentioned earlier in this review, Grimes *et al.* performed a comprehensive large-scale analysis of phosphorylation, acetylation, and methylation in 45 lung cancer cell lines compared with normal and drug treated cell-lines [103]. They used a cluster-filtered network (CFN) approach to analyze MS data and a *t*-distributed stochastic neighbor embedding (t-SNE) method to identify PTMs clusters. Their approach was to integrate two different kinds of information in the computational data analysis: clustering based on statistical relationships among various PTMs and PPI data from public databases, outlining relevant signal transduction pathways.

A summary of key features of some of the tools described above and that we can recommend for the study of PTM crosstalk are shown in Table **2**.

Table 2. Summarized features of some available bioinformatic tools for the study of PTM crosstalk.

Bioinformatic tool	Major purpose(s)	Notable features	URL/Reference(s)
PTM-X	PTM crosstalk prediction	Intra- and inter-protein prediction for two sites	http://bioinfo.bjmu.edu.cn/ptm-x/ [119]
SAPH-ire	PTM hotspots prediction and ranking	Focus on biological and functional impact	[120]
PTMScape	PTM site prediction and crosstalk mapping	Open source tool; optimized for large-scale crosstalk mapping; R package	http://137.132.97.109:59739/CSSB_LAB/[122]
dbPTM	Integrated PTM resource	Both a platform (>30 external tools) and a PTM data repository	http://dbptm.mbc.nctu.edu.tw/ [124]
PTMCode	Database for PTM associations	Interactive web interface; several evidence channels	http://ptmcode.embl.de/[129]
PTMeta	Computational pipeline for peptide PTM	Full pipeline; optimized for large-scale analysis of multiple PTM types	[134]
MaxQuant	MS data analysis software package	User-friendly; familiar for most proteomics researchers; not optimized for large-scale PTM crosstalk analysis	[141]
Byonic	Mass spectrometry search engine/ software	Suitable for PTM data analysis; commercial; not optimized for PTM crosstalk	[136]

5. PTM CROSSTALK DESCRIBES PROTEIN FUNCTIONS AND INTERPRETS SIGNALING

Although PTM crosstalk is at an early stage, several reports producing MS-generated data from PTM-enriched samples are now available. One of these studies has described the lysine-acetylome and lysine-phosphoproteome of HCT-8 and HCT-116 colon cancer cells after treatment with histone deacetylase (HDAC) inhibitor romidepsin [142]. Modified proteins were involved in spliceosome, nucleoplasm activity, mitotic prometaphase, cell cycle, histone core interactions, mRNA catabolism, RNA biosynthesis and histone modifying MlL1-WDR5 complexes. Examples of PTM crosstalk based on positive regulation between the acetylome and phosphoproteome were described, and 274 proteins, *i.e* HNRNP (heterogeneous nuclear ribonucleoproteins) A1, HNRNP U and the CDH4 complex among them, showed both lysine acetylation and lysine phosphorylation modifications. Another study using HCT-116 colon cancer cells, here treated with dichloroacetate, which is a pyruvate dehydrogenase kinase inhibitor, also showed a positive correlation between acetylated and succinylated sites, identifying simultaneously acetylation and succinylation on proteins involved in glycolysis such as PKM (pyruvate kinase muscle), PGK1 (phospho-glycerate kinase 1), LDHB (lactate dehydrogenase B) and ENO1 (enolase 1) [143].

PTM crosstalk has also been characterized in single proteins. An impressive PTM crosstalk study on histone H3 tails purified from wild type and engineered mouse embryonic stem cells, which were knocked out in components of the epigenetic machinery (SUZ12, RING1A and RING1B) and in three DNA methyltransferases (DNMT1, DNMT3A and DNMT3B), found a positive crosstalk between adjacent monomethylated and acetylated lysine, and a negative crosstalk among nearly all the seven identified di- and trimethylated lysines in H3 tails [144]. The study identified arginine methylation events that do not co-occur with other PTMs in the H3 tail. Notably, R2me1 and R2me2 were found to antagonize K4me3; R8m2 appeared to antagonize K9 methylation, K4me3, K9ac and K14ac (K and R stands for lysine and arginine; me and ac stands for methylated and acetylated, respectively, in the PTM description of this study). This well-defined crosstalk pattern strongly suggested R2- and R8-methylations as indicators of chromatin states. In addition, integration of RNAseq data with the H3 tail PTM data found correlation between monomethylations and K9-methylations and their modifying enzymes.

Thus, it becomes clear that the greatest challenge in the study of simultaneous PTMs is the elucidation of crosstalk mechanisms from interpretation of MS-based data. While crosstalk discovery might be feasible in single proteins or protein families, crosstalk characterization of biological samples of high complexity remains challenging. It has been suggested that a quantitative strategy based on systems biology in combination with mathematical modelling might

unravel complex PTM crosstalk [145]. As the precursors of protein PTMs are mostly small molecules (except in the case of ubiquitination), the integration of metabolomics data with the PTM-omics data becomes necessary to understand PTM changes and their crosstalk [146].

6. AML COMPLEXITY CAN BE DECODED BY PTM CROSSTALK

Recent studies have revealed the importance of dysregulated epigenetics in the pathogenesis of AML, mainly affecting cytosine methylation and modifications of histone and non-histone chromatin-binding proteins. The most studied protein epigenetic modifications in AML are histone acetylation and methylation. Mutations on histone lysine acetyltransferases (*KAT)* and *HDAC* are rare in AML [147]. HDAC activity rather seems to be influenced by interaction with myeloid oncoproteins such as EVI1 and PML-RARA [148-149]. Methylation of histone lysines is processed by lysine methyltransferases (KMT) and reverted by lysine demethylases (KDM). Although histone methylation can both activate and repress gene transcription, several methylation sites have already been associated with either gene activation (H3K4, H3K36 and H3K79) or repression (H3K9, H3K27 and H4K20). In AML, both KMT and KDM are involved in fusion proteins of aberrant methylation and demethylation activities [147].

Due to their reversibility, epigenetic modifications are attractive drug targets. Several inhibitors of DNA methyltranferases, IDH1/2, HDAC, histone KAT, histone acetyl "readers", histone KMT, histone KDM and histone arginine methyltransferases have been tested in clinical trials and some of them got approval for clinical use [147,150-153].

However, in order to design novel targets of epigenetic modifications, the identification of epigenetic PTMs and their crosstalk as well as the corresponding modifying proteins becomes relevant. An MS-based quantitative profiling of the most prevalent PTMs in sera from AML, breast cancer and non-small cell lung cancer patients (lysine acetylation and arginine monomethylation) was recently carried out with 250 µl (app. 15 mg of total protein amount) of serum samples, employing a sequential IAP workflow with lysine-acetyl and arginine-methyl antibody beads [154]. The study identified 796 acetylation and 808 methylation sites from the three cancer types. Functional classifications of the proteins identified in the enrichment of acetylated peptides showed that most of the sites were found in serum-abundant and secreted proteins. Conversely, many arginine-methylated sites were identified in RNA processing proteins and transcription regulators, and few in serum-abundant proteins. Hierarchical clustering of quantitative data for both enrichments from all the cancer samples showed that samples from the same cancer type clustered together when using

methylation enrichment data in the analysis, and that AML and breast cancer samples showed the highest correlation. Conversely, when acetylation enrichment data were used, not all the patients from the same cancer type clustered together, showing a high heterogeneity of the acetylproteome of the cancer types investigated in this study. While the acetylproteome of AML samples was enriched for acetyl modifications on proteins of the complement and coagulation cascades, the monomethyl-arginine sites were localized on proteins involved in the mRNA surveillance pathway.

Twenty histone modifying proteins (HMP) were analyzed by a reverse phase protein array comprising a total of 232 primary antibodies in a large cohort of 205 AML patients using the protein expression of the CD34+ cells as reference. Higher expression of HMP, KDM1A, HNRNPK, NCL, HDAC2, ASH2L, SIRT1, WTAP, CLPP, BRD4 and NPM1, mainly in *FLT3* mutated patients, was associated with poorer outcome. Inhibition of transcriptional repressor KDM1A seems to increase H3K4 marks and re-expression of aberrantly silenced genes such as tumor suppressor gene *CDH1* encoding for the e-cadherin protein in AML cell lines [155]. NCL (nucleolin) appears to affect epigenetic regulation by acting as a histone chaperone and as a transcription regulator of RNA polymerase 1. Upregulation of this polymerase is related to altered apoptosis, differentiation and appearance of leukemic cells [156-157].

To our knowledge, there are no global or large scale, *i.e.* MS-based, characterization studies of other PTMs, beside phosphorylation, and PTM crosstalk in AML blasts. The characterization of other PTM types, PTM crosstalk and the corresponding modifying proteins turns into a necessary strategy to understand AML complexity.

CONCLUSION

The availability of enrichment methods at peptide level for a substantial number of protein PTMs as well as increasingly sensitive LC-MS/MS equipment encourage researchers to investigate the profiling of multiple PTM types in a proteome. MS-based proteomics technologies can now provide in-depth and unbiased quantitative data on PTMs on a global scale. Nevertheless, the amounts of cell lysate required for the enrichment of many PTMs, mostly the IAP-based enrichments, represent a limitation to the study of clinical samples, like those from AML patients. In addition, new and/or improved bioinformatics tools are required for data analysis, interpretation and presentation of the study outcome.

Although the era of the discovery of multiple PTM types has just started and still represents a young research field with several challenges to solve, we need to move beyond studies that focus on single PTM types and instead focus on PTM crosstalk, that might determine the function(s) of proteins. In AML, several phosphoproteome studies have well

characterized key phosphorylation events. However, as phosphorylation positively and negative crosstalks with other PTMs, it becomes necessary to investigate several PTM types simultaneously to properly define the interaction mechanisms of targetable proteins.

We hope this review will introduce the PTM crosstalk field to the AML research community and encourage, using the current MS-based sample preparation protocols and bioinformatics tools, the finding of the multiple functions of the AML proteome. The discovery of the different proteoforms determined by their PTM status will lead to the selection of biomarkers of higher predictability and the design of more effective therapeutic strategies.

CONSENT FOR PUBLICATION

Not applicable.

CONFLICT OF INTEREST

The authors declare no conflict of interest, financial or otherwise.

ACKNOWLEDGEMENTS

MHV is supported by the Western Norway Regional Health Authority and the Norwegian Cancer Society. AG is supported by the Research Council of Norway. FB is supported by the Western Norway Regional Health Authority. RW is supported by the Norwegian Cancer Society.

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