REVIEW ARTICLE

Utilization of Analytical Omics Tools in the Molecular Diagnostics of Multiple Myeloma

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Abstract: Multiple myeloma (MM) is characterized by the clonal proliferation of malignant plasma B-lymphocytes and even as of today, it is an incurable disease. MM accounts for approximately 10% of all hematologic cancers. Its molecular pathogenesis is poorly understood, but the bone marrow microenvironment of tumor cells and genetic factors have apparent roles in the process. Accurate diagnosis is important to properly identify and stratify the disease, however, MM identification steps are time-consuming and expensive. Thus, development of early molecular diagnostic methods is of high importance in order to start proper therapies as early in the disease progression as possible, given the nature of the poor survival rates/remission periods. Molecular diagnostics via analytical omics represents one of the promising toolsets to speed up the diagnostic process. In this paper, we critically review the utilization of state of the art, high sensitivity analytical omics approaches (genomics, proteomics, metabolomics, lipidomics and glycomics) in MM diagnostics at the molecular level.

Keywords: Multiple myeloma, molecular diagnostics, genomics, proteomics, metabolomics, lipidomics, glycomics.

1. INTRODUCTION

Multiple myeloma is a hardly diagnosable disease in its early stage due to the presence of malignant plasma cells only in the bone marrow [1]. Symptoms are triggered by the excess of plasma cells in the bone marrow, producing a large amount of immunoglobulins, causing weakness, confusion, fatigue, anemia or hypercalcemia. In the meantime the associated headache, visual changes and retinopathy may be caused by the hyperviscosity of the blood, due to its high paraprotein content (when the disease is advanced). Full understanding of the molecular mechanism behind MM would help in the development of modern therapeutic alternatives [2]. Multiple myeloma is the second most frequent malignant disease of the blood in most parts of the World and its incidence varies depending on geographic location and race distribution [3]. The occurrence rate in the United States was 6.1/100,000 between 2007 and 2011, with the African American population twice as likely that of in the white Caucasian race, which latter shows only a 4/100.000 incidence rate [4, 5]. African Americans are more likely to suffer MM, in this ethnic group is lower risk to develop IgH translocations than their European counterparts [6, 7]. Regarding the sexual distribution, it is slightly more common in men. The average age of the first diagnosis is about 61 years for women and 62 years for men [8] but 2% of the patients were younger than 40. An average of 20 000 new cases are diagnosed every year and the median age of death is about 75. Introduction of new therapeutic drugs such as thalidomide, lenalidomide, bortezomib increased the survival and decreased the mortality rate with 2% per year [9]. The anemia caused by multiple myeloma, manifested by the inhibition of normal red blood cell production (hematopoiesis), is the results of the replacement of normal bone marrow by infiltrating tumor cells. Renal insufficiency may occur both acutely and chronically, however, the most common cause of renal failure is the large amount of paraproteins secreted by malignant plasma cells in MM patients [10]. Multiple myeloma cells produce different types of monoclonal proteins, most commonly immunoglobulins, paraproteins, Bence-Jones proteins (BJPs), free immunoglobulin light chains, *etc*. and these species are abnormally high in the bloodstream [11]. These proteins mostly excrete through the kidneys and damage the renal tubules of the nephron. By all means, the total immunoglobulin level is significantly increased in MM, and the majority of these antibodies are inefficient monoclonal forms and paraproteins from the malignant plasma cells.

There are various blood tests to diagnose multiple myeloma, *e.g*., erythrocyte sedimentation rate (ESR), serum electrophoresis as well as serum levels of β2-microglobulin, calcium, albumin, urea and creatinine. If ESR is abnormal, it can be a sign of various illnesses such as infections or inflammation but also for multiple myeloma [12]. A full blood cell count can also be

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conducted to check the levels of the different types of blood cells. Abnormal blood cells can be an indication of MM. Blood calcium level also is elevated in MM, especially when the malignant cells are in the hard outer cover of the bones [13]. Urine tests can reveal abnormal proteins like BJP as one type of paraproteins, which the body eliminates into the urine. Half of newly diagnosed MM patients have reduced creatinine clearance and ~9% of the patients have obligatory dialysis due to severe renal failure in case of cast nephropathy [14]. The severe kidney destruction induced by these pathological processes is one of the primary causes of death for MM patients [15].

Nonsecretory multiple myeloma (NSMM) is a rare form that accounts for 1 - 5% of all cases. In NSMM, the plasma cells probably fail to secrete immunoglobulins, thus the absence of abnormal amount of paraproteins makes the diagnosis difficult [16]. Monoclonal gammopathy in blood and pathological paraproteins in urine are not identifiable, thus it is hard to establish the accurate diagnosis [17]. Morphological examinations such as bone marrow aspiration, magnetic resonance imaging (MRI) or computational tomography (CT) can help to set up an accurate diagnosis.

The International Myeloma Working Group (http://imwg.myeloma.org/) suggests that a patient with presumed MM should undergo bone marrow aspiration and/or biopsy, and the diagnosis is verified when the detected plasma cells are 10% or greater [18, 19] (Table **1**). If superabundance of monoclonal antibodies occurs in patients but neither any organ deviation is identified, nor the histological examination of bone marrow shows any results, the status would indicate monoclonal gammopathy of undetermined significance (MGUS) [20]. MGUS is a status where the body produces myeloma proteins (M-proteins) [21], an abnormal immunoglobulin fragment or immunoglobulin light chains in excess by the abnormal clonal proliferation of plasma cells. Other terms for such proteins found in blood and urine are as follows: M protein, M component, spike protein, or paraprotein. It is important to note that MGUS can transform into 'smoldering' myeloma or myeloma multiplex blood diseases [20-22].

2. MULTIPLE MYELOMA CLASSIFICATION SYSTEM

Classification of MM patients starts by examining the "CRAB" levels (calcium elevation, renal damage, anemia, bone destruction) and the conventional radiography method by measuring against the Durie Salmon (D/S) criterions (Table **2**). D/S staging system is based on the amounts of abnormal monoclonal immunoglobulins, calcium and hemoglobin found in the blood, moreover on the seriousness of bone damage based on X-ray examination [23, 24]. However, the recently modified Durie Salmon Plus (D/S P) system utilizes modern imaging technologies [25], thus, early MM statuses of I, II and III can be efficiently isolated. Multiple and complex chromosomal abnormalities are present in MM and using cytogenetics or genetics techniques such as PCR or interphase fluorescence in situ hybridization (FISH) makes possible to reveal that chromosomal abnormalities are nearly universal and early events in MM [26]. Considering the above, the International Myeloma Working Group approved the International Staging System (ISS) in 2005 [27]. Since then, serum β2-microglobulin (β2M) was introduced as a potential prognostic factor in MM by univariate and multivariate techniques, thus, the ISS enables easy comparison of clinical trial results and more reproducible than the D/S staging method. The latter one cannot be used for therapeutic risk prediction and not exact enough for precise estimation of tumor malignancy [19]. Therefore, ISS is by far the most appropriate classification method of MM patient diagnosis. Please note that high β2M level reflects not only MGUS [28], asymptomatic MM or MM patients and renal dysfunction, but also other unknown parameters, such as diabetes mellitus, hypertonia and compromised immune functions, that cannot be classified into the stages specified above [27]. Although, both the D/S P and the ISS systems provide accurate classification for MM, future staging systems should account for tumor genetics and post-translational modifications of protein biomarkers to better assess the disease burden [7, 29].

3. GENOMICS

Genetic markers are frequently used as diagnostic tools in multiple myeloma. DNA mutation is detectable by both high resolution comparative genomic hybridization array (CGH) or fluorescence in situ hybridization (FISH), which also demonstrated their importance in the molecular classification of MM. These molecular biology techniques are available in clinical blood tests [31-33]. Cytogenetic methods can provide useful prognostic information by separating hyperploid and nonhyperploid multiple myeloma cases [34, 35]. FISH has been frequently utilized in multiple

Table 1. Traditional diagnostic procedures currently used to identify patients with multiple myeloma.

Protein Analysis	Morphology	Imaging Techniques
Serum Electrophoresis	Bone marrow aspiration	X-ray
Immunofixation	Trephine biopsy	MRI
Serum-free light chain assay	Immunophenotyping	CT/PET-CT
Serum-heavy light chain assay	Genetics and cytogenetics	

Table 2. Comparison of the Durie-Salmon [25] Durie-Salmon PLUS [30] and the International Staging Systems [27].

Stage	Durie-Salmon Staging System	Durie-Salmon PLUS Staging System (MRI, FDG PET/CT)	International Staging System
I	All of the following: Hemoglobin value >10.5 g/dL Serum calcium value normal or ≤12 mg/dL X-ray studies of bone, normal bone structure (scale 0) or solitary bone plasmacytoma only Low monoclonal-component production rate—IgG value <5 g/dL; IgA value <3 g/dL Bence Jones protein <4 g/24 hours	Can have a plasmacytoma and/or at least < 5 focal lesions; mild diffuse disease	Serum $beta_2$ -microglobulin <3.5 mg/L and albumin ≥3.5 g/dL
II	Neither stage I nor stage III A = No renal failure (creatinine ≤2 mg/dL) B = Renal failure (creatinine >2 mg/dL)	5–20 focal lesions; moderate diffuse disease	Serum $beta_2$ -microglobulin <3.5 mg/L and albumin <3.5 g/dL or $beta_2$ -microglobulin 3.5 to 5.5 mg/L
III	Hemoglobin value <8.5 g/dL Serum calcium value >12 mg/dL X-ray studies of bone, >3 lytic bone lesions High monoclonal-component production rate—IgG value >7 g/dL; IgA value >5 g/dL Bence Jones protein >12 g/24 hours	> 20 focal lesions; severe diffuse disease	Serum $beta_2$ -microglobulin ≥5.5 mg/L

myeloma diagnosis to detect non-random abnormalities in interphase nuclei [36]. In order to determine if MM contributes to recurrent molecular cytogenetic changes as a response to bortezomib therapy, Chang *et al*. used fluorescence in situ hybridization combined with cytoplasmic immunoglobulin light chain staining (clg-FISH) and found to be independent of returning genomic abnormalities [37-39]. Hyperdiploidity is identified by FISH in about 50% of multiple myeloma patients including several trisomies of the odd-numbered chromosomes. Hyperdiploid MM has a longer overall survival rate and a favorable prognosis. The oligo-based comparative genomic hybridization array analysis (oaCGH) is valuable in detecting prognostically relevant genomic aberrations. Combination of oaCGH data with ISS staging might help to define new sub-groups in MM [40]. However, translocation and/or partial or complete deletion of chromosome 13 associated with other genetic markers of malignancy indicated poor progression for patients with MM [41]. As a general consideration, chromosome 13 deletions/translocations are prerequisites for the clonal expansion of MM. In myeloma, the deletion of 17p13 most probably leads to loss of TP53 heterozygosity, which is a well-known tumor suppressor gene and regulates cell-cycle progress and apoptosis. This abnormality is associated with poor outcome and it can be detected by FISH [41, 42]. Nonhyperdiploid myeloma (NHMM) patients are described by a frequency of over 85% of immunoglobulin heavy chain (IgH) chromosome translocation. Translocation of the IgH oncogene has effects on 14q32, 4p16, 6p21, 11q13 and 16q23 localizations [43]. NHMM patients (a very aggressive form of the disease) showed short remission phase and poor overall survival. Patients with NHMM have a higher prevalence of genetic occurrences associated with progression, which includes chromosome 13, 14 and 17 abnormalities [44]. One of the most frequent genetic abnormalities is t(11;14)(q13;q32) translocation (Table **3**), which causes increased gene expression of the oncogene cyclin D1 (CCND1). Indeed, cyclin D1 expression has been discerned in absence of t(11;14) in MM patients carrying hyperdiploid karyotypes and chromosome 11 polysomy [45, 46]. In MM the t(11;14)(q13;q32) translocation, occurs in about 15% of the patients and seems to be associated with the fibroblast growth factor receptor (FGFR3), and the MM SET domain (MM SET, also known as WHSC1) dysregulation. The t(4;14)(p16.3;q32) translocation is cytogenetically latent [46-48] but FISH can identify the presence of this rearrangement. The t(4;14) redistribution involves the IGH gene and two protein-coding genes located at 4p16.3, multiple myeloma SET domain (MMSET) and the FGFR3, an oncogenic receptor tyrosine kinase [49, 50]. The t(14;16)(q32;q23) translocation is associated with increased c-maf expression in about 5% of MM patients [51]. The t(6;14)(p21;q32) involves the gene CCND3 at 6p21, resulting in up-regulation and it is often seen on a backdrop of a complex karyotype, and the derivative chromosome 14 can be present in multiple copies [46, 52].

Chromosome 1 abnormalities are common structural mutations in MM mostly involving deletions and/or amplifications in 1p, which indicates a poor prognosis [53]. TP53 deletion of the short arm of chromosome 17 leads to the loss of heterozygosity of TP53, which is associated with a more aggressive type of MM. It shows poor prognosis for MM patients, however, it can be detectable with FISH [54]. Chromosome 14 abnormalities frequently mean aberrations in hypoploid MM and associated with poor prognosis, but it is detectable only by FISH. Amplifications and/or translocations of the MYC

Table 3. The most recurrent IgH translocations in multiple myeloma are reported with the frequency, main molecular target and the normal gene functions.

Abnormal Chromosome Number	Translocation	Gene(s)	Frequency	Signature	Gene Function	Prognostic Relevance
14 [45, 46]	t(11;14)(q13;q32)	CCND1	15-30%	cyclin D1 upregulation	Cell cycle G1/S transition	Good
14 [46-50]	t(4;14)(p16.3;q32)	FGFR3, MMSET	15-20%	MMSET/FGFR3/Cyclin D2 upregulation	Bone development and maintenance Oncogene, overexpression results in proliferation and anti-apoptotic effects	Poor
16 [51]	t(14;16)(q32;q23)	MAF	5%	c-maf/cyclin D2 upregulation	Oncogene, enhances tumor stroma interactions	Poor
12 [46, 52]	t(6;14)(p21;q32)	CCND3	3-4%	cyclin D3 upregulation	Cell cycle G1/S transition	Good

oncogene appeared in 45% MM patients [55]. Karyotype, FISH, PCR or RNA-based micro arrays have been able to detect gene expression profile of patients with MM, thus accurately predict the prognosis. Deletions in the p53 locus (17p13) and activation of RAS mutations have been identified in 10% and 35–50% of MM patients, respectively. The presence of these abnormalities is presumably associated with poor prognosis [56, 57]. Therefore, it can be speculated that such anomalies play critical roles in the progression of the disease. [46] Glucocorticoid resistance has been triggered by low expression/mutation of NR3C1, which decreases therapeutic options, but thalidomide keeps patients responsive to the therapy [58-60]. Melphalan resistance [59] has been triggered by t(4;14) and it proved to be ineffective for patients with p53/MM SET [61].

Gene expression profiling can identify high-risk MM disease. Shaughnessy *in vitro*. detected 70 genes using 17 probe sets capable of predicting high-risk prognostic significance, a specific technology that was later developed to a Myeloma Prognostic Risk Score (MyPRS) [7, 62, 63]. Genome-wide association studies (GWASs) investigate frequent genetic variants between groups of individuals known to have a disease and similar individuals without the disease. The MyelomA Genetics International Consortium (MAGIC) has determined alterations that might predispose an individual to MM, such as a genetic variation in the 8q24 region [7, 64, 65]. A preliminary report from that investigation has already presented that African-American patients with MM have different disease characteristics than Caucasian patients with MM [66]. The advent of new technologies, such as gene-expression profiling (GEP), has provided the necessary tools to study MM in unprecedented detail. Pioneering studies using microarray technology by Zhan *et al*. have identified novel MM-associated genes suggesting a genomics-based classification system for MM [7, 67].

4. PROTEOMICS

Analytical proteomics comprises a combination of techniques that are designed to profile, quantitate, and determine proteins or peptides at the global level. Each proteomics technologies has different advantages, disadvantages, and limitations and the technologies shown below should be considered as complementary tools [68] (Fig. **1**). Two-dimensional gel electrophoresis (2-DE) has been the classical technique of choice for the separation of complex protein mixtures [69, 70]. Its advantages are the unparalleled resolution at the protein level, the efficiency in separating protein isoforms, and the capability of generating a proteome ‘‘snapshot’’ of immediate visual impact [46, 71, 72]. Rees-Unwin *et al*. applied global protein expression analysis to describe the pathways of dexamethasone-mediated apoptosis and resistance in MM [73] by analyzing MM.1S cells with 2-DE and identified a series of proteins that were up- and down-regulated after dexamethasone treatment. As a development, 2-D DIGE (Difference Gel Electrophoresis) solves some of the problems related with traditional 2-DE such as samples can be easily compared or the use of an internal standard for the comparison of several biological replicates and allows more accurate and sensitive quantitative proteomic studies [74, 75]. As a separation, detection, and quantification technique, DIGE is an important tool, especially for clinical laboratories involved in the determination of protein expression levels and biomarker discovery [76]. Serum protein electrophoresis (SPE) is utilized in the detection of serum protein disorders. It is inexpensive and widely available in hospitals and clinics to separate serum proteins, such as albumin and immunoglobulins, based on their size and charge, and readily detects paraproteins. While, SPE is an accepted technique for monitoring the clinical course of MM [77], there are some disadvantages due to unseen M-proteins and co-migration with other serum proteins [78]. Positive SPE predicts the high likelihood of plasma cell neoplasm, while a negative result suggests the absence of MM. Patients with MM may be identified with serum protein electrophoresis, although it should be combined with UPE (urine protein electrophoresis), IFE (immunofixation electrophoresis) and FLC (serum-free light chain) assays to minimize diagnostic errors [79]. IFE is a standard clinical technique for identification

and immunochemical characterization of monoclonal immunoglobulins (M-proteins) [80]. It proved to be more sensitive than SPE, however, IFE is not quantitative. Immunoglobulin heavy/light chain immunoassays ('Hevylite', HLC) is a recently developed tool, which separately measures κ and λ light chain types of IgG and other immunoglobulins, thus, provides information about each tumor clones and give quantitative data about the immune suppression of each non-tumor related immunoglobulins [81]. HLC ratios provided a quantitative addition to IFE [82]. The new form of flow cytometry, fluorescence-activated cell sorting (FACS) can reveal global immune dysfunctioning by screening and analyzing cell surface antigens in blood circulation [83]. This method indicates IgG abnormalities in MM patients. In liquid chromatography (LC) based proteomics, mixtures of proteins are mainly digested to peptides by proteases and then separated by one or two dimensional LC, and coupled with MS or MS/MS [84]. The samples separated by chromatography are injected into the mass spectrometer via online electrospray ionization or spotted to MALDI (matrix assisted laser desorption ionization) target plates for further analysis by MS/MS. MS-based quantification methods are based on labeling proteins or peptides prior to the MS analysis. These include: stable-isotope labeling by amino acids (SILAC) in MM cell culture, isotope-coded affinity tags, iTRAQ (isobaric Tags for Relative and Absolute Quantitation) and ^{18}O labeling [76, 85, 86]. Stable-isotope labeling by amino acids in cell culture for human MM cell lines NCI-H929, MM1S, U266, KMS11, or PRMI 8226 [87] strategy has several advantages with respect to ^{15}N labeling. In SILAC, the expected mass differences are known before peptide identification, simplifying quantitation. A SILAC study was the first attempt to provide a comprehensive map of changed protein expression profile accompanying transformation of MM to sPCL in a single patient, identifying numerous candidate proteins that can be targeted by currently available small molecule drugs [88]. Stable-isotope labeling by amino acids in cell culture has the advantages of a predictable mass shift and the incorporation of the tags during cell growth, prior to sample preparation [68, 76, 89]. The iTRAQ solution labels the N-terminus of tryptic peptides and the amino group side chain of lysine residues. The labeled samples are analyzed by mass spectrometry [85]. Collision induced dissociation of iTRAQ-labeled peptides creates peptide sequence information and relative quantification data between the samples [90, 91]. de la Luz-Hernández *et al*. also applied the iTRAQ technology to investigate different protein levels in adapted and non-adapted NS0 multiple myeloma cell lines. This study discovered changes in lactate production rate with respect to glucose consumption [92]. iTRAQ holds the promise as an efficient analytical proteomics based biomarker discovery tool [93]. ^{18}O labeling is a method where proteolytic labeling and stable isotope incorporation happens simultaneously during proteolytic digestion step [68, 94]. In addition to comparative (relative) quantitation [95], this method has also been applied to provide labeled peptides for absolute quantitation [96] and identify disease-specific biomarkers [97-99], quantify N-glycosylation site occupancy [100], and detect changes in phosphorylation [68, 100]. In one study, synthesized benzoic acid-d_0 N-succinimidyl ester (BzOSu) and benzoic acid-d_5 N-succinimidyl ester (d-BzOSu) were used as light and heavy isotope solutions for stable isotope quantification for the comparative analysis of glycopeptides. Using this technique provided enhanced ionization efficacy in both positive and negative ionization modes by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS). Control and myeloma glycopeptide samples were quantitatively analyzed by mixing Bz-tagged IgG1 glycopeptides with d-Bz-tagged human serum IgG glycopeptides [101]. Selected reaction monitoring (SRM) is a new promising technique that does not apply labeling and stable isotopes to obtain quantitative information [68, 102].

Investigation of clinical samples by proteomic fingerprinting technology combining magnetic beads with MALDI-TOF MS to analyze serum from MM patients has been recently reported [76, 103]. Concerning biofluid proteomics, a work introducing the use of surface-enhanced laser desorption/ionization time-of-flight mass spectrometry (SELDI-TOF MS) in profiling MM serum has also been described. Furthermore, SELDI-TOF technology was also applied in a recent investigation of the interaction between MM cells and osteoclasts [46]. Hong *et al*. developed a technique to assess the quality of SELDI-TOF data based on a correlation matrix [104]. The correlation matrix approach was used to MM related lytic bone disease study to effectively detect low-quality spectra prior to post-analysis [68].

Phosphoproteomics is an effective MS-based procedure that detects phosphorylation related post-translation modifications (PTMs) [105-107]. There are several studies using the phosphoproteomics approach to investigate MM. In one study, label-free quantitative phosphoproteomics method was applied to detect and quantify phosphotyrosine (pY) sites modulated by FGFR3 activation and inhibition in MM cells [108, 109]. This study also demonstrated the potential utility of pY-directed phosphoproteomics to measure drug pharmacodynamics since it provided a measure of drug-target modulation and gave an insight into drug mode of action [68, 110]. This same group [111] described that the phosphorylation stoichiometries of two phosphorylation sites on Lyn kinase could be identified in human MM-derived cell lines and xenograft tumors. Lyn kinase is the prevalent Src family protein-tyrosine kinase in B cells and is included in B cell-related malignancies including MM [76, 112].

5. METABOLOMICS AND LIPIDOMICS

The metabolome represents the collection of all metabolites in a biological system [113]. Kvitvang *et al*. quantified over 30 metabolites in JJN-3 multiple myeloma cell extracts by using a new preparation

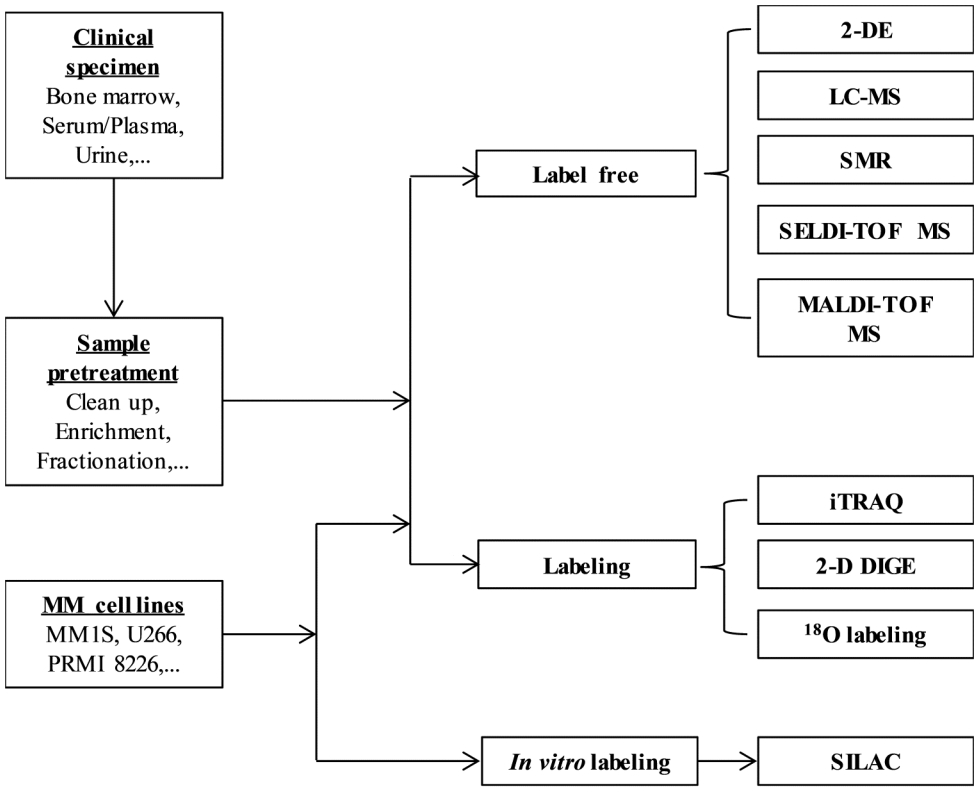

Fig. (1). Schematic representation of various proteomic approaches available to study multiple myeloma at the molecular level. According to the origin of the sample and the designed experimental approach, samples undergo a pretreatment process and analyzed by different techniques.

protocol with subsequent capillary ion exchange chromatography - tandem mass spectrometry (capIC–MS/MS) analysis [114]. Another study found that metabolic profiles obtained by 1H proton nuclear magnetic resonance (1H-NMR) in detecting multiple myeloma biomarkers may be effective to differentiate multiple myeloma and healthy patients and monitor response to treatment [115]. Zaal *et al*. investigated the metabolic mechanisms of bortezomib resistance by mass spectrometry-based metabolomics and proteomics in bortezomib-sensitive and bortezomib–resistant MM cell lines, as well as in a set of CD138+ cells obtained from MM patients. This group described that bortezomib resistance [116] in MM cells was associated with the expression of 3-phosphoglycerate dehydrogenase (PHGDH), which catalyzed the rate-limiting step of serine synthesis. PHGDH expression of bortezomib resistant cells was increased in multiple myeloma. Using tracer-based metabolomics, it was shown that the serine synthesis pathway was significantly increased in bortezomib resistant MM [117]. Other research groups analyzed multiple myeloma samples using liquid chromatography with quadrupole time-of-flight mass spectrometry (LC-MS) and found that in MM patients, pyrimidine metabolism, carbon metabolism, and bile secretion pathways were potentially affected by the disease [118]. Tavel *et al*. developed unbiased 1H High-Resolution Magic Angle Spinning (HR-MAS) nuclear magnetic resonance (NMR) metabolomics to study biopsy specimens of osteolytic lesions from pathological fractures caused by MM. This proof-of-principle study revealed integrated metabolomics and histomorphology as a promising approach for the targeted study of osteolytic lesions [119]. It was also demonstrated that nanoflow ultra-performance liquid chromatography system coupled to a high-resolution mass spectrometry offered the required sensitivity and specificity for global metabolomics studies. These data demonstrated that up to ~6500 authentic metabolite features could be identified from several milligrams of multiple myeloma cell [120]. Preliminary experiments confirmed that 5-aminoimidazole-4-carboxamide-1-β-riboside induced apoptosis in MM cell lines but, as previously reported [121], rapamycin only induced G1 arrest. A possible explanation for inhibition of UMP synthase activity by this substrate of UMP synthase was a decrease of 5-phosphoribosyl-alpha-pyrophosphate in cellular levels. These data revealed that the pyrimidine biosynthesis pathway can be a potential molecular target for future multiple myeloma therapeutics [122].

LC-MS fingerprinting of serum metabolites of multiple myeloma and chronic lymphocytic leukemia [60, 123] patient samples demonstrated the presence of the same metabolites (*e.g*., fatty acids, acylcarnitines, sphingolipids, phospholipids, phenylalanyl-phenylalanine, and isoprene) as well as those, which render them different (*e.g*., lysophosphatidylcholines, monoacylglycerols, aminocaproic acid, phenylacetyl-glutamine) in common for both malignancies [124]. Lodi

et al. studied serum and urine samples by NMR and detected carnitine and acetylcarnitine as potential biomarkers of the active disease both in initial diagnosis and relapse as well as a mediator of MM related pathologies [125]. Kumazoe *et al*. showed that metabolic profiling was an efficient chemical-mining approach for detecting botanical drugs with therapeutic potential against MM. They demonstrated the polyphenol eriodictyol significantly contributes to apoptosis induction by epigallocatechin-3-O-gallate (EGCG) *in vitro* and in a mouse tumor model by amplifying EGCG-induced activation of the 67-kDa laminin receptor signaling pathway [126].

Lipidomics is the large-scale study of pathways and networks of cellular lipids in biological systems [127-129]. It is a relatively new field that has been driven by rapid advances in technologies such as mass spectrometry (MS), nuclear magnetic resonance (NMR) spectroscopy, fluorescence spectroscopy, dual polarization interferometry and computational methods used to recognize the role of lipids in many metabolic diseases such as obesity, atherosclerosis, stroke, hypertension and diabetes. Lipidomics data on multiple myeloma showed an overall reduction in lipid biosynthesis and fatty acid incorporation with a significant decrease in lysophospholipid levels [130]. Mechanistically, the importance of membrane lipids in contributing to the endoplasmic reticulum stress levels and inducing the unfolded protein response activation in myeloma cells has not been examined until now. More efficient SK2 inhibitors and modulators of sphingolipid signaling are currently in development and expected to further highlight the unappreciated role of sphingolipid biology in myeloma, which will ultimately lead to future therapeutic options [131].

6. GLYCOMICS

Glycoproteins contain oligosaccharide chains (glycans) covalently attached to their polypeptide backbone. Glycosylation is not template driven; it is known to be associated with the co- and post-translational modifications occurring during protein synthesis and processing [132]. The N-linked glycans are attached to the carboxamido nitrogen on asparagine (Asn) residues co-translationally and modified post-translationally. The O-linked glycans are attached to the hydroxyl of serine and threonine side-chains. Proteins destined to be on the cell surface typically undergo glycosylation in the endoplasmic reticulum-Golgi pathway, where a sequence of regulated events occur involving glycosidases and glycosyltransferases to create highly complex but specific glycan structures [133]. Most secreted extracellular proteins are also often glycosylated, which may change in several diseases [134, 135]. N-linked glycosylation of glycoproteins plays important roles in biological processes, programmed cell death and differentiation. Detection of specific N-linked glycans can be potentially be used in disease diagnosis and prognosis [132]. A large number of publications reported on changes in the glycosylation pattern of transmembrane proteins and circulating glycoproteins in malignant diseases such as lung cancer, colorectal cancer, breast cancer, esophageal adenocarcinoma, liver carcinoma, or multiple myeloma [136-139]. Such changes include increase in branching, changes in sialylation and fucosylation, *etc*. [140].

N-glycosylation of immunoglobulin (IgG) 1 molecules is mostly located at the highly conserved Asn^{297} site of the C_H2 domain in the fragment crystallizable region (Fc) region. This is important in MM because most patients are IgG paraprotein producers [141-143]. Analysis of the N-glycosylation profiles of paraproteins may have prognostic value for MM patient treatment.

N-glycans can be removed from the glycoproteins by an enzymatic procedure utilizing peptide-N-glycosidase F or other endoglycosidases [144-146] (Fig. 2). Another option is hydrazine release [147], which while technically more challenging provides an orthogonal evaluation option that should always be contrasted at least once with new samples, particularly those of non-mammalian origin.

Analysis of N-glycans by techniques, such as liquid chromatography, mass spectrometry, or capillary electrophoresis helped to identify new and accurate glyco-biomarkers. Glavery *et al*. demonstrated that ST3GAL6 gene knockdown resulted in significant reduction in the levels of alpha 2,3–linked sialylation on the surface of MM cells with an associated reduction in adhesion to MM bone marrow stromal cells and fibronectin along with reduced trans-endothelial migration *in vitro* [148]. Knockout of the ST3GAL6 gene significantly reduced homing and engraftment of MM cells in the bone marrow niche *in vivo*, decreased tumor burden and improved survival. This study pointed out the importance of changed glycosylation, particularly sialylation in MM cell adhesion and migration [148]. It was shown that IgG B cell receptor in myeloma is more heavily sialylated that of normal control cells and the increased sialylation of IgG B cell receptor is associated with higher levels of tyrosine phosphorylation of the IgG B cell receptor supramolecular complex [149]. Chen *et al*. identified two N-glycan structures (FA2G1, FA2BG2), which held diagnostic value using fluorophore-assisted capillary electrophoresis (DSA-FACE) [150] (Fig. 3). Jefferis *et al*. isolated unusual mono-antennary oligosaccharides in IgG2 and IgG3 proteins with serum of MM patients. These results suggested a lower activity for N-acetyl-glucosaminyl transferase in IgG2-subclass-producing and IgG3-subclass-producing plasma cells [151]. The IgGs of multiple myeloma patients had highly sialylated, fucosylated, and bisected GlcNAc (N-acetylglucosamine) containing biantennary sugar chains at their Fab fragments as was shown by lectin-blotting techniques [152]. Farooq *et al*. reported that the N-glycosylation of IgG3 paraproteins changes during chemotherapy, thus can distinguish remission patients from patients with active disease [153]. Another study showed that purified serum IgG N-glycans are useful for isolating control and multiple

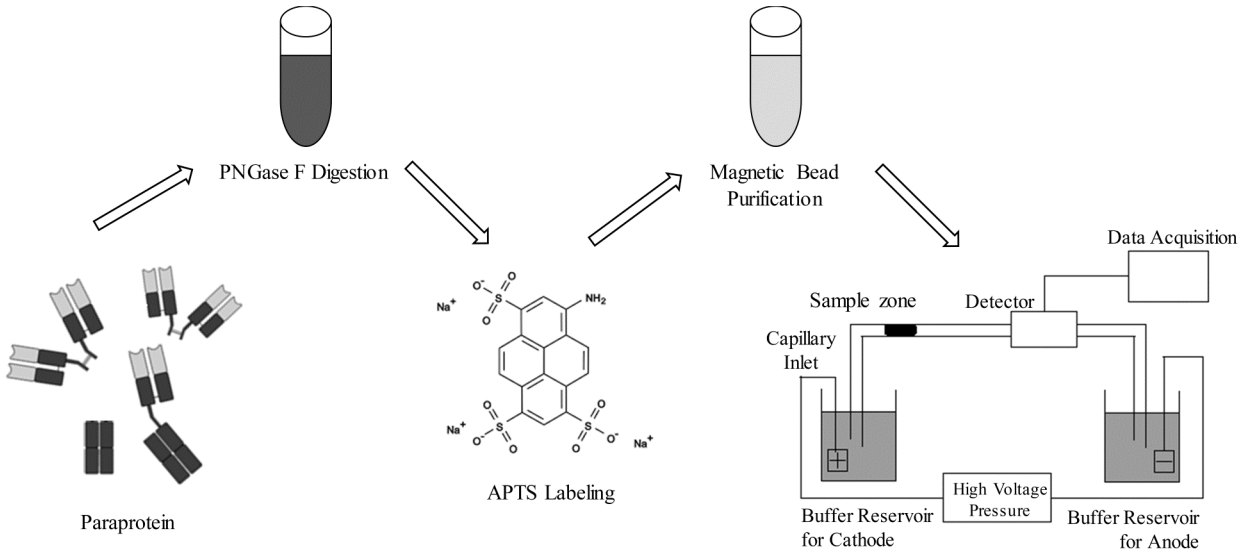

Fig. (2). Scheme of a full sample preparation workflow for N-glycosylation analysis utilizing specially functionalized magnetic beads.

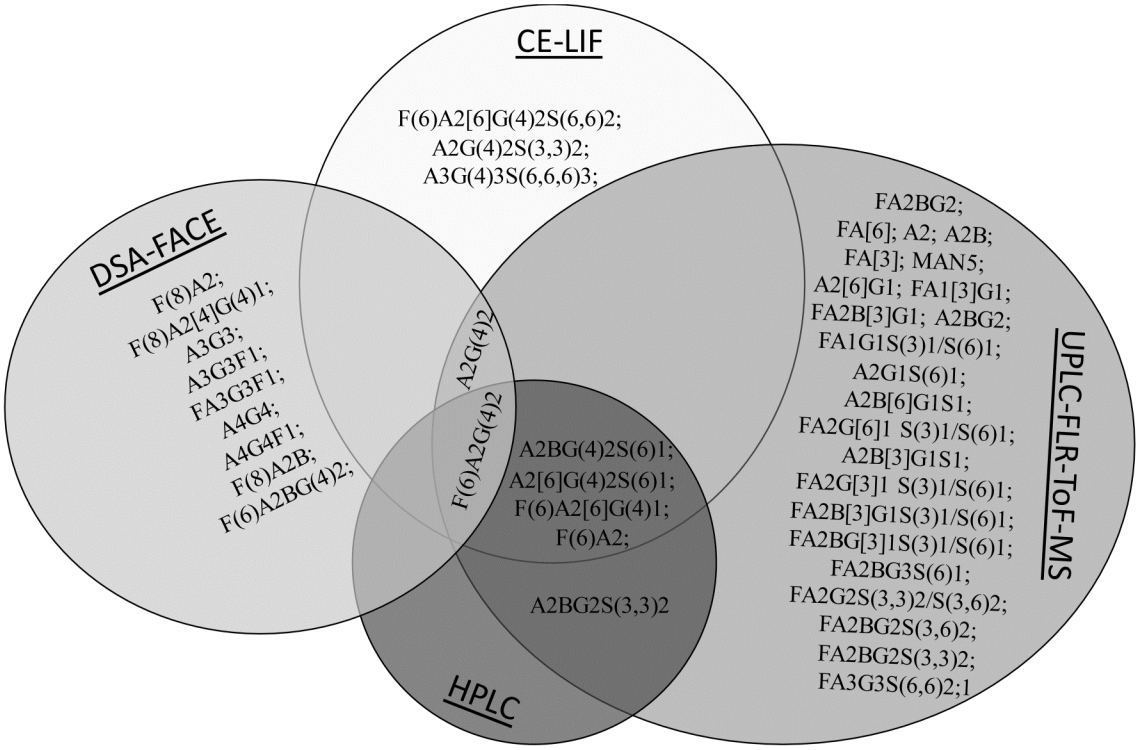

Fig. (3). N-glycans detected in multiple myeloma serum samples by various methods. DSA-FACE – DNA sequencer-aided fluorophore-assisted carbohydrate electrophoresis, CE-LIF – capillary electrophoresis analysis with laser-induced fluorescence detection, UPLC-FLR-ToF-MS – ultra performance liquid chromatography coupled with photodiode array detector and time-of-flight mass spectrometry, HPLC – high-performance liquid chromatography. Glycan structures are given by their nomenclature suggested by Harvey *et al*. [155].

myeloma patients as well as for patient classification [154].

Fleming *et al*. discovered modifications in sialylated and neutral glycan structure ratios in MGUS and multiple myeloma [156]. Our group used CE-LIF (capillary electrophoresis analysis with laser-induced fluorescence detection) for the analysis of the N-glycans after endoglycosidase (PNGase F) mediated sugar digest and aminopyrenetrisolfonate fluorophore labeling. While characteristic N-glycosylation pattern differences were found between normal control and untreated, treated and remission stage MM patient samples at the global serum level, less distinctive

changes were observed at the immunoglobulin level [157]. Other methods, like HILIC-UPLC-FLR (hydrophilic interaction liquid chromatography on an ultra performance liquid chromatography system with fluorescence detection) were also used for the separation of multiple myeloma patients serum samples [154].

CONCLUSION AND FUTURE PROSPECTIVES

Multiple myeloma (MM) is a currently immedicable hematological disease of human plasma cells, producing a large amount of abnormal antibodies [123, 158] (referred to as paraproteins) that leads to serious health issues and death. Regarding the analytical omics based endeavors to diagnose the disease, many genomics based studies have been reported, some of which are already being used in the practice of multiple myeloma diagnosis. While a plethora of proteomics approaches have been described in the literature, only a few publications were published in multiple myeloma diagnostics by metabolomics and lipidomics. Glycomics, on the other hand, has already shown a correlation between glycosylation modifications and MM stages, thus may offer important information for patient treatment. By all means, using high-resolution, state of the art analytical omics tools can provide very accurate and fast molecular diagnostic information, helping to choose the best treatment therapy for multiple myeloma patients.

ABBREVIATIONS

MM	=	multiple myeloma
BJP	=	Bence-Jones proteins
ESR	=	erythrocyte sedimentation rate
MGUS	=	monoclonal gammopathy of undetermined significance
M-proteins	=	myeloma proteins
D/S	=	Durie Salmon
D/S P	=	Durie Salmon Plus
ISS	=	International Staging System
β2M	=	serum β2-microglobulin
CGH	=	comparative genomic hybridization array
FISH	=	fluorescence in situ hybridization
cIg-FISH	=	cytoplasmic immunoglobulin light chain staining
oaCGH	=	oligo-based comparative genomic hybridization array analysis
NHMM	=	nonhyperdiploid myeloma
CCND1	=	oncogene cyclin D1
FGFR3	=	fibroblast growth factor receptor
MMSET	=	multiple myeloma SET domain
MyPRS	=	Myeloma Prognostic Risk Score
GWASs	=	genome-wide association studies
MAGIC	=	MyelomA Genetics International Consortium
GEP	=	gene-expression profiling
2-DE	=	two-dimensional gel electrophoresis
2-DIGE	=	difference gel electrophoresis
SPE	=	Serum protein electrophoresis
UPE	=	urine protein electrophoresis
IFE	=	immunofixation electrophoresis
FLC	=	serum-free light chain
'Hevylite', HLC	=	immunoglobulin heavy/light chain immunoassays
FACS	=	fluorescence-activated cell sorting
LC	=	liquid chromatography
MS	=	mass spectrometry
MALDI	=	matrix-assisted laser desorption ionization
SILAC	=	stable-isotope labeling by amino acids
iTRAQ	=	isobaric Tags for Relative and Absolute Quantitation
SRM	=	Selected reaction monitoring
MALDI-TOF	=	Matrix-assisted laser desorption/ionization time-of-flight
SELDI-TOF	=	Surface-enhanced laser desorption/ionization time-of-flight
PTMs	=	post-translation modifications
pY	=	quantify phosphotyrosine
capIC-MS/MS	=	capillary ion chromatography - tandem mass spectrometry
HR-MAS	=	High-Resolution MagicAngle Spinning
PHGDH	=	3-phosphoglycerate dehydrogenase
EGCG	=	epigallocatechin-3-O-gallate
DSA-FACE	=	DNA sequencer-aided fluorophore-assisted carbohydrate electrophoresis
CE-LIF	=	capillary electrophoresis analysis with laser-induced fluorescence detection
HILIC-UPLC-FLR	=	hydrophilic interaction liquid chromatography on an ultra performance liquid chromatography system with fluorescence detection
GlcNAc	=	N-acetylglucosamine

CONSENT FOR PUBLICATION

Not applicable.

CONFLICT OF INTEREST

The authors confirm that this article content has no conflicts of interest.

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