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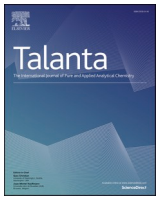

# Dithiothreitol-based protein equalisation in the context of multiple myeloma: Enhancing proteomic analysis and therapeutic insights

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ABSTRACT

In this study, we employed the dithiothreitol-based protein equalisation technique and analytical proteomics to better understand myeloma diseases by comparing the proteomes of pellets and supernatants formed upon application of DTT on serum samples. The number of unique proteins found in pellets was 252 for healthy individuals and 223 for multiple myeloma patients. The comparison of these proteomes showed 97 dysregulated proteins. The unique proteins found for supernatants were 264 for healthy individuals and 235 for multiple myeloma patients. The comparison of these proteomes showed 87 dysregulated proteins. The analytical proteomic comparison of both groups of dysregulated proteins is translated into parallel dysregulated pathways, including chaperone-mediated autophagy and protein folding, addressing potential therapeutic interventions. Future research endeavours in personalised medicine should prioritize refining analytical proteomic methodologies using serum dithiothreitol-based protein equalisation to explore innovative therapeutic strategies. We highlight the advanced insights gained from this analytical strategy in studying multiple myeloma, emphasising its complex nature and the critical role of personalised, targeted analytical techniques in enhancing therapeutic efficacy in personalised medicine.

## 1. Introduction

Multiple myeloma (MM) is a slowly growing and complex haematological malignancy characterized by abnormal proliferation of plasma cells in the bone marrow, leading to bone destruction, monoclonal gammopathy (i.e. increased immunoglobulin synthesis), and potential complications such as renal failure and anaemia [1]. Despite advancements in treatment modalities, MM remains associated with substantial morbidity and mortality rates [2]. The global incidence of MM is significant, with approximately 160,000 new cases and 106,000 deaths reported annually [2]. In the United States, the incidence rate from 2003 to 2016 was 8.47 per 100,000 persons, with variations across different racial and ethnic groups [3].

In the field of analytical proteomics, significant strides have been made in understanding, detecting, and monitoring MM. Anderson et al. [4] employed plasma membrane profiling of primary human myeloma

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cells, identifying SEMA4A as a crucial protein for myeloma cell growth and a potential immunotherapy target. Kumar et al. [5] used single-cell proteomics and RNA sequencing to explore clofazimine's efficacy against drug-resistant myeloma, identifying novel pathways associated with clofazimine sensitivity. Tierney et al. [6] combined ex-vivo drug sensitivity testing with proteomics, identifying proteins that could modulate cell adhesion-mediated drug resistance. As a further example, Ye et al. [7] demonstrated the potential of proteomic analysis in discovering novel subtype-specific protein biomarkers for MM using a combinatory strategy involving nanoscale proteomics and machine learning.

Despite advanced techniques like high-resolution mass spectrometry and bioinformatics tools to improve the sensitivity and specificity of proteomic analyses in MM, applying proteomics to serum samples for MM research encounters significant challenges. The most notable challenge is the dynamic range of protein concentrations in serum. This is because the most abundant proteins, such as albumin and immunoglobulins, can overshadow the detection of less abundant, disease-specific proteins. In this context, the work done by our group [8–13] on dithiothreitol (DTT)-based protein equalisation in bladder cancer highlights the potential of this method to underscore biochemical pathways and identify biomarkers for cancer comprehensively. This method is fast, cheap, and easy to apply.

In this study, we employed the DTT-based protein equalisation technique and analytical proteomics to better understand myeloma diseases by comparing the pellet's proteomes and supernatant formed upon application of DTT on serum samples. Our secondary objective was to elucidate the biochemical pathways involved in the disease as reflected in the blood serum and to identify potential new biomarkers and therapeutic targets. This work demonstrates that DTT-based protein equalisation is instrumental in revealing the intricate molecular mechanisms underlying multiple myeloma (MM), thereby contributing to developing more effective diagnostic and treatment strategies. This highlights DTT as a universal analytical proteomics strategy for serum samples to underpin the understanding of disease mechanisms.

## 2. materials and methods

### 2.1. Experimental study design

The serum samples from 16 participants were categorized into healthy individuals and multiple myeloma patients. The group of healthy individuals comprises 5 females and 6 males, while the group of myeloma patients presents 5 males. Data about the study cohort is presented in Supplementary Material 1 Table 1. All patients were informed about the study and signed an informed consent according to the policies of the Garcia de Orta Hospital Ethics Committee, which approved our study. The ethics approval is presented in Supplementary Material 2. Exclusion criteria were patients suspected of Human Immunodeficiency Virus (HIV) or other viral infections such as Hepatitis B or C. Samples were taken after diagnosis and before the beginning of any treatment.

### 2.2. Serum sample preparation

Blood samples were collected in red glass vacutainer tubes without anticoagulants or preserves. The samples were allowed to clot at room temperature, followed by centrifugation at 2000×*g* for 10 min at room temperature (RT). After centrifugation, serum was aliquoted and stored at −80 °C.

### 2.3. Tryptophane fluorescence emission assay for proteome and proteome digest quantification

Proteome and proteome digest concentrations were determined using the tryptophan fluorescence emission assay [14]. In this method, a standard calibration curve was created using 0.0102 μg/μL tryptophan dissolved in 8 M urea in 0.1M Tris-HCl pH 8 to span a linear detection range from 0 to $5.1 \times 10^{-3}$ μg/μL as described by Domingos et al. [15]. For measurement, 75 μL of calibration solutions were transferred to a quartz-bottom 96-well plate. A dilution protocol was applied to align with the assay's linear range to assess the concentration of proteome and proteome digest in unknown samples. This involved adding 5 μL of each sample in duplicate to the plate wells, followed by adding 70 μL of the 8 M urea in 0.1M Tris-HCl pH 8. Measurements were performed with an excitation wavelength set at 280 nm, with a long pass dichroic mirror at 309 nm and an emission wavelength of 350 nm, utilizing a bandwidth of 20 nm. The quantification of proteome and proteome digest was derived from fluorescence intensity measurements, which applied an average tryptophan weight content assumption of 1.17 % for human proteins [14].

### 2.4. Serum proteome equalisation

We selected serum as the biological fluid for analysis because multiple myeloma is predominantly a haematological malignancy, with the majority of its biomarkers and pathological manifestations detectable in blood. For the processing of raw serum samples, an initial volume of 20 μL was treated with 2.2 μL of 500 mM DTT and incubated for 30 min at a temperature of 37 °C [8–13]. This procedure was performed in triplicate. After incubation, samples were centrifuged at 20,000×*g* for 20 min. Subsequently, the supernatant was carefully transferred to new Eppendorf tubes, while the residual pellet was gently rinsed with 10 μL of Milli-Q water. This rinse was then combined with the initially collected supernatant. The total protein in the combined supernatant sample (SN) was quantified via tryptophan emission, as described in section 2.3. Quantification of the protein content in the pellet was done using the following formula:

$$\mu g\ of\ Protein\ in\ Pellet = \mu g\ of\ Protein\ in\ Raw\ Serum\ -\ \mu g\ of\ Protein\ in\ SN$$

### 2.5. Proteome reduction, alkylation, and digestion

Sample preparation was performed as described previously, with optimization for the supernatant and pellet samples [8]. Initially, supernatant samples were diluted to achieve a target protein concentration of 103 ± 8 μg per 20 μL of sample volume. This step was followed by adding 5 μL of a Reduction/Alkylation solution consisting of 10 mM Tris (2-carboxyethyl)phosphine (TCEP), 40 mM CAA, 0.1M Tris-HCl pH 8.8, to each sample. The mixture was then incubated for 30 min at 37 °C. The pellets were solubilized with 150 μL of 70 mM Tetraethylammonium bromide (TEAB) followed by probe ultrasonication for 1 min (Ultrasonic frequency: 30 kHz, Ultrasonic Amplitude: 100 %, Cycle time: 0,8 s). Afterwards, 100 ± 2 μg of the pellets' proteins (final volume of 20 μL) were reduced with 5 μL of Reduction/Alkylation solution. Before trypsin digestion, samples were diluted to 150 μL with 70 mM TEAB.

For the proteome digestion phase, 5 μL of a Trypsin/Lysine-C solution, at a concentration of 0.67 μg/μL prepared in 70 mM TEAB, was added to each reduced and alkylated sample. The samples were left to digest overnight at 37 °C. Following digestion, the resultant peptide mixtures were concentrated by drying in a speed vacuum concentrator. Before downstream analysis, peptides were resolubilized in 150 μL of 3 % (v/v) Acetonitrile (ACN) in 0.1 % (v/v) aqueous formic acid (FAaq), followed by 10 min of ultrasonication using an ultrasonic bath at 100 % ultrasonic amplitude.

### 2.6. LC-MS/MS analysis

LC-MS/MS analysis was performed using UltiMate 3000 ultra-high performance liquid chromatograph from Thermo Scientific, coupled to Ultra High-Resolution Quadrupole Time-of-Flight (UHR-QTOF) IMPACT HD mass spectrometer from Bruker. 0.5 μL of the sample with a total

peptide concentration of 0.6 μg/μL were loaded onto a μPAC™ Trapping column and desalted for 2.7 min with 1 % (v/v) ACN in 0.1 % FAaq at a flow rate of 15 μL $min^{-1}$. Then the peptides were separated using an analytical column (200 cm μPACTM PharmaFluidics) with a linear gradient at 500 nL $min^{-1}$ (mobile phase A: FAaq 0.1 % (v/v); mobile phase B: 99.9 % (v/v) ACN and 0.1 % (v/v) FAaq) 0–2 min from 3 % to 5 % of mobile phase B, 5–76 min from 5 % to 17 % of mobile phase B, 76–104 17 % to 25 % B, 104–121 25 %–35 % B. Chromatographic separation was carried out at 35 °C. MS acquisition was set to MS (2 Hz) cycles, followed by MS/MS (8–32Hz), cycle time 3.0 s, active exclusion, exclude after one spectrum, release after 2 min. The precursor was reconsidered if its current intensity was 3.0 higher than the previous intensity and intensity threshold for fragmentation of 2500 counts.

### 2.7. Bioinformatics data analysis and functional enrichment

Raw LC−MS/MS data were processed in MaxQuant (V.1.6.10.43) for protein identification and label-free quantification using standard settings [16]. Peptide lists were searched against the human Uniprot FASTA database. A contaminant database generated by the Andromeda search engine [17] was configured with cysteine carbamidomethylation as a fixed modification and N terminal acetylation and methionine oxidation as variable modifications. We set the false discovery rate (FDR) to 0.01 for protein and peptide levels with a minimum length of seven amino acids for peptides, and the FDR was determined by searching a reverse database. Enzyme specificity was set as C-terminal to arginine and lysine, and a maximum of two missed cleavages were allowed. Peptide identification was performed with an allowed initial precursor mass deviation of up to 25 ppm and an allowed fragment mass deviation of 25 ppm. Proteins matching to the reversed database were filtered out. LFQ was performed with a minimum ratio count of 2. Data processing and statistical analysis were performed using Perseus (version 1.6.10.50) with default settings [18,19]. All proteins matching the reversed database were filtered out. We employed a Multiple-sample ANOVA test (Permutation-based FDR set at 0.05, S0 = 0) and a Two-sample T-test (Permutation-based FDR set at 0.05, S0 = 0).

ClueGO v 2.5.10, a plugin of Cytoscape 3.10.1, was used to unveil the most significant pathways. The following analysis parameters were defined for ClueGO: function; two sample lists were loaded, one per cluster; the organism selected was Homo sapiens, and the type of IDs used was Accession ID. Clusters were used to obtain a deep comparison between the different clusters; The Ontologies/Pathways selected were: GO_BiologicalProcess and Reactome_pathways. “All Evidence codes” box was chosen; regarding network specificity, it was set as “detailed”; the box of “Use GO Term Fusion” and “Show only pathways with p-value ≤0.05” was also chosen. GO Tree Interval was set to Min Level 3 and Max Level 8. Selection criteria for the terms that have associated genes from cluster 1 were set as a minimum of 3 genes per term and a minimum of 4 % from all genes related to the term.

## 3. Results and discussion

### 3.1. Serum total protein quantification in raw serum, supernatant, and pellet

MM patients present increased levels of total serum proteins compared to healthy individuals (HI). This hallmark results from the excessive production of monoclonal proteins or immunoglobulins in the malignant plasma cells [20]. Our quantitative analysis is consistent with this trait and demonstrates this pattern, as evidenced in Fig. 1a, highlighting a significant difference in the total protein levels between MM and HI. This variation is evident in the supernatant and pellet after DTT-based serum protein equalisation. Fig. 1a also depicts the higher variability of protein levels in MM patients, highlighting phenotypical diversity. A deeper exploration into the specific proteins involved, achievable through proteomic analysis, is crucial for a more comprehensive understanding of the molecular dynamics that play in MM. Such exploration is explained below.

### 3.2. Dithiothreitol-based serum protein equalisation

After equalising the proteome using DTT, we observed a comparable pattern regarding protein depletion between healthy and MM samples (Fig. 1a). The protein depleted was *c.a.* 64 % of the total protein content in healthy samples and 57 % in MM samples. There was a more significant variability in the depletion percentage among the MM samples, ranging from 39 % to 75 %, compared to a narrower range of 59 %–71 % observed in the healthy samples.

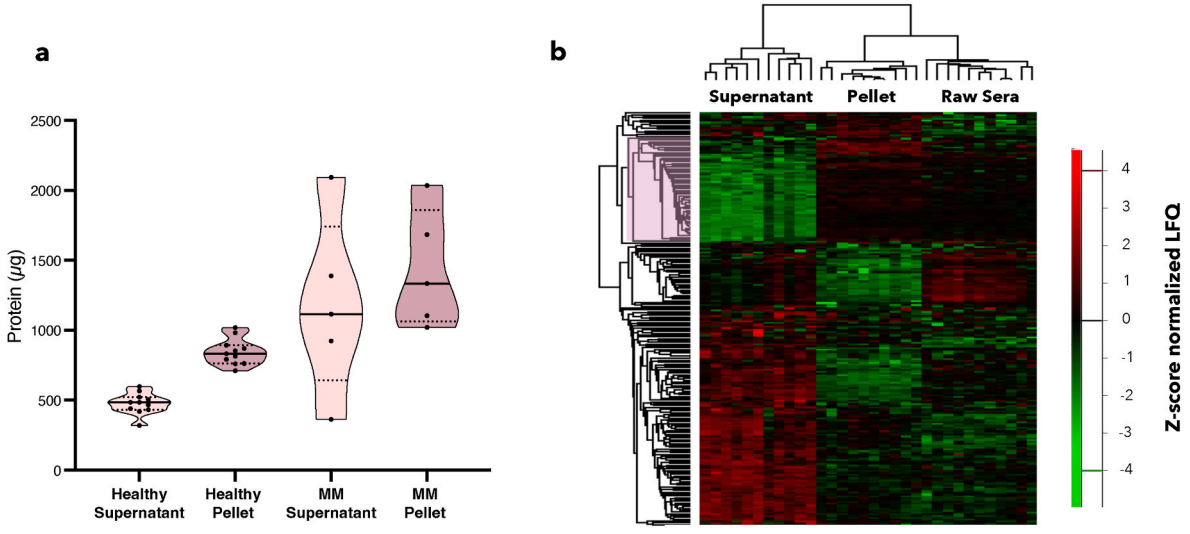


**Fig. 1.** – Proteomic profiling in healthy individuals and multiple myeloma patients. (a) Violin plot illustrating the data distribution of total protein quantification in supernatant and pellet samples from healthy individuals (n = 11) and Multiple Myeloma patients (n = 5). Quantification was performed using the tryptophan emission assay, with each sample analyzed in duplicate. The plots were generated using Prism version 10.1.1, where each point represents an individual patient. The solid bar in the middle indicates the median, while the dashed line represents the quartile. (b) Proteome comparison between raw, depleted (n = 11) and pellet sera in healthy individuals (n = 10). The heatmap was generated with Perseus software (version 1.6.10.50). Log2-transformed Label-Free Quantitation (LFQ) data were normalized using Z-score normalization, followed by a Multiple-sample ANOVA test (Permutation-based False Discovery Rate [FDR] at 0.05, S0 = 0).

Fig. 1b illustrates the impact of the equalisation process on protein concentrations in the supernatant and pellet derived from serum samples. In the supernatant, highlighted in green, a specific set of proteins show reduced intensities compared to their concentrations in raw serum.

These proteins, which include highly abundant ones such as albumin, serotransferrin, α-1-antitrypsin, plasminogen, complement-associated proteins, and various apolipoproteins, are found in increased abundance in the pellet. This observation implies their effective removal from the supernatant, addressing the efficiency of the depletion process. In addition, a distinct set of proteins, highlighted in red, exhibited higher intensities in the supernatant than in the raw serum, signalling an effect attributed to the equalisation process. Moreover, the proteomic profiles of the samples displayed a tendency to cluster by group, confirming the unique proteomic fingerprint of each cohort, raw, pellet and supernatant.

### 3.3. Proteomic comparison of healthy versus multiple myeloma's supernatants

Our methodology, which employed quantitative proteomics on serum pellets, enabled us to quantify 264 proteins in individuals without health issues and 235 in patients with MM. The levels of the standard proteins between both groups were compared through a heatmap shown in Fig. 2a. The comparison addressed 32 upregulated and 65 downregulated proteins in MM.

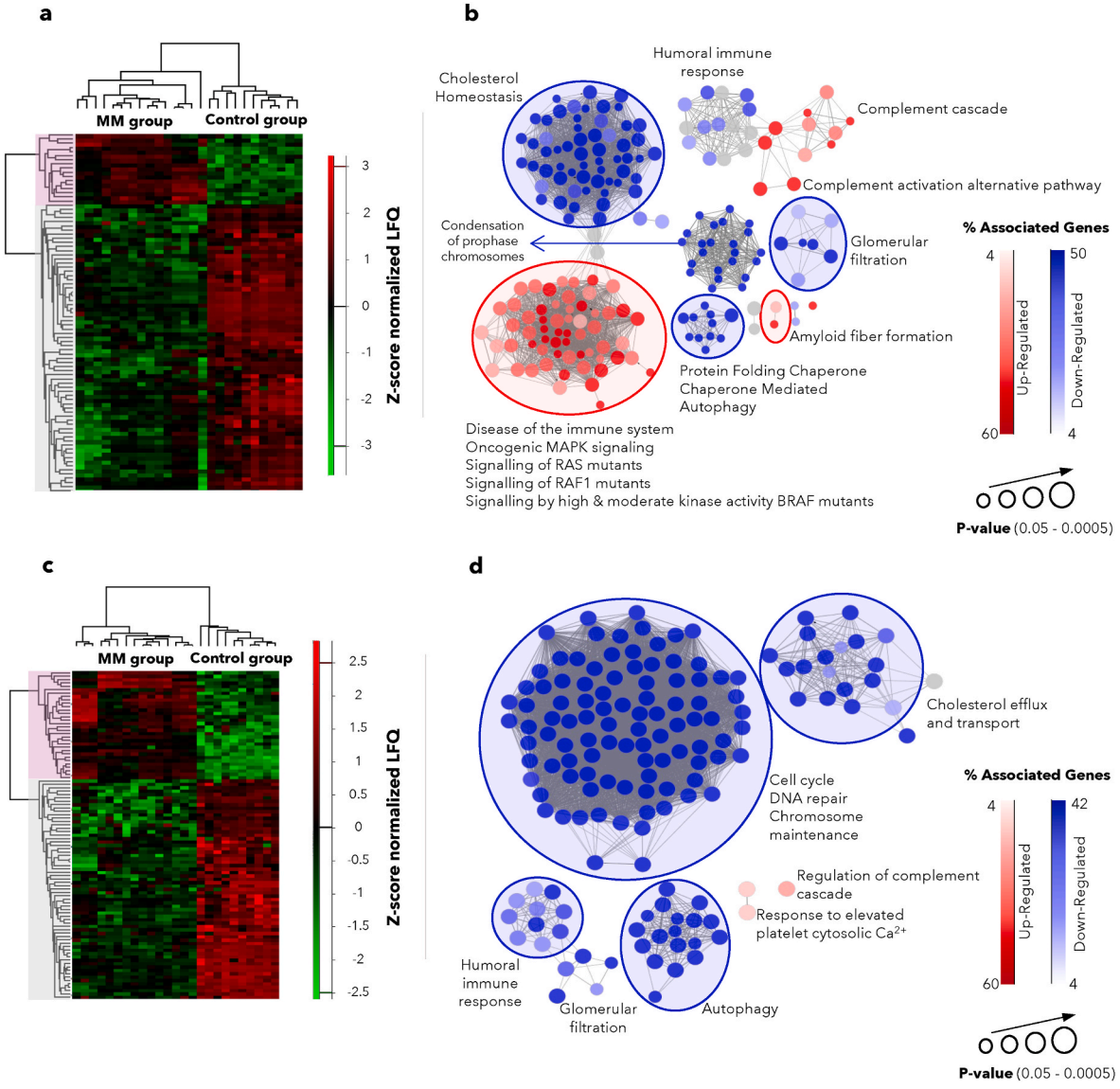


**Fig. 2.** Comparative proteomic analysis between MM patients and healthy individuals. (a) Comparison of supernatant proteins between healthy individuals (n = 11) and the patient group (n = 15). The heatmap was generated using Perseus, with Log2-transformed LFQ data normalized by Z-score and a Two-sample T-test (Permutation-based FDR at 0.05, S0 = 0). (b) ClueGO analysis of up- and downregulated proteins in serum from MM patients (n = 5). Red indicates GO/pathway terms specific to upregulated proteins, and blue indicates terms specific to downregulated proteins. The functionally grouped network displays terms as nodes linked based on their kappa score level (≥0.4). Only the most significant term label per group is shown, with node size indicating significance for term enrichment. (c) Comparison of pellet proteins between healthy individuals (n = 11) and the patient group (n = 5). The heatmap was generated with Perseus, using Log2-transformed LFQ data normalized by Z-score, followed by a Two-sample T-test (Permutation-based FDR at 0.05, S0 = 0). (d) ClueGO analysis of up- and downregulated proteins in serum from MM patients (n = 5). GO/pathway terms for upregulated proteins are shown in red, and those for downregulated proteins are in blue. The functionally grouped network displays terms as nodes, linked by their kappa score level (≥0.4), with only the label of the most significant term per group displayed. Node size reflects term enrichment significance.

Table 2 of Supplementary Material 1 presents the complete list of proteins. To better understand the pathways where these 87 (17 + 70) dysregulated proteins are involved and how they are interconnected, we ran a gene ontology and Reactome pathway analysis using the ClueGo plug-in in Cytoscape. The results are shown in Fig. 2b.

Fig. 2b reveals that in MM patients, there is a notable downregulation in Chaperone-mediated Autophagy (CMA) and in the activities of protein-folding chaperones, highlighted by the low group p-value = $2.3 \times 10^{-3}$. These pathways are vital for cellular balance, ensuring proteins are correctly folded and preventing the harmful accumulation of misfolded proteins. The noted reduction in these pathways suggests a compromised cellular capability in managing misfolded proteins, increasing the risk of their aggregation. In conjunction with the MM characteristic overproduction of light chains, the impaired function of these chaperone systems leads to an increased formation of amyloid fibrils, a process which is presented upregulated (coloured red, p-value = $3.4 \times 10^{-5}$) in MM patients (see Fig. 2b). Amyloid fibrils are challenging to degrade and can deposit in various organs, disrupting normal function. The kidneys, for example, are particularly vulnerable to the impacts of amyloid fibrils' accumulation. Amyloid deposits can compromise the kidneys' filtration structures, impeding glomerular filtration and possibly leading to renal failure [21]. The malfunction of the glomerular filtration pathway (p-value = $3.7 \times 10^{-11}$) in our MM patients corroborates this observation.

The proliferation of malignant plasma cells in the bone marrow can suppress the growth and function of normal B cells [22]. This suppression can decrease the production of other essential antibodies, further weakening the humoral immune response as we have found in our MM patients, (Fig. 2b, p-value = $2.3 \times 10^{-23}$). Furthermore, in MM patients, the up-regulation of the complement cascade we found (p-value = $5.3 \times 10^{-22}$), especially the alternative pathway, might be a compensatory mechanism to compensate for the weakening of the humoral immune response. With the downregulation of the humoral immune response, the body might be trying to boost its innate immune defences by enhancing the complement system's activity. Typically, the classical complement pathway is activated by antibodies bound to antigens. Considering the impaired humoral response observed in MM, fewer antibodies might be available to trigger the classical pathway, leading the body to rely more on the alternative pathway, as evidenced by our data. Although the complement system is a defensive mechanism, its excessive activation may result in unintended tissue harm [23]. The byproducts of this overactive complement cascade can trigger inflammation and damage tissues, a situation that becomes more critical when the activation is persistent. This issue is of particular concern for organs such as the kidneys, which are already at risk due to the deposition of amyloid fibrils in cases of MM.

The significant up-regulation of the MAPK pathway (p-value = $2.5 \times 10^{-18}$), as depicted in Fig. 2b, plays a pivotal role in the progression of the disease. This pathway, essential for cellular growth, differentiation, and survival, becomes abnormally active in MM. This increases cell proliferation and decreases programmed cell death (apoptosis). A vital aspect of this dysregulation involves the Signalling of RAS mutants. This signalling cascade continuously transmits growth signals from receptors to the cell's nucleus. Additionally, mutations in RAF1 further enhance the MAPK signalling. Another component, BRAF, also shows mutations in MM. As reported in the literature, those with moderate kinase activity contribute to cell growth, while mutations in high kinase activity suggest a more aggressive disease variant [24].

### 3.4. Proteomic comparison of healthy versus multiple myeloma's pellets

For serum pellets, we quantified 252 proteins in individuals without health issues and 223 in patients with MM. The levels of the standard proteins between both groups were compared through a heatmap shown in Fig. 2c. The comparison addressed 32 upregulated and 65 downregulated proteins in MM. Table 3 of Supplementary Material 1 presents the complete list of proteins.

The Reactome pathway analysis of the 97 proteins (32 + 65) presented in Fig. 2d illustrates a decrease in the activity of several critical biochemical processes in MM patients, specifically highlighting a notable reduction in the Humoral immune response, glomerular filtration, and autophagy. This indicates that the body's immune system functions sub-optimally, and the kidneys do not effectively filter the blood. Interestingly, this reduction in activity mirrors what we observed in the supernatant of the same samples. Furthermore, a marked downregulation in the cell cycle, DNA repair, and Chromosome maintenance pathways (p-value = $1.0 \times 10^{-11}$) was also observed. This reduction suggests that the cells might have compromised abilities in repairing DNA damage and preserving chromosomal integrity. Such impairments can lead to genomic instability, a common hallmark of many cancers. When cells cannot effectively repair DNA, they accumulate mutations and chromosomal abnormalities. Over time, these genetic alterations can provide growth advantages to cancer cells, potentially leading to unchecked proliferation and tumour formation [25]. In the context of MM, this downregulation could be contributing to the progression of the disease and the development of more aggressive cancerous clones. Furthermore, the observed up-regulation of cytosolic $Ca^{2+}$ in platelets (p-value = $1.5 \times 10^{-15}$) carries significant implications. This is particularly relevant as hypercalcemia, which is characterized by elevated calcium levels in the bloodstream, frequently emerges as a complication in individuals diagnosed with MM [26]. This process is driven by myeloma cells secreting factors that stimulate osteoclasts, the specialized cells that break bone tissue. Dysregulated calcium signalling can lead to an increase in calcium release from bones and a reduction in calcium excretion by the kidneys, thereby contributing to the development of hypercalcemia [27]. Moreover, the disruption of calcium signalling pathways in multiple myeloma cells could have a broader impact on the immune system [25], potentially leading to immune dysfunction and indirectly facilitating disease progression. This highlights the critical need to target these pathways in developing potential therapeutic strategies.

Finally, we observed a down-regulation in the cholesterol efflux process and transport pathways (p-value = $1.6 \times 10^{-9}$). The cholesterol removal process involves removing extra cholesterol from cells, particularly those lining the walls of blood vessels. This process is crucial for preventing the build-up of cholesterol in artery walls, which can lead to the development of atherosclerosis and heart diseases. Cholesterol transportation, on the other hand, refers to the movement of cholesterol molecules through the bloodstream with the help of specialized carriers like low-density lipoprotein (LDL) and high-density lipoprotein (HDL) [28]. In the context of MM, the decrease in these critical processes indicates issues in the mechanisms responsible for efficiently removing cholesterol from cells and transporting it to the liver. This alteration can have significant implications for maintaining a healthy cholesterol balance, affecting overall health.

### 3.5. Insights gained from analytical proteomics of multiple myeloma patients

Although the number of patients involved limits the insights gained from this study, some trends aligned with the known disease course of MM. We detected a significant upregulation in the amyloid fibre formation pathway. Considering that 10–15 % of multiple myeloma patients eventually develop amyloidosis, this observation suggests the possibility that these patients may already exhibit early amyloid fibre formation. Consequently, this underscores the potential for earlier detection and management of amyloidosis through proteomic longitudinal monitoring of MM individuals. This possibility is further confirmed by data presented in Fig. 3, where the levels of HPS90-like proteins are lower in patients than in healthy individuals. Furthermore, other key proteins in amyloid fibril formation, namely B2M, CLU and GSN, are presented at higher levels in patients than in healthy individuals. To the

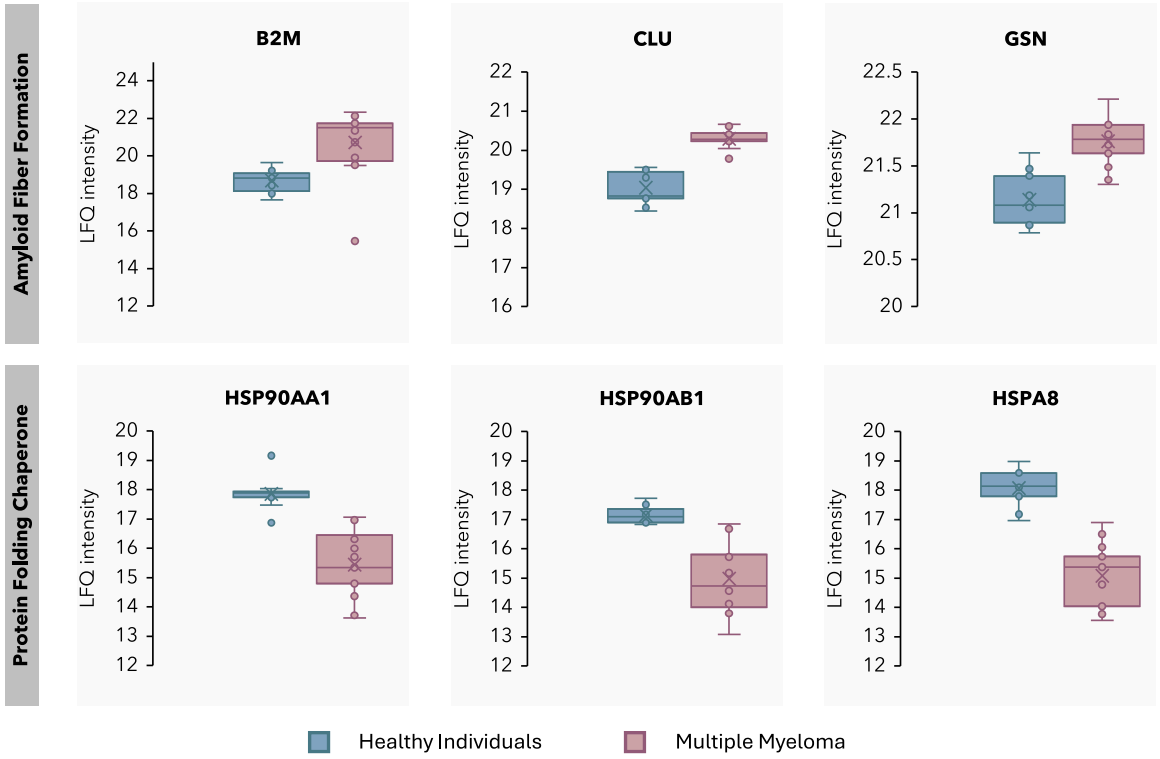


**Fig. 3.** Representative boxplot of Label-free quantification data intensity between healthy individuals (n = 11) and multiple myeloma patients (n = 5). Within the amyloid fibre formation group, data is depicted for beta-2 microglobulin (B2M), Clusterin (CLU), and Gelsolin (GSN); for the Protein folding chaperone group, data is shown for the chaperone proteins 90 kDa (HSP90 AA1 and HSP90 AB1) and 70 kDa chaperone protein (HSPA8). Each boxplot shows individual data points, with the boxes representing the interquartile range (IQR, from the first quartile (Q1) to the third quartile (Q3)), the horizontal line within the box indicating the median (Q2), and the whiskers extending to 1.5 times the IQR. Data points beyond this range are shown as outliers.

best of our knowledge, no evidence of GSN increased concentrations has been described to date among MM patients. Only three patients have been described in the literature as affected by ocular amyloid and multiple myeloma [29].

Additionally, the observed down-regulation of protein-folding chaperones and chaperone-mediated autophagy pathways (critical for proper protein folding), alongside the up-regulation of amyloid fibre formation, underscores the importance of the proteostasis network in cellular health and the severe impact of its dysregulation. Targeting the dysregulated pathways involved in protein folding, aggregation, and degradation is currently done by several first and second-generation drugs, such as bortezomib and lxazomib. These treatments, however, often cause adverse reactions due to their non-specific action on cancer cells [30]. Alternatively, drugs that inhibit Heat Shock Protein 90, a molecular chaperone involved in the proper folding and function of many oncogenic proteins, could be beneficial [31].

Additionally to proteostasis in MM, other dysregulated pathways identified in our proteomic analysis, including (i) oncogenic MAPK signalling, (ii) activation of the complement cascade, (iii) abnormal calcium homeostasis, (iv) suppression of the humoral immune response, and (v) genomic instability (cell cycle, DNA repair, chromosome maintenance), open a broad spectrum of potential therapeutic targets for MM patients. Targeted therapies, such as mitogen-activated protein kinase (MEK) inhibitors like Trametinib or Cobimetinib, might effectively inhibit the oncogenic MAPK signalling pathway, thereby reducing cell proliferation and inducing apoptosis in malignant cells [32]. Specifically for MM patients with BRAF mutations, drugs such as Vemurafenib or Dabrafenib, which specifically target mutated forms of the BRAF protein, could be considered to inhibit the MAPK pathway downstream [33].

The observed up-regulation of the alternative complement pathway in MM suggests that targeting complement system components could offer therapeutic benefits by reducing inflammation and tissue damage associated with excessive complement activation. Eculizumab, a monoclonal antibody designed to inhibit the C5 component of the complement pathway, emerges as a promising candidate for repurposing in the treatment of MM. This approach is underpinned by Eculizumab's success in managing complications such as carfilzomib-induced thrombotic microangiopathy, sometimes encountered in MM therapy [34]. However, further research is needed to understand its impact on disease progression and overall patient outcomes.

Abnormal calcium homeostasis, especially hypercalcemia of malignancy, is a common finding typically found in patients with advanced-stage cancers [35]. Thus, bisphosphonates can manage hypercalcemia. Similarly, calcimimetics drugs could be explored for their ability to lower serum calcium levels by increasing the sensitivity of the calcium-sensing receptor on parathyroid cells.

The downregulation of the humoral immune response suggests using immunomodulatory drugs like Lenalidomide and Pomalidomide. These drugs enhance the immune system's response against MM cells by modulating the activity of immune cells and inhibiting the proliferation of MM cells [36,37]. PARP Inhibitors for targeting DNA repair pathways, PARP inhibitors like Olaparib and Niraparib, which are effective in cancers with defective DNA repair mechanisms, could be investigated in MM, especially in patients showing significant down-regulation in DNA repair pathways [38,39].

It is important to note that while these compounds have shown potential benefits in various studies, their clinical effectiveness in MM patients, optimal dosages, and possible side effects must be thoroughly evaluated in clinical trials.

Finally, employing proteostasis enhancers that positively modulate the proteostasis network, particularly by enhancing the degradation of misfolded proteins via the ubiquitin-proteasome system (UPS) or the autophagy-lysosome pathway, may prove advantageous. This approach

aims to clear accumulated protein aggregates and restore cellular function. To the best of our knowledge, this strategy has not been tried yet in MM.

## 4. Conclusions

The dithiothreitol-based serum protein equalisation process revealed a pattern of protein depletion that differs between healthy and MM samples, with a more significant variability observed in MM samples. This suggests differences in the proteomic profiles between the two groups, which could be crucial for understanding MM's molecular dynamics. Our findings also contribute to a growing body of evidence supporting the use of serum proteomic analysis and the dithiothreitol-based serum protein equalisation method as valuable tools for monitoring disease progression and for identifying patients at increased risk of developing amyloidosis, potentially leading to more timely and targeted interventions. Notably, the conclusions retrieved from the serum's pellet and supernatant proteomics analysis are complementary.

This study confirms that MM patients exhibit significantly higher levels of total serum proteins than healthy individuals, a hallmark of MM due to the overproduction of monoclonal proteins or immunoglobulins by malignant plasma cells. This difference is evident in the serum's supernatant and pellet fractions after protein equalisation, highlighting the phenotypical diversity among MM patients.

Our study has notably identified an up-regulation in the amyloid fibre formation pathway alongside a down-regulation of mechanisms responsible for managing protein aggregates. This underscores the potential for the early detection of amyloidosis, which addresses a fundamental aspect of disease progression in MM. It also points to the use of proteostasis enhancers and other various drug classes for MM treatment. Based on our data we propose that using proteostasis to promote the degradation of misfolded proteins would represent a potentially effective strategy. This approach seeks to eliminate protein aggregates and restore normal cellular functionality. In conjunction with the proteostasis enhancers, our findings highlight the therapeutic promise of various drug classes for MM treatment. These include MEK inhibitors, complement system inhibitors, bisphosphonates, calcimimetics, immunomodulatory drugs, and PARP inhibitors, which emerged as promising candidates to restore balance to the identified dysregulated pathways.

## CRediT authorship contribution statement

**Ines F. Domingos:** Writing – review & editing, Writing – original draft, Visualization, Methodology, Investigation, Formal analysis, Data curation. **Luis B. Carvalho:** Writing – review & editing, Visualization, Methodology, Investigation, Formal analysis, Data curation. **Carlos Lodeiro:** Writing – review & editing, Visualization, Resources, Project administration, Methodology, Funding acquisition. **Rita Gerivaz:** Writing – review & editing, Conceptualization. **Gali Prag:** Writing – review & editing. **Emanuele Micaglio:** Writing – review & editing. **Eli Muchtar:** Writing – review & editing. **Hugo M. Santos:** Writing – review & editing, Writing – original draft, Visualization, Supervision, Resources, Project administration, Methodology, Investigation, Funding acquisition, Formal analysis, Data curation, Conceptualization. **Jose L. Capelo:** Writing – review & editing, Writing – original draft, Visualization, Supervision, Resources, Project administration, Methodology, Investigation, Funding acquisition, Conceptualization.

## Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

## Data availability

Data will be made available on request.

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## Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.talanta.2024.126589.

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