Acta Scientific CANCER BIOLOGY (ISSN: 2582-4473)

Volume 10 Issue 1 January 2026

Conceptual Paper

Designing a Proteomic Study Protocol in Multiple Myeloma: Methodological Framework, Technical Workflow, Data Analysis and Translational Considerations

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Received: February 04, 2026

Published: March 04, 2026

Abstract

Background: Multiple myeloma (MM) is a biologically heterogeneous plasma cell malignancy with variable clinical presentation and treatment response. Genomic studies incompletely explain disease behavior, highlighting the need for proteomic characterization, particularly in treatment-naïve patients.

Objectives: To perform comprehensive proteomic profiling of malignant plasma cells from treatment-naïve MM patients; to correlate proteomic signatures with clinicopathological parameters and survival outcomes; and to identify molecular mechanisms underlying varied clinical presentation and suboptimal therapeutic response.

Methods: This prospective study included 48 newly diagnosed, treatment-naïve MM patients and 4 healthy controls. $CD138^+$ plasma cells were isolated from bone marrow aspirates using magnetic-activated cell sorting and validated by flow cytometry. Proteomic analysis was performed using iTRAQ-based quantitative mass spectrometry and label-free LC-ESI-MS/MS platforms. Differentially expressed proteins were identified using statistical thresholds (log2 fold change and adjusted p-values), followed by principal component analysis (PCA), Gene Ontology (GO), KEGG pathway enrichment, and protein–protein interaction network analysis. Proteomic profiles were compared between patients and controls, and between responders and non-responders to standard therapy.

Results: PCA demonstrated clear separation between MM patients and controls, confirming a distinct disease-associated proteomic signature. Fifty significantly dysregulated proteins were identified, predominantly involving proteasomal function, cytoskeletal organization, and adhesion signaling. Key downregulated proteins included ADRM1 and ILK, implicating disrupted proteostasis and altered plasma cell–microenvironment interactions. Functional enrichment revealed involvement of proteasome, focal adhesion, PI3K-AKT signaling, and oxidative phosphorylation pathways. Comparative analysis between responders and non-responders (n=3) showed upregulation of FABP6 and NUP205 in resistant cases, suggesting metabolic rewiring and altered nuclear transport as mechanisms of resistance. Network analysis indicated proteomic plasticity and pathway redundancy in non-responders.

Conclusion: Treatment-naïve MM exhibits distinct proteomic alterations characterized by dysregulated proteostasis and adhesion pathways. Metabolic reprogramming and nuclear transport alterations appear central to therapeutic resistance. Proteomic profiling offers valuable insights into MM biology and may facilitate identification of predictive biomarkers and novel therapeutic targets for personalized disease management.

Keywords: Multiple Myeloma; Proteomics; Plasma Cells; Signalling Pathways; Disease Biology

Abbreviations

BM: Bone Marrow; BMIF: Bone Marrow Interstitial Fluid; CHSY1: Chondroitin Synthetase; FISH: Flourescent in Situ Hybridisation; GEP: Gene Expression Profiling; IGF: Insulin Like Growth Factor; ISS: International Staging System; LDH: Lactate Dehydrogenase; MGUS: Monoclonal Gammopathy of Undetermined Significance; MM: Multiple Myeloma; MS: Mass Spectroscopy; MZB1: Marginal Zone B and B1 Cell Specific Protein; PC: Plasma Cell; PTM: Post Translational Modification; SDF: Stromal Derived Growth Factor; SMM: Smoldering multiple Myeloma; WM: Waldenstrom Macroglobulinemia; XPO1: Exportin 1; 2-DE: 2 Dimensional gel electrophoresis

Introduction

Multiple myeloma (MM) is a clonal plasma cell (PC) malignancy characterized by uncontrolled proliferation of terminally differentiated B cells within the bone marrow (BM), leading to monoclonal protein production and a spectrum of end-organ damage including hypercalcaemia, renal dysfunction, anaemia, and bone disease. MM accounts for approximately 10–15% of all haematological malignancies and represents the second most common blood cancer worldwide [1]. Over the past two decades, the therapeutic landscape of MM has been transformed by the introduction of proteasome inhibitors, immunomodulatory drugs, monoclonal antibodies, and cellular therapies, resulting in substantial improvements in progression-free and overall survival. Nevertheless, MM remains largely incurable, with most patients eventually relapsing and developing drug-resistant disease [2].

Multistep pathogenesis and disease heterogeneity

MM is not a biologically uniform entity but rather a heterogeneous disease with marked interpatient and intrapatient variability. The widely accepted multistep model of myelomagenesis proposes that MM evolves from precursor plasma cell disorders, namely monoclonal gammopathy of undetermined significance (MGUS) and smouldering multiple myeloma (SMM). MGUS is a relatively common premalignant condition characterized by the presence of a monoclonal protein without end-organ damage, with an annual progression risk of approximately 1%. SMM represents an intermediate asymptomatic stage with a higher risk of progression to overt MM. Despite shared genomic alterations across MGUS, SMM, and MM, only a subset of patients progress to symptomatic disease, suggesting that additional biological factors beyond genetic lesions govern disease evolution [3].

Extensive genomic studies have identified recurrent chromosomal abnormalities in MM, including hyperdiploidy, translocations involving the immunoglobulin heavy chain (IGH) locus, and secondary genetic events such as MYC activation, RAS mutations, and TP53 deletions. While these abnormalities contribute to clonal evolution and prognosis, genomic changes alone do not fully explain disease behaviour, clinical heterogeneity, or differential therapeutic response. Indeed, gene expression profiling (GEP) studies have demonstrated that the transcriptional differences between MGUS and MM are relatively modest, reinforcing the concept that post-transcriptional and post-translational mechanisms play a pivotal role in disease progression [4].

Role of the bone marrow microenvironment

The BM microenvironment is increasingly recognized as a critical determinant of MM pathogenesis, progression, and therapeutic resistance. Myeloma cells reside within a highly specialized niche composed of stromal cells, osteoblasts, osteoclasts, endothelial cells, immune cells, extracellular matrix components, and a complex network of cytokines, chemokines, and growth factors. Bidirectional interactions between malignant PCs and the BM microenvironment promote tumour cell survival, proliferation, angiogenesis, immune evasion, and resistance to apoptosis [5].

Key signalling pathways activated through cell–cell contact and soluble mediators include the NF-κB, PI3K/AKT, MAPK, and JAK/STAT pathways. Cytokines such as interleukin-6 (IL-6), vascular endothelial growth factor (VEGF), insulin-like growth factor-1 (IGF-1), and stromal cell–derived factor-1 (SDF-1) have been shown to support MM cell growth and protect against drug-induced cell death. Furthermore, BM-mediated drug resistance remains a major barrier to curative therapy, as myeloma cells can persist within protective niches despite exposure to potent anti-myeloma agents [6].

Importantly, the functional consequences of these interactions are largely mediated at the protein level. Protein abundance, post-translational modifications (PTMs), protein–protein interactions, and protein localisation ultimately dictate cellular phenotype and

behaviour. Thus, a comprehensive understanding of MM biology requires investigation beyond the genome and transcriptome, extending into the proteome.

Limitations of genomic and transcriptomic approaches

Although high-throughput genomic and transcriptomic technologies have revolutionized MM research, these approaches have inherent limitations. mRNA expression does not always correlate with protein abundance due to regulatory processes such as alternative splicing, mRNA stability, translational efficiency, and PTMs. Moreover, proteins are the primary effectors of cellular function and drug targets; therefore, alterations at the proteomic level may more accurately reflect disease state, therapeutic vulnerability, and resistance mechanisms [7].

GEP-based risk stratification models have been developed and validated in MM, providing valuable prognostic information. However, their utility in guiding individualized therapy remains limited, particularly in real-world clinical settings. Additionally, GEP does not capture dynamic changes in protein signalling networks induced by the BM microenvironment or therapeutic pressure [8]. These limitations highlight the need for complementary proteomic approaches to achieve a more integrated and functionally relevant understanding of MM biology.

Proteomics as a tool in cancer research

Proteomics refers to the large-scale study of proteins, encompassing their expression levels, structures, functions, interactions, and modifications. Proteomic profiling is one of the most universally applied strategies for cancer biomarker development. Blood and plasma circulate in the body and effectively sample most tissues and can contain proteins and peptides. Proteomics allows the studies of global protein expression, post translational modifications, protein –protein interactions, and ultimately protein function. Advances in mass spectrometry (MS)-based technologies have enabled high-throughput, sensitive, and quantitative analysis of thousands of proteins from biological samples. Proteomic profiling can be performed on cells, tissues, and biofluids such as serum and plasma, making it particularly attractive for biomarker discovery [9].

In oncology, proteomics has emerged as a powerful strategy for identifying diagnostic, prognostic, and predictive biomarkers, elucidating oncogenic signalling pathways, and uncovering novel therapeutic targets. Unlike genomic alterations, which may be static, the proteome is highly dynamic and responsive to environmental cues, disease stage, and treatment exposure. This dynamic nature makes proteomic analysis particularly relevant for studying treatment-naïve disease, where baseline protein expression patterns may reflect intrinsic disease biology rather than therapy-induced changes [10].

Proteomic studies in multiple myeloma

Proteomic investigations in MM have provided important insights into disease mechanisms, including dysregulated protein synthesis, unfolded protein response activation, metabolic reprogramming, and immune modulation. Early proteomic studies identified differential expression of acute-phase proteins, complement components, and immunoglobulin fragments in MM patient sera. Subsequent analyses using advanced MS platforms have expanded the myeloma proteome, identifying proteins involved in cell cycle regulation, apoptosis, oxidative stress, and BM interaction.

Importantly, proteomic profiling has revealed potential biomarkers associated with disease stage, prognosis, and therapeutic response. Alterations in proteasome subunits, chaperone proteins, adhesion molecules, and signalling mediators have been linked to sensitivity or resistance to proteasome inhibitors and immunomodulatory drugs. However, many existing studies have focused on relapsed or treated patients, where prior therapies may confound interpretation of proteomic changes [11].

Rationale for studying treatment-Naïve MM

Studying newly diagnosed, treatment-naïve MM patients provides a unique opportunity to characterize the intrinsic proteomic landscape of the disease before therapeutic selection pressures alter tumour biology. Baseline proteomic signatures may help identify biologically distinct subgroups, correlate with established clinico-pathological parameters such as International Staging System (ISS) stage, cytogenetic risk, tumour burden, renal function, and bone disease, and potentially predict early treatment response and long-term outcomes.

Furthermore, treatment-naïve proteomic profiling may uncover early biomarkers of aggressive disease biology, enabling

improved risk stratification and personalized treatment strategies. Integration of proteomic data with clinical and pathological variables can facilitate a more holistic understanding of MM heterogeneity and guide rational therapeutic decision-making.

Clinical and translational relevance

The identification of reliable biomarkers remains a major challenge in MM management. Current prognostic models rely on clinical parameters, cytogenetics, and limited molecular markers, which may not fully capture disease complexity. Proteomic biomarkers detectable in BM plasma cells or peripheral blood could offer minimally invasive tools for diagnosis, prognosis, monitoring disease response, and detecting minimal residual disease.

Proteomics in MM research thus may contribute to the discovery of diagnostics, prognostic, and predictive biomarkers, e.g., to the identification of novel regulatory mechanisms of MM development and the development of novel anti-MM therapeutic strategies.

International status

Mitsiades CS., *et al*. published their first proteomic study in which analysis of signalling pathways in samples of MM versus Waldenstrom macroglobulinemia (WM) was done. Significant overlap between the proteomic profiles of two cell types was uncovered. The absence of expression of the germinal centre kinase (GCK), a serine threonine protein kinase, in both MM and WM cells was considered indicative of the post germinal centre ontogeny of both MM and WM. It was also found that proteasome inhibitor bortezomib down regulated the expression of several proteins involved in DNA repair. The authors highlighted how proteomic technologies such as two-dimensional gel electrophoresis (2-DE) and mass spectrometry enable the simultaneous analysis of thousands of proteins, allowing identification of disease-specific protein expression patterns, signaling pathway alterations, and protein modifications. In WM, proteomic profiling helped characterize aberrant survival pathways, interactions between malignant cells and the bone marrow microenvironment, and dysregulated stress-response and apoptosis-related proteins. These findings underscored biological similarities and distinctions between WM and other plasma cell disorders, particularly multiple myeloma [12].

A key emphasis of the study was the ability of proteomics to identify novel biomarkers for diagnosis, prognosis, and therapeutic monitoring. This study provided insights into mechanisms of drug resistance, especially those mediated by cytokines and adhesion molecules within the tumor microenvironment.

Yin., *et al*. (2005) investigated the role of chondroitin synthase-1 (CHSY1) in mediating interactions between multiple myeloma (MM) cells and osteoclasts, a critical process underlying myeloma-associated bone disease. Myeloma bone disease is characterized by increased osteoclast activity and suppressed osteoblast function, resulting in osteolytic lesions and skeletal-related events. While several cytokines and adhesion molecules were known contributors, the molecular mechanisms governing myeloma–osteoclast crosstalk remained incompletely understood.

Using gene expression profiling and functional assays, the authors identified CHSY1 as being significantly upregulated in myeloma cells in the presence of osteoclasts. CHSY1 is an enzyme involved in the biosynthesis of chondroitin sulfate glycosaminoglycans, which are key components of cell-surface and extracellular matrix proteoglycans. The study demonstrated that increased CHSY1 expression enhanced chondroitin sulfate production on myeloma cells, thereby promoting stronger adhesion and interaction with osteoclasts.

Functional experiments showed that disruption of chondroitin sulfate synthesis—either by enzymatic degradation or by inhibiting CHSY1—significantly reduced myeloma cell adhesion to osteoclasts and impaired osteoclast-mediated myeloma cell growth and survival. These findings indicated that CHSY1-dependent chondroitin sulfate chains are essential for sustaining the reciprocal signaling between myeloma cells and osteoclasts that fuels disease progression and bone destruction.

Importantly, the study linked CHSY1-mediated interactions to activation of downstream survival pathways in myeloma cells, highlighting how the bone marrow microenvironment confers growth advantage and resistance to apoptosis. By identifying CHSY1 as a molecular regulator of tumor–microenvironment interactions, the authors provided mechanistic insight into how myeloma cells exploit osteoclasts to enhance their survival.

In conclusion, Yin., *et al*. established chondroitin synthase-1 as a key mediator of myeloma–osteoclast interactions and a potential therapeutic target. Targeting CHSY1 or chondroitin sulfate biosynthesis may represent a novel strategy to disrupt myeloma-

induced osteoclast activation and mitigate myeloma-related bone disease [13].

Proteomics studies can be used to investigate the effect of drug treatment on cell lines and can provide relevant information regarding the mechanisms behind its efficacy. It can also give insights into the modifications that accompany drug resistance. Rees-Unwin., *et al*. (2007) conducted a comprehensive proteomic evaluation of dexamethasone-mediated apoptosis and resistance in multiple myeloma (MM), aiming to elucidate molecular pathways that determine glucocorticoid sensitivity. Although dexamethasone is a cornerstone of MM therapy, intrinsic and acquired resistance significantly limit its clinical efficacy. The molecular basis of this resistance was incompletely understood, prompting the use of proteomic approaches to identify key regulatory proteins and pathways. The authors employed two-dimensional gel electrophoresis (2-DE) coupled with mass spectrometry to compare protein expression profiles between dexamethasone-sensitive and dexamethasone-resistant myeloma cell lines, both at baseline and following drug exposure. This unbiased proteomic strategy enabled the identification of proteins differentially expressed in response to glucocorticoid-induced apoptosis.

Proteomic analysis revealed that dexamethasone-sensitive cells demonstrated significant modulation of proteins involved in apoptotic signaling, stress response, protein folding, and redox regulation. Key pathways implicated included mitochondrial apoptosis, with altered expression of proteins regulating oxidative stress and energy metabolism. In contrast, dexamethasone-resistant myeloma cells showed upregulation of chaperone proteins, antioxidant enzymes, and cytoprotective stress-response proteins, suggesting enhanced cellular defense mechanisms that counteract glucocorticoid-induced cell death. Notably, resistance was associated with dysregulation of proteins involved in proteasomal function, cytoskeletal organization, and metabolic adaptation, highlighting the multifactorial nature of glucocorticoid resistance. The study also demonstrated that dexamethasone exposure induces distinct proteomic signatures rather than uniform apoptotic responses, underscoring biological heterogeneity among myeloma cells.

The authors emphasized the translational significance of these findings, proposing that proteomic signatures could serve as biomarkers of dexamethasone sensitivity and identify novel therapeutic targets to overcome resistance. Integrating proteomic data with genomic and functional studies was suggested as a strategy to guide combination therapies that restore glucocorticoid responsiveness [14].

Ge., *et al*. (2009) investigated the molecular mechanisms underlying arsenic-induced apoptosis in human multiple myeloma cells using integrated proteomic and functional analyses. Recognizing the therapeutic potential of arsenic compounds in hematologic malignancies, the study aimed to define the intracellular pathways responsible for arsenic-triggered cell death. Comparative proteomic profiling, employing two-dimensional electrophoresis and mass spectrometry, identified numerous proteins differentially expressed following arsenic exposure. The analysis revealed a dual molecular mechanism of apoptosis induction. First, arsenic disrupted mitochondrial function, leading to altered expression of proteins involved in oxidative stress, energy metabolism, and mitochondrial integrity, ultimately activating the intrinsic apoptotic pathway. Second, arsenic modulated endoplasmic reticulum stress and protein homeostasis, characterized by changes in chaperone proteins and regulators of protein folding, contributing to apoptosis through stress-mediated signaling pathways.

Functional validation experiments confirmed the involvement of reactive oxygen species generation, mitochondrial membrane depolarization, and caspase activation. Collectively, the findings demonstrated that arsenic induces myeloma cell apoptosis through coordinated mitochondrial and stress-response pathways, providing mechanistic rationale for arsenic-based therapeutic strategies in multiple myeloma [15].

Paradeisi., *et al*. (2025) performed a proteomic analysis of bone marrow $CD138^{+}$ plasma cells from patients with MM to identify protein signatures associated with response to commonly used therapeutic regimens. Using high-resolution mass spectrometry–based proteomics, the study compared protein expression profiles between treatment responders and non-responders, aiming to uncover molecular determinants of therapeutic sensitivity and resistance.

The analysis revealed distinct differentially expressed proteins involved in key biological processes, including protein homeostasis,

cellular metabolism, stress response, immune regulation, and apoptosis. Several proteins were associated with pathways linked to drug action and tumor cell survival, suggesting mechanistic relevance to treatment outcomes. Importantly, proteomic patterns were shown to reflect clinical response more closely than single biomarkers, highlighting the heterogeneity of MM at the protein level. The authors propose that proteomic profiling of $CD138^{+}$ cells can support predictive biomarker discovery, improve patient stratification, and guide personalized treatment strategies. Overall, the study underscores the clinical utility of proteomics in understanding therapeutic response and resistance in multiple myeloma [16].

Wu., *et al*. (2025) conducted a serum proteome profiling study to identify noninvasive biomarkers associated with relapsed multiple myeloma (MM). Using high-throughput mass spectrometry–based proteomics, the authors compared serum protein expression patterns between patients with relapsed MM and those with newly diagnosed or stable disease. The analysis revealed distinct proteomic signatures linked to disease relapse, reflecting altered tumor biology and systemic responses. Among the differentially expressed proteins, thrombospondin-1 (TSP-1) and lactoferrin emerged as key candidate biomarkers. Both proteins showed significantly elevated serum levels in relapsed MM patients and were further validated using targeted assays. Functional pathway analysis suggested that these proteins are involved in angiogenesis, immune modulation, inflammation, and tumor–microenvironment interactions, processes known to contribute to myeloma progression and therapeutic resistance.

The study highlights the potential clinical utility of serum-based proteomic biomarkers for early detection of relapse, disease monitoring, and risk stratification. Overall, the findings support serum proteomics as a promising approach for improving management of relapsed MM [17].

National status

Incidence data regarding MM from India is scanty attributing to inadequate cancer registries. Incidence of MM in Indian population has been reported to be less than that in the Western population [18]. MM constitutes 1.1% of all haematological malignancies in India as compared to 13-15% of all haematological malignancies reported globally [19,20]. Various unique features have been reported in Indian MM patients like higher proportion of anemia, bony abnormalities, high serum creatinine, higher stage of the disease and frequent extra medullary presentation, etc. Similarly, epidemiological variations have also been reported from Indian subset of patients like earlier age at onset as compared to western population and a greater incidence of MM in age < 40 years [21-23]. Limited data is available regarding the cytogenetics abnormalities in MM patients from India. A retrospective study of fluorescent in situ hybridisation (FISH) analysis in MM patients stated that the frequency of chromosomal aberrations is different and much less in India. Another similar study from Tata Memorial Centre Mumbai reported low frequency of chromosome 13 aberrations and t(11:14) as compared to western population, probably indicating ethnic diversity [24,25]. These variations may have vital implications as they may be a surrogate of differential pathogenesis. It may be related to diverse ethnicity, demographic profile and environmental conditions.

As regards proteomic studies in myeloma form Indian continent, there is a scarcity of data. Chanukuppa., *et al*. investigated the molecular mechanisms underlying bortezomib resistance in multiple myeloma using a quantitative proteomic approach. Bortezomib-sensitive and bortezomib-resistant myeloma cells were compared using high-resolution mass spectrometry–based proteomics to identify differentially expressed proteins associated with drug resistance. The analysis revealed significant dysregulation of proteins involved in nuclear transport, proteostasis, apoptosis, and stress response pathways. Notably, exportin-1 (XPO1), a key nuclear export protein, was found to be markedly overexpressed in bortezomib-resistant cells and patient samples with poor response to therapy. Functional validation demonstrated that inhibition of XPO1 restored bortezomib sensitivity, enhanced apoptosis, and reduced survival signaling in resistant myeloma cells. These findings establish XPO1 as a critical mediator of bortezomib resistance and highlight nuclear export as an important therapeutic vulnerability in multiple myeloma. The study provides a strong rationale for combining XPO1 inhibitors with proteasome inhibitors to overcome drug resistance and improve clinical outcomes [26].

Chanukuppa., *et al*. performed a comprehensive proteomic analysis of multiple myeloma using paired bone marrow interstitial fluid (BMIF) and serum samples from patients and healthy controls. High-throughput quantitative proteomic platforms, including 2D-DIGE, iTRAQ, and SWATH-MS, were employed to

identify differentially abundant proteins associated with disease biology. The analysis revealed extensive proteomic remodeling, with 279 proteins significantly altered in BMIF and 116 proteins in serum. Dysregulated proteins were mainly involved in immune regulation, inflammation, extracellular matrix remodeling, angiogenesis, and lipid metabolism, reflecting the complex tumor–microenvironment interactions in multiple myeloma. Importantly, several overlapping proteins between BMIF and serum, such as haptoglobin, kininogen-1, transferrin, apolipoprotein A1, and albumin, were proposed as potential circulating biomarkers. This study is one of the first from India to systematically compare BMIF and serum proteomes in multiple myeloma, providing valuable insights into disease pathogenesis and offering a foundation for developing minimally invasive diagnostic and prognostic biomarkers [27]. Further they employed an integrated proteomic and functional approach to identify novel biomarkers involved in the pathogenesis of multiple myeloma. Comparative shotgun proteomic analysis was performed on mononuclear cells derived from multiple myeloma patients and healthy controls using high-resolution mass spectrometry. The study identified a distinct set of differentially expressed proteins linked to endoplasmic reticulum stress, protein folding, and plasma cell survival pathways. Among these, marginal zone B and B1 cell specific protein (MZB1) was significantly overexpressed in myeloma samples. Further functional validation demonstrated that MZB1 promotes myeloma cell proliferation, survival, and immunoglobulin secretion, highlighting its role in disease progression. Elevated MZB1 expression was also associated with adverse clinical features. The findings suggest that MZB1 plays a critical role in multiple myeloma biology and may serve as a potential diagnostic or prognostic biomarker. This study provides mechanistic insights into plasma cell dysregulation and underscores the value of proteomics in biomarker discovery for MM [28].

Despite these important contributions, proteomic research in MM in India remains at a nascent stage. Existing studies are limited in number, involve relatively small cohorts, and are largely confined to a few specialized centers. Comprehensive, multicentric proteomic analyses integrating clinical outcomes, treatment response, and longitudinal sampling are still lacking. Given the biological heterogeneity of multiple myeloma and the distinct demographic and disease characteristics of Indian patients, there is a clear unmet need for expanded proteomics-driven research. Systematic proteomic profiling has the potential to uncover population-specific biomarkers, mechanisms of drug resistance, and novel therapeutic targets. Strengthening this research area could significantly advance personalized myeloma care and bridge existing knowledge gaps between Indian and global datasets.

Importance of the proposed study in the context of current status

At our centre , we have observed that

- A sizeable number of our patients present to us at young age (under 50 years) which is contrary to the usual presentation of MM, in 6^{th} or 7^{th} decade of life.
- Extra-medullary presentation of disease is being encountered more frequently at initial diagnosis.
- Response to standard treatment therapy is suboptimal.

Therefore, it becomes necessary to study the disease biology using the novel proteomics technology as it may contribute to the discovery of diagnostics, prognostic, and predictive biomarkers, e.g., to the identification of novel regulatory mechanisms of MM development and the development of novel anti-MM therapeutic strategies.

Expected outcomes

- The proposed study would throw light on various signalling pathways associated with the pathogenesis and chemo-resistance of MM.
- Proteomic profiling will be able to decipher the molecular mechanism behind the high observation of extra-medullary presentation and suboptimal response to standard treatment protocols in this part of country.
- It is expected that proteomics would lead us to discover novel MM bio markers and anti-MM therapeutic strategies associated with various stages of MM development and chemo-resistance.

Aim of the study

Comprehensive proteomic profiling of malignant plasma cells from MM treatment naïve patients

Objectives of the study

- To study the correlation between various clinic-pathological parameters and the proteomic profile
- To study the correlation between proteomic profile and survival outcomes.
- To decipher the molecular mechanism behind the varied clinical presentation and suboptimal response to therapy.
- To identify potential novel biomarkers which can subsequently be studies for novel anti-MM therapeutic targets

Materials and Methods

The study was conducted at the All India Institute of Medical Sciences , Rishikesh, Uttarakhand, with the Central Drug Research Institute, Lucknow serving as the collaborating institute for plasma cell sorting procedures. The study population comprised newly diagnosed, treatment-naïve multiple myeloma (MM) patients who meet the predefined eligibility criteria. A total sample size of 52 participants were included, consisting of 48 MM patients and 4 control subjects, over a study duration of three years. Adult patients aged 18–75 years with a confirmed new diagnosis of MM and who provide written informed consent were enrolled. Patients with any concurrent malignancy or those unwilling to provide consent were excluded.

All enrolled patients underwent detailed clinical history assessment and physical examination. Peripheral blood samples were collected for laboratory investigations, including complete hemogram with peripheral smear (automated analyzer), renal and liver function tests, serum β2-microglobulin, lactate dehydrogenase (LDH), serum protein electrophoresis, serum immunofixation electrophoresis, serum free light chain assay, and 24-hour urine protein electrophoresis. Additionally, unilateral bone marrow aspiration and biopsy were performed for cytogenetic analysis, FISH myeloma panel testing, and proteomic studies.

Sample preservation

Cryopreservation of the marrow aspirate sample was performed using 10% DMSO and 90% FBS. To prevent protein degradation, G6521 protease inhibitor cocktail (50X) was added. Samples were preserved at −80 °C. Samples were transported to CDRI Lucknow for plasma cell sorting.

Isolation of CD138$^+$ PC from BM samples

Reagents

- CD138 MicroBeads (human) – Miltenyi Biotec (130-051-301)
- MACS MS Columns – Miltenyi Biotec (130-042-201)
- MACS Buffer: 1× PBS supplemented with 0.5% BSA and 2 mM EDTA; filtered (0.22 µm) and stored at 4 °C
- FACS Buffer: 1× PBS supplemented with 2% FBS and 2 mM EDTA; filtered (0.22 µm) and stored at 4 °C
- RBC Lysis Buffer – Sigma (R7757)
- Anti-human CD138-APC antibody – Miltenyi Biotec (130-127-998)

Preparation of bone marrow cells

Frozen bone marrow biopsy samples were thawed on ice and washed twice with cold 1× PBS by centrifugation at 350 ×g for 5 minutes. The cell pellet was resuspended in 1 mL RBC lysis buffer and incubated for 3 minutes at room temperature to remove erythrocytes. Lysis was stopped by immediately placing the cells on ice and adding 10 mL of cold MACS buffer. Cells were centrifuged at 350 ×g for 5 minutes, the supernatant discarded, and the pellet resuspended in 1 mL MACS buffer. Viable cells were counted using a hemocytometer under light microscopy.

Magnetic-activated cell sorting (MACS) of CD138$^+$ cells

For every 2 × 10^7 total cells, the suspension was centrifuged at 300 ×g for 10 minutes and the supernatant completely removed. The cell pellet was resuspended in 80 µL MACS buffer, followed by the addition of 20 µL CD138 MicroBeads. After gentle mixing, cells were incubated for 15 minutes at 2–8 °C.

Cells were washed with 1 mL MACS buffer per 2 × 10^7 cells and centrifuged at 300 ×g for 10 minutes. The pellet wass resuspended in 500 µL buffer (up to 10^8 cells) and subjected to magnetic separation.

An MS column was placed in the magnetic field of a MACS separator and pre-rinsed with 500 µL buffer. The cell suspension was applied to the column. Flow-through containing unlabeled cells was collected. The column was washed three times with buffer, adding fresh buffer only when the reservoir is empty.

The column was then removed from the separator, placed over a collection tube, and labeled $CD138^+$ cells were eluted by flushing with 1 mL buffer using the plunger. For higher purity, the eluted fraction was subjected to a second round of magnetic separation using a new column.

Flow Cytometric Confirmation of CD138 expression

The enriched $CD138^+$ fraction was counted and centrifuged at 300 ×g for 10 minutes. Up to 1×10^6 nucleated cells were resuspended in 98 µL FACS buffer, followed by addition of 2 µL anti-CD138-APC antibody. Cells were incubated for at least 10 minutes at 2–8 °C in the dark.

After staining, cells were washed with 1 mL buffer and centrifuged at 300 ×g for 10 minutes. The supernatant was discarded, and the cell pellet was resuspended in an appropriate volume of buffer for flow cytometric analysis.

Sample preparation for proteomics platforms

Isobaric tags for relative and absolute quantitation (iTRAQ)

All reagents were from the iTRAQ 8-plex kit (Applied Biosystems, ThermoFisher Scientific, Waltham, MA, USA). Lysates from PC pellets were prepared according to the manufacturer's protocol. Each iTRAQ 8-plex experiment consisted of peptides from 0.5 x 106 MM.1S cell lysates labelled with reagents. Half of the remaining reagents were used to label samples from patients and the remaining half to label the normal samples. The labelled peptide mixture was fractionated on a strong cation exchange (SCX) MicroSpin column with sequential elution of bound peptides. The eluate was dried and reconstituted in 01% trifluoroacetic acid (TFA) and then separated by reversed phase chromatography using a Zorbax 300 SB C18 column. Column effluent was mixed with matrix-assisted laser desorption/ionization (MALDI) matrix and spotted onto 192-well MALDI target plates which was latter analysed by tandem MS. The MS and MS/MS spectra were acquired on an Applied Biosystems 4800 Proteomics Analyser [time-of-flight (TOF)/TOF; AB SCIEX, Framingham, MA, USA] in positive ion reflection mode. Single-stage mass spectra for all samples were collected first and in each sample well MS/MS spectra will be acquired from the 12 most intense peaks above the signal-to-noise ratio threshold of 60. Protein identification and quantification was carried out using PROTEINPILOT software v2.0.1 (AB SCIEX) and the Celera protein sequence database (https://www.celera.com/), which comprised sequences from NCBI Refseq, Swiss-Prot, and TrEMBL data-bases.

Label-free quantitation

Pellets containing 0.5 x 106 PC from the same patients that were to be analysed by iTRAQ were lysed in radioimmunoprecipitation assay (RIPA) buffer (Pierce, Thermo Fisher Scientific). Denatured samples were separated on 4–12% Bis-Tris gels, which are to be stained with Gel-Code Blue (ThermoFisher Scientific). After each lane was cut into 22 equal pieces, gel plugs are to be de-stained with30% methanol, washed with 25 mM ammonium bicarbonate in 50% acetonitrile (ACN), reduced with 10 mM dith-iothreitol (ThermoFisher Scientific), alkylated using 50 mM iodoacetamide and digested with trypsin. Peptides were extracted first with 01% TFA in60% ACN and subsequently with 01% TFA in 100% ACN. Each supernatant was collected, pooled and dried, then reconstituted in solvent prior to liquid chromatography (LC)/MS analysis. Samples were analyzed via liquid chromatography-electrospray ionization-tandem mass spectrometry (LC-ESI-MS/MS) on a linear ion trap mass spectrometer (LTQ; ThermoFisher Scientific) coupled to an Agilent Technologies (Santa Clara, CA, USA) nano LC system and a C18 reversed phase LC column (Micro-Tech Scientific, Orange, CA, USA). Data Dependent Analysis (DDA) mode was utilized on the LTQ to perform MS on all ions above an ion count of 1000. Tandem MS spectra from the LTQ were searched with SEQUEST (http://fields.scripps.edu/sequest/index.html) against the human IPI protein database appended with an equal number of decoy (reversed) protein sequences (for FDR estimation).

Results and Discussion

Global proteomic profiling and data quality assessment

Principal Component Analysis (PCA) demonstrated clear clustering between healthy controls and myeloma patients, indicating robust biological separation and high-quality proteomic discrimination. Minimal overlap suggests that the disease state produces a distinct proteomic signature.

In the responder vs non-responder PCA, separation was less pronounced but still appreciable, reflecting shared disease biology with distinct resistance-associated alterations in the non-responder subset (Figure 1).

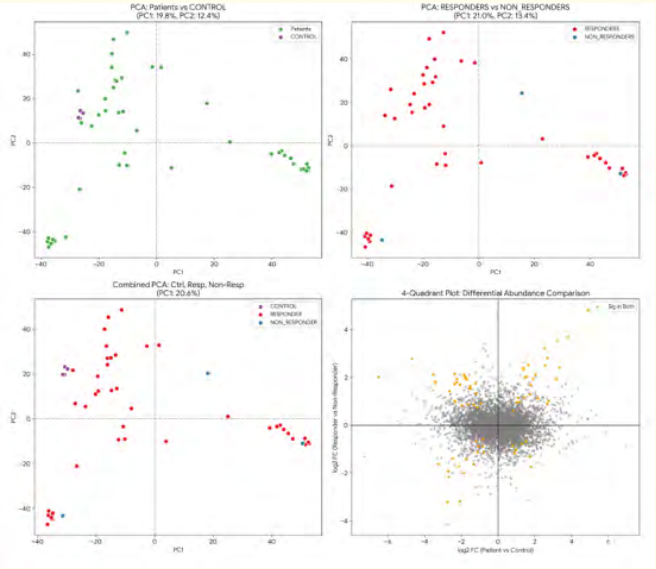

Figure 1: Principal Component Analysis plot.

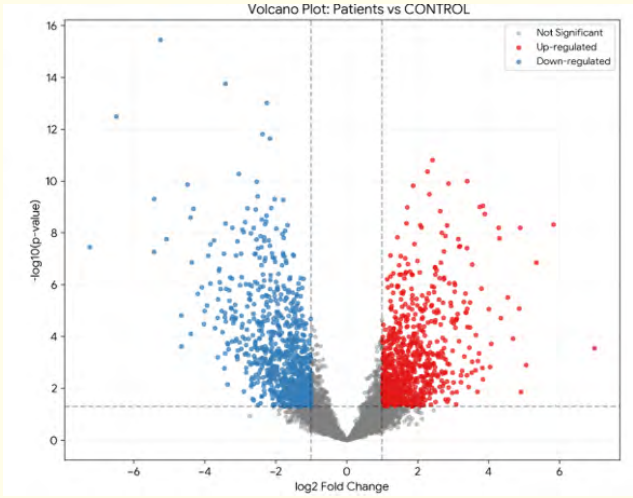

Figure 2: Volcano plot: patient vs control.

Differential expression: Patients vs healthy controls

The volcano plot analysis revealed multiple significantly dysregulated proteins. Among the top 50 differentially expressed proteins.

Key downregulated proteins

- ADRM1 (Adhesion Regulating Molecule 1) – log2FC: -5.25
- ILK (Integrin-linked kinase) – log2FC: -3.42
- CNN2 (Calponin-2)
- PDLIM7 (PDZ and LIM domain protein 7)
- BIN2 (Bridging integrator 2)

The predominance of downregulated cytoskeletal and adhesion-associated proteins suggests disruption of cell-structural integrity and altered plasma cell–stromal interactions (Figure 2).

Biological interpretation

- **Proteasomal Pathway Alterations**: ADRM1 is involved in ubiquitin-mediated proteasomal degradation. Its marked downregulation suggests dysregulation of protein turnover, a central feature of myeloma biology, particularly relevant in the context of proteasome inhibitor therapy.
- **Cell Adhesion and Microenvironmental Signaling:** ILK and PDLIM7 are integrin-associated signaling molecules. Their dysregulation reflects altered adhesion-mediated drug resistance (CAM-DR), a known mechanism in myeloma.
- **Cytoskeletal Remodeling**: Downregulation of CNN2 and BIN2 indicates structural reprogramming, potentially facilitating disease dissemination within the marrow niche.

Functional enrichment analysis

Gene Ontology (GO) Enrichment

GO analysis demonstrated significant enrichment in:

- Cytoskeletal organization
- Proteasome-mediated protein catabolism
- Cell adhesion and extracellular matrix interaction
- Immune modulation pathways

This supports the concept that myeloma progression involves structural remodeling, proteostasis dysregulation, and immune evasion (Figure 3).

KEGG pathway enrichment

Enriched pathways included:

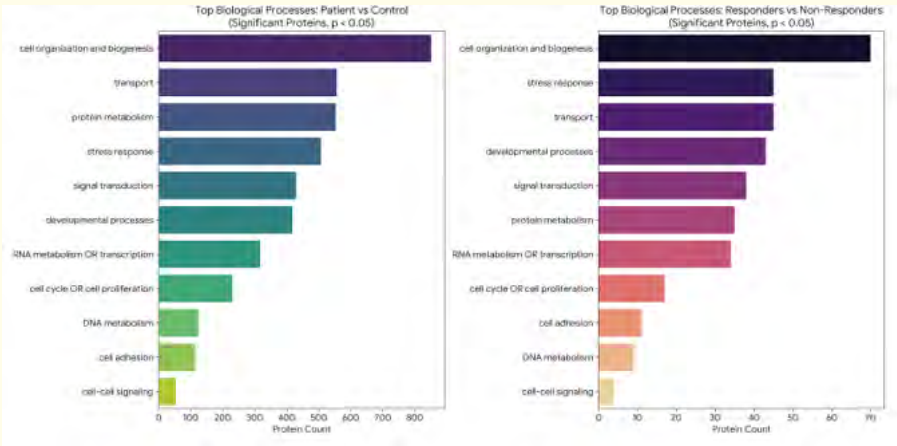

Figure 3: Gene Ontology enrichment.

- Proteasome pathway
- Focal adhesion
- PI3K-AKT signaling
- Oxidative phosphorylation

These findings confirm activation of survival signaling pathways and altered metabolic reprogramming in myeloma cells (Figure 4).

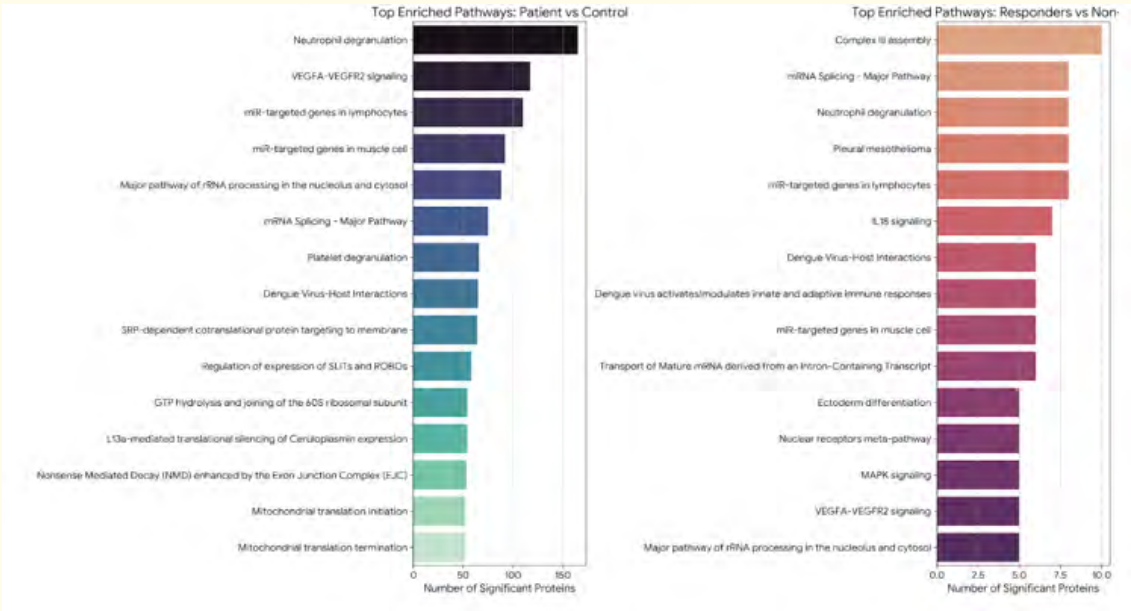

Figure 4: Kyoto Encyclopedia of Genes and Genomes enrichment.

Responder vs non-responder proteomic comparison

Among the 48 patients, 3 were non-responders. Although the sample size of non-responders is small, significant proteomic differences were observed (Figure 5).

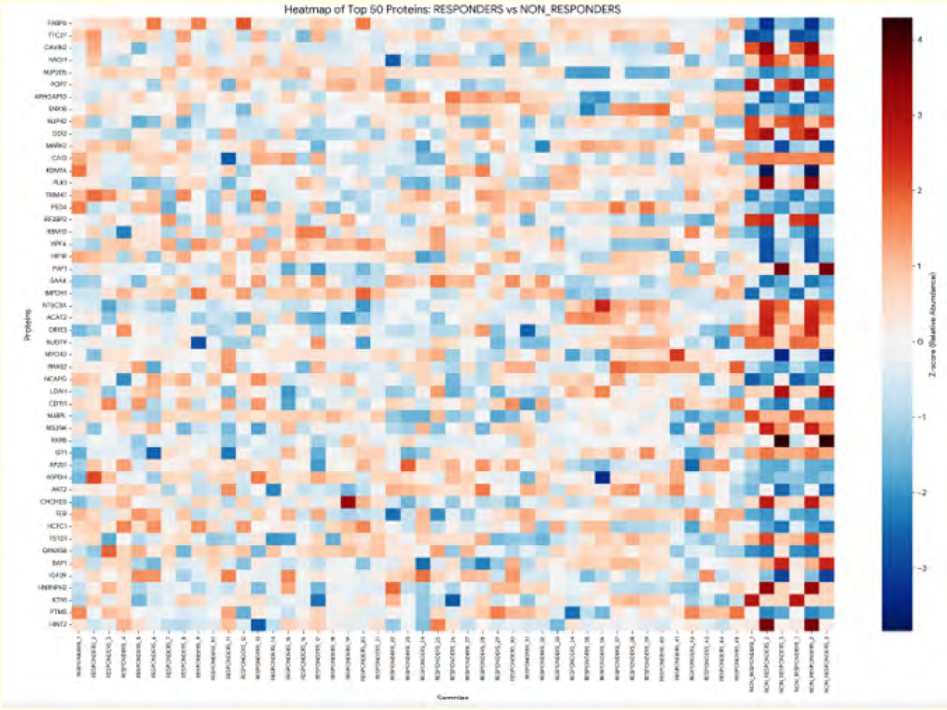

Figure 5: Responders vs non-responders heatmap.

Key Upregulated Proteins in Non-Responders:

- FABP6 (Fatty Acid Binding Protein 6) – log2FC: +3.66
- TTC27 (Tetratricopeptide Repeat Protein 27) – log2FC: +3.23
- NUP205 (Nuclear Pore Complex Protein 205) – log2FC: +4.64

Key Downregulated Proteins in Non-Responders:

- CAVIN2 (Caveolae-associated protein 2)
- HAGH (Hydroxyacylglutathione hydrolase)

Biological interpretation of treatment resistance

- **Metabolic Reprogramming:** Upregulation of FABP6 suggests enhanced lipid transport and fatty acid metabolism in non-responders. This indicates metabolic adaptation as a resistance mechanism. Lipid metabolic rewiring may provide alternative energy sources under therapeutic stress.
- **Nuclear Transport and Transcriptional Regulation**: NUP205 upregulation suggests altered nuclear-cytoplasmic trafficking. Dysregulated nuclear pore complex activity may enhance oncogenic transcriptional programs and survival signaling.
- **Stress Detoxification Pathways**: Downregulation of HAGH, involved in detoxification of reactive metabolites, suggests altered oxidative stress handling. Resistant clones may adopt alternative antioxidant mechanisms.
- **Membrane Signaling and Caveolae Dynamics**: CAVIN2 downregulation indicates altered membrane microdomain organization. Caveolae are involved in receptor signaling; their disruption may modify drug uptake or survival signaling.

Network analysis

Protein–protein interaction network analysis revealed tightly interconnected clusters involving:

- Proteostasis machinery
- Adhesion signaling complexes
- Nuclear transport proteins
- Metabolic enzymes

In responders, network topology demonstrated more centralized signaling hubs. In non-responders, networks were more dispersed, suggesting proteomic rewiring and pathway redundancy — hallmarks of therapeutic resistance.

Integrated interpretation

The proteomic data suggest that:

- Myeloma cells exhibit profound dysregulation of structural, proteasomal, and adhesion pathways compared to healthy plasma cells.
- Treatment response is associated with preservation of regulated proteostasis and adhesion signaling.
- Non-responders demonstrate:
- Enhanced lipid metabolism
- Altered nuclear transport
- Membrane microdomain remodeling
- Adaptive metabolic rewiring

These findings support a model in which resistant clones survive via metabolic flexibility and proteomic plasticity rather than reliance on a single dominant oncogenic pathway.

Clinical implications

- Potential Biomarkers of Resistance: FABP6 and NUP205 may serve as predictive biomarkers for poor therapeutic response.
- Therapeutic Targeting Opportunities:
- Targeting lipid metabolism
- Inhibiting nuclear transport mechanisms
- Combination strategies addressing metabolic rewiring

Personalized Proteomic Stratification: Proteomic profiling may complement genomic risk models to identify high-risk or refractory patients early.

Limitations of the study

The present study has certain limitations that should be acknowledged. The small number of non-responders (n = 3) substantially limits the statistical power of comparative analyses and restricts the generalizability of conclusions regarding proteomic determinants of treatment resistance. In addition, the absence of longitudinal proteomic assessment—particularly paired pre- and post-treatment sampling—precludes dynamic evaluation of therapy-induced molecular alterations and clonal evolution. Finally, the findings are based primarily on discovery-level proteomic data and therefore require independent validation, including functional in vitro and in vivo studies, to establish mechanistic relevance and confirm the biological significance of the identified differentially expressed proteins.

Conclusion

This comprehensive proteomic analysis demonstrates distinct molecular signatures separating multiple myeloma patients from healthy controls and identifies biologically meaningful differences between treatment responders and non-responders. The data emphasize:

- Proteostasis and adhesion dysregulation as core disease mechanisms.
- Metabolic reprogramming and nuclear transport alterations as key resistance pathways.

Proteomics provides a powerful functional layer of disease characterization and holds promise for identifying predictive biomarkers and novel therapeutic vulnerabilities in multiple myeloma.

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