ORIGINAL ARTICLE

Comparative proteomic profiling of sera from patients with refractory multiple myeloma for predicting response to bortezomib-based therapy

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KEY WORDS

biomarkers, bortezomib, multiple myeloma, proteomics, response

ABSTRACT

INTRODUCTION In the era of implementing novel agents in multiple myeloma (MM) regimens, drug resistance has become a key factor undermining the results of treatment. Identifying biomarkers allows the prediction of therapy outcomes with specific agents and may lead to the avoidance of resistance.

OBJECTIVES This study aimed to identify biomarkers in the pretreatment sera of patients with refractory/relapsed MM that differ from those in the sera of patients who achieved a better depth of response with bortezomib-containing therapy.

PATIENTS AND METHODS Pretreatment serum samples were obtained from 61 proteasome inhibitor-naive, transplant-eligible patients who were eligible for salvage PAD (bortezomib, doxorubicin, and dexamethasone) or VTD (bortezomib, thalidomide, and dexamethasone) chemotherapy. Based on their response to therapy, patients were classified into 3 groups: complete or very good partial response, partial response, and progressive or stable disease. A comparative proteomic analysis of the groups was performed.

RESULTS The analyzed groups significantly differed in terms of both overall survival and progression-free survival. In total, 632 proteins were identified. The proteomic signature revealed 54 proteins that differentiated each analyzed experimental group. Functional analysis revealed that the main identified pathways (17 proteins) involved the regulation of hydrolase activity and cellular response to stimuli. The identified proteins included apolipoprotein C1, complement components, and sulfhydryl oxidase 1.

CONCLUSIONS Our results demonstrated that the label-free proteomic analysis is a useful method for describing proteins differentially expressed in the sera of patients with MM. Further studies are needed to analyze the use of identified proteins as biomarkers.

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INTRODUCTION Rapid progress following the introduction of new drugs in multiple myeloma (MM) treatment has completely changed the landscape of this neoplasm. These advances have led to a significant improvement in the outcomes of MM therapy by doubling the estimated time of overall survival (OS) compared with that in the 1990s. However, the disease remains incurable, mostly due to the inevitable progression of cell clones with acquired resistance to the implemented treatment.[1,2] With so many therapeutic options available, the question of choosing appropriate agents for individual patients has become more important than ever.

Numerous studies have proved the importance of achieving deep responses (complete response [CR] or very good partial response [VGPR]) to the initial treatment, underlining the

importance of targeting the disease in the initial phase, which is considered to be the most sensitive.[3-5] Current approaches to choosing treatment regimens, described in guidelines such as Stratification of Myeloma and Risk-adapted Therapy, focus on de-escalation of therapy in newly diagnosed patients with a favorable cytogenetic profile of MM rather than on choosing specific drugs adjusted to an individual patient's profile.[6] Efforts are still being made to make therapy more individualized. With this goal in mind, different techniques are used to identify biomarkers that allow for the prediction of patient response to treatment.[7]

In this study, a comparative proteomic profiling of pretreatment sera was performed. The analysis of the global expression of proteins makes proteomics a very useful technique for the search of yet undiscovered biomarkers. Our group has recently proved that proteomics can be successfully used to describe proteins that differentiate the bone marrow plasma cells of patients who have a deep response (ie, CR or VGPR) to bortezomib-based therapy from those of patients who are refractory to the treatment.[8] This led to the description of pathways potentially responsible for resistance. However, obtaining plasma cells from bone marrow biopsy makes this approach too inefficient to identify biomarkers that can be used in clinical practice. Performing proteomic analysis on serum may help overcome this obstacle, because the concentration of the defined biomarkers can easily be assessed in a specimen obtained from such a noninvasive procedure as blood sample collection.[9]

The goal of this study was to establish a proteomic profile that can predict the achievement of at least VGPR to bortezomib-based chemotherapy administered in the setting of refractory MM, therefore identifying specific proteins that can be further analyzed as potential biomarkers of response.

PATIENTS AND METHODS

Patients and samples

Serum samples were obtained from 61 proteasome inhibitor-naive, transplant-eligible patients before the beginning of salvage PAD (bortezomib, adriamycin, and dexamethasone—40 patients) or VTD (bortezomib, thalidomide, and dexamethasone—21 patients) chemotherapy due to resistance to the first-line induction regimen, CTD (cyclophosphamide, thalidomide, and dexamethasone). After therapy completion, each patient's response to the treatment was evaluated according to the International Myeloma Working Group criteria.[10] The study protocol conformed to the Ethical Guidelines of the World Medical Association Declaration of Helsinki and was approved by the Bioethical Commission of the Poznan University of Medical Sciences, Poznań, Poland. Progression-free survival (PFS) and OS were analyzed using the PRISM software version 5.0 (GraphPad Software, La Jolla, California, United States).

Nano-liquid chromatography–tandem mass spectrometry

Serum proteins for label-free analysis were prepared as described previously.[11] For each run, 1.5 μg of the digested serum protein samples were subjected to nano-liquid chromatography–tandem mass spectrometry (nano LC-MS/MS) analysis using a Dionex UltiMate 3000 RSLC nano System and a Q-Exactive Orbitrap mass spectrometer (Thermo Fisher Scientific, Waltham, Massachusetts, United States). All samples were prepared separately for digestion in triplicate, and every prepared sample was then injected randomly into the liquid chromatography system in duplicate. Every 73 hours, a system calibration was performed.

Quantitative analysis of proteomic data

The data obtained were quantitatively analyzed with MaxQuant[12,13] version 1.5.1.2 using the UniProt Complete Proteome Set of Humans (123,619 sequences) with the following parameters: a tolerance level of 10 ppm for MS and 0.05 Da for MS/MS. Trypsin was used as the digesting enzyme and 2 missed cleavages were allowed. The carbamidomethylation of cysteines was set as a fixed modification, and the oxidation of methionines was allowed as a variable modification. Proteins were identified using a minimum false discovery rate of 1%. The quantitative analysis of the serum samples was based on the label-free quantification (LFQ) intensities. The data were evaluated and the statistics were calculated using Perseus (version 1.4.1.3, Max Planck Institute of Biochemistry, Martinsried, Germany) and Statistica v. 12.0 (StatSoft, Inc., Kraków, Poland) software.

Comparative analyses were performed between 3 different experimental groups: patients who achieved a CR or VGPR (CR/VGPR), those who achieved partial response (PR), and those who achieved progressive or stable disease (PD/SD). The mean LFQ intensities with standard deviations were calculated for all groups. The fold changes in the level of the proteins were assessed by comparing the mean LFQ intensities among all groups.

For multiple comparisons, the one-way analysis of variance (ANOVA) with the Bonferroni correction for multiple testing was performed. For comparisons between 2 groups, *t* tests were used. A protein was considered to be differentially expressed if the difference was statistically significant ($P < 0.05$), the fold change was a minimum of ±1.5, and it was identified with a minimum of 2 peptides with confidence exceeding 99%. Regression and correlation analyses were also performed for the obtained results. The reproducibility of technical and biological replicates was assessed by scatter plotting and correlation coefficient determination based on LFQ signals. Correlations between variables were assessed using the Pearson (Perseus) and Spearman (Statistica) coefficients. Multivariate analyses were carried out by an untargeted principal component analysis (PCA). The obtained data were analyzed using

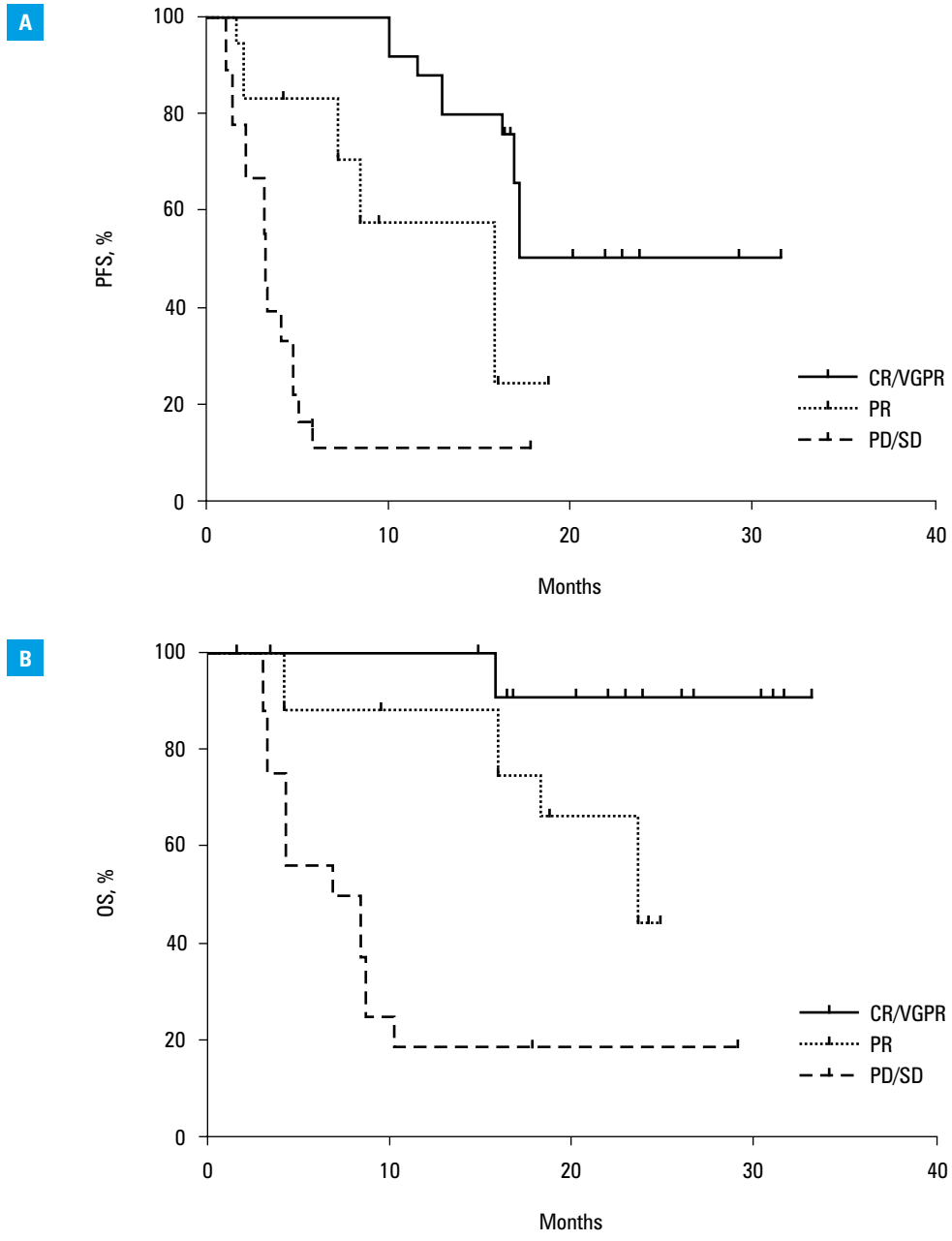

FIGURE 1
A – Kaplan–Meier estimates of progression-free survival (PFS); **B** – overall survival (OS) according to response to therapy (complete or very good partial response [CR/VGPR] vs partial response [PR] vs progressive or stable disease [PD/SD]) (n = 61, log-rank for PFS, 25.55, $P < 0.0001$; log-rank for OS, 29.33, $P < 0.0001$)

annotation tools and the Fisher exact test to find enriched Gene Ontology (GO) annotations in the 54 differentially expressed proteins. The differential proteins were classified based on their respective molecular function, biological processes, and physiological pathways. Annotation analyses were performed using Perseus and Protein ANalysis THrough Evolutionary Relationships (PANTHER) tools (available online: http://pantherdb.org/)[14] for identifying enriched functions and physiological pathways.

Enzyme-linked immunosorbent assay validation An enzyme-linked immunosorbent assay (ELISA) was used to validate the differential expression of C-reactive protein (CRP) in serum samples from patients treated with the PAD regimen. The serum protein level was measured using a commercially available sandwich ELISA kit (Cusabio, College Park, Maryland, United States) according to the manufacturer's instructions.

RESULTS Patients were classified into 3 groups based on their response to a median of 6 cycles of PAD (range, 1–6) or VTD (range, 1–6): 24 patients who achieved CR/VGPR, 19 patients who achieved PR, and 18 patients who achieved PD/SD. There was no difference in the distribution of the stage of the disease according to the Salmon–Durie staging system or the International Staging System in all 3 groups (data not shown). Patients in the CR/VGPR group had significantly prolonged PFS and OS when compared with patients from the PR group (median PFS for CR/VGPR not reached [NR] vs 15.8 months for PR [hazard ratio [HR], 0.17; 95% CI, 0.04–0.66], median OS for CR/VGPR NR vs 23.7 months for the PR group [HR, 0.17; 95% CI, 0.06–0.5]) and from the PD/SD group (median PFS for PD/SD 3.2 months [HR, 0.08; 95% CI, 0.03–0.2], median OS for the PD/SD group 7.6 months [HR, 0.04; 95% CI, 0.01–0.13]). There was also a significant difference between PR and PD/SD patients in terms of

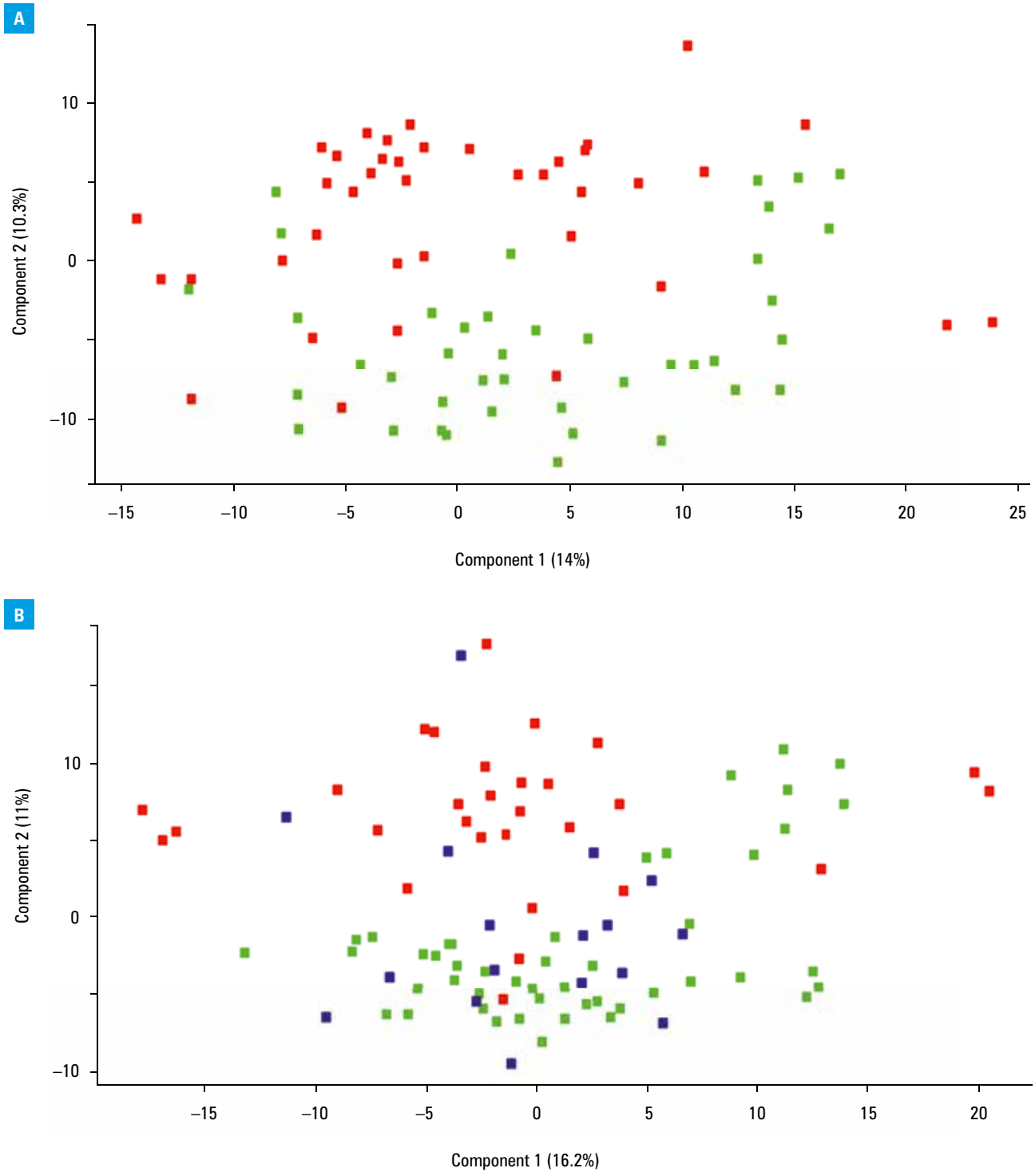

FIGURE 2 **A** – principal component analysis (PCA) of the label-free quantification (LFQ) intensities obtained from the serum of complete or very good partial response (CR/VGPR) (green) and progressive or stable disease (PD/SD) (red) patients; **B** – PCA of the LFQ intensities for CR/VGPR (green), partial response (blue) and PD/SD (red) patients. Calculations were performed with Perseus.

both PFS (HR, 0.28; 95% CI, 0.12–0.66) and OS (HR, 0.19; 95% CI, 0.07–0.5) (FIGURE 1).

The sera were collected from patients without any fractionation and digested in solution with trypsin and analyzed by nano-LC-MS/MS in one batch. The correlation analysis based on the LFQ intensities between technical replications revealed Pearson coefficients between 0.98 and 0.99. The reproducibility of the biological replicates revealed Pearson coefficients between 0.82 and 0.90. The lowest reproducibility was shown for serum proteins obtained from patients in the PR group.

A total of 632 proteins were identified with a false discovery rate of 1%. The PCA clearly differentiated the CR/VGPR and PD/SD groups (FIGURE 2A). When the PR group was also analyzed, separation of each experimental group was not evident (FIGURE 2B).

The proteomic signature revealed 54 proteins that differentiated all analyzed experimental

TABLE 1 A list of selected, differentially expressed proteins associated with response to PAD or VTD therapy

Protein names	ANOVA *P* value	Fold change CR/VGPR vs PD/SD	Fold change CR/VGPR vs PR	Fold change PR vs PD/SD
Thyroxine-binding globulin	3.81E-04	3.56	2.50	1.42
Kallistatin	8.02E-03	3.35	2.54	1.32
Platelet factor 4	1.30E-03	3.19	1.37	2.33
Corticosteroid-binding globulin	4.19E-03	3.12	2.29	1.36
Adiponectin	2.19E-02	2.94	1.13	2.60
Complement component C7	3.80E-02	2.37	2.47	0.96
Complement C4-B	2.90E-02	2.36	2.56	0.92
Phosphatidylinositol-glycan-specific phospholipase D	1.08E-03	2.29	1.66	1.38
C-X-C motif chemokine	2.17E-02	2.28	1.37	1.66
Attractin	3.67E-02	2.08	1.53	1.35
Apolipoprotein C1	3.67E-03	2.01	1.35	1.48
Coagulation factor X	1.64E-02	1.94	1.67	1.16
Heparin cofactor 2	2.99E-02	1.82	1.96	0.93
Gelsolin	1.02E-02	1.77	1.32	1.33
CD5 antigen-like	4.21E-02	1.71	0.60	2.84
Insulin-like growth factor-binding protein	2.00E-02	1.70	1.28	1.33
C4b-binding protein alpha chain	3.16E-03	1.64	2.14	0.77
Phosphatidylcholine-sterol acyltransferase	1.80E-02	1.62	1.52	1.07
Ficolin 3	3.37E-02	1.57	1.33	1.18
Alpha-2-macroglobulin	2.89E-02	1.57	1.19	1.31
Peptidase inhibitor 16	1.55E-02	1.52	1.06	1.44
Fetuin B	2.19E-02	1.51	1.08	1.40
Alpha-1B-glycoprotein	2.39E-05	0.61	0.69	0.88
Inter-alpha-trypsin inhibitor heavy chain H3	7.11E-03	0.57	0.80	0.70
HLA class I histocompatibility antigen, alpha	2.38E-03	0.56	0.87	0.64
Inter-alpha-trypsin inhibitor heavy chain H4	1.88E-04	0.55	0.77	0.72
Complement factor H-related protein 5	7.47E-03	0.50	0.61	0.82
Sulfhydryl oxidase 1; sulfhydryl oxidase	2.68E-04	0.46	0.68	0.68
Complement component C9	2.00E-03	0.46	0.57	0.81
PDZ and LIM domain protein 1	2.46E-03	0.42	0.48	0.87
Thymosin beta-4	2.97E-02	0.42	0.90	0.47
C-reactive protein	2.03E-02	0.42	1.02	0.41
Serum amyloid A-2 protein	1.29E-02	0.36	0.12	3.08
Serum amyloid A-1 protein	1.48E-02	0.17	0.10	1.72
Vitamin D-binding protein	5.48E-03	0.13	0.46	0.27

A fold change of ± 1.5 and a *P* value of less than 0.05 indicate significant changes.

Abbreviations: CR/VGPR, complete or very good partial response; PAD, bortezomib, doxorubicin, and dexamethasone regimen; PR, partial response; PD/SD, progressive or stable disease; VTD, bortezomib, thalidomide, and dexamethasone

groups (ANOVA significant). The most differential proteins revealed a difference between the CR/VGPR (optimal responders) and the PD/SD groups (51 proteins), confirming the result obtained by the PCA. A total of 16 upregulated and 35 downregulated proteins were identified in the sera of patients derived from the PD/SD group compared with the CR/VGPR group. Twenty-six proteins differentiated the CR/VGPR and the PR groups; among these, 14 proteins revealed increased accumulation and 12 revealed a decreased abundance in the PR group compared with the CR/VGPR group. A comparison between the PR and the PD/SD groups showed 19 differential proteins, 5 of which were increased and 14 decreased in the PD/SD group when compared with the PR group. The abundance of 16 differential proteins linearly decreased from the CR/VGPR group across the PR group to the PD/SD group. On the other hand, the accumulation of 11 proteins linearly increased from the CR/VGPR group, across the PR group to the PD/SD group. Some of these differential proteins are presented in TABLE 1 and FIGURE 3. A complete list of differentially expressed proteins, with detailed information, is presented in Supplementary material online.

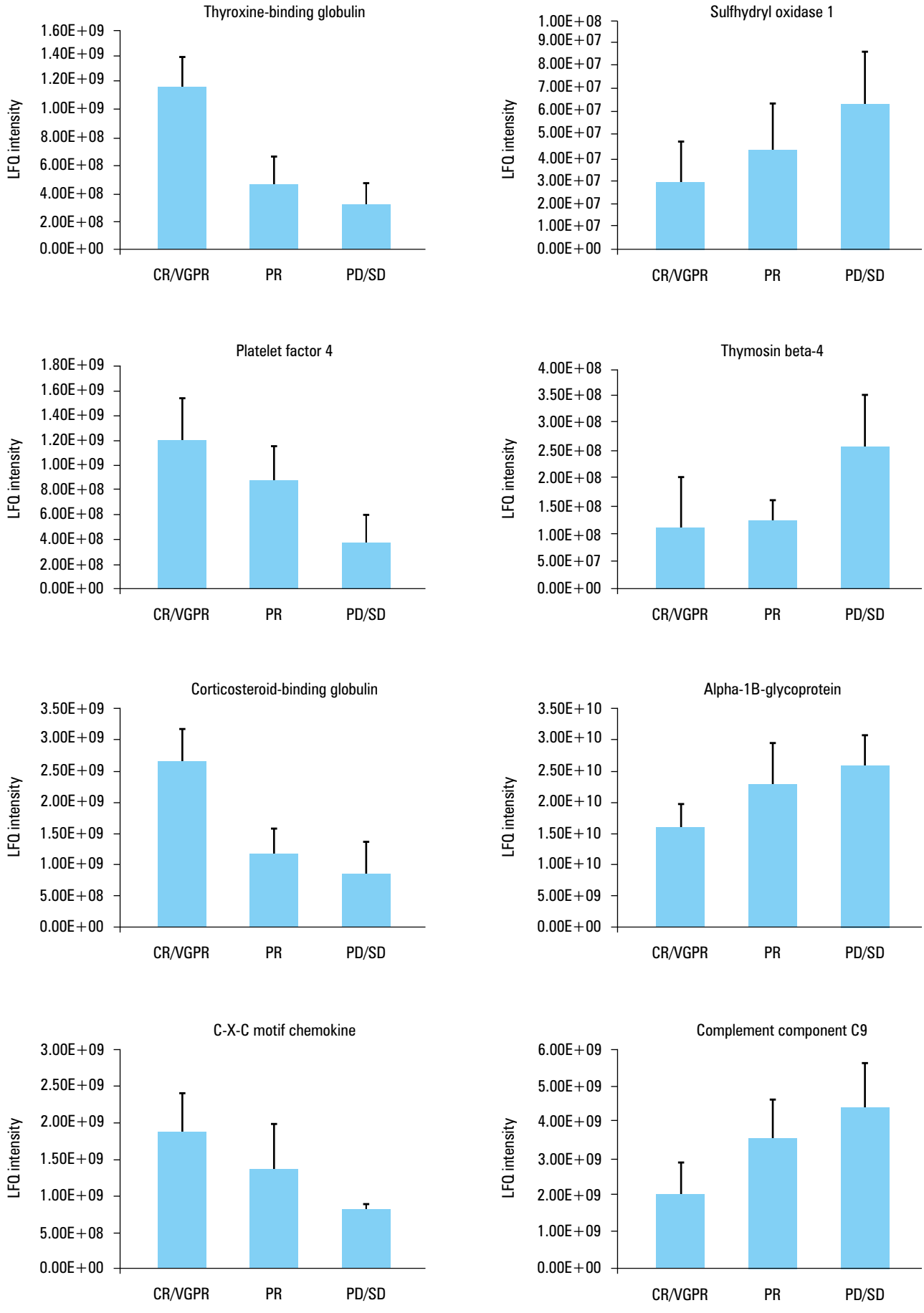

FIGURE 3 Relative abundance based on label-free quantification (LFQ) intensities of selected differentially expressed proteins revealed linear up- or downregulation in complete or very good partial response (CR/VGPR), partial response (PR), and progressive or stable disease (PD/SD) groups. The charts show the mean and SD for all analyzed groups.

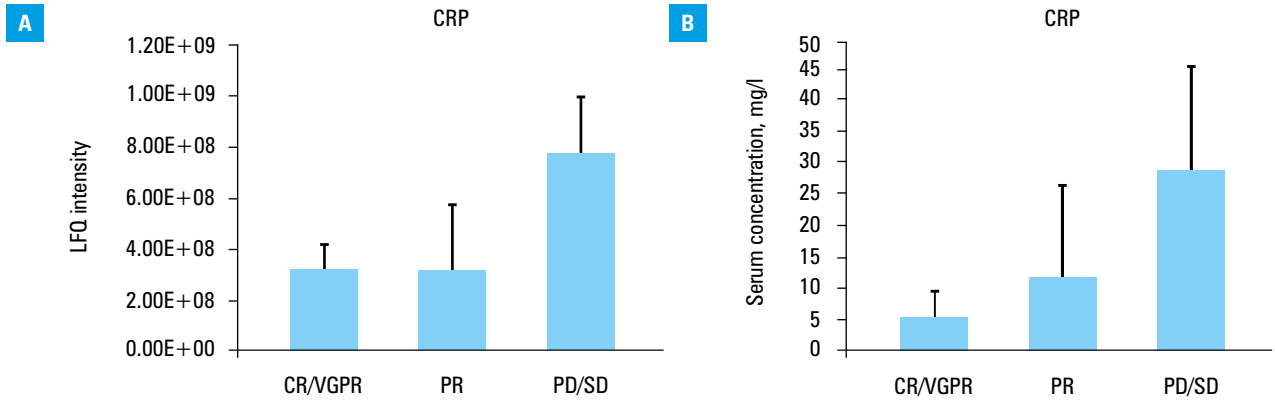

FIGURE 4 **A** – relative abundance of the C-reactive protein (CRP) in complete or very good partial response (CR/VGPR), partial response (PR), and progressive or stable disease (PD/SD) based on label-free quantification (LFQ) intensities; **B** – concentration of the CRP in the serum based on the immunoenzymatic assay. Charts show the mean and SD for all analyzed groups.

The functional analysis revealed that the most enriched category in terms of biological processes or molecular function was the regulation of cellular processes (17 proteins), particularly the regulation of hydrolase activity (Benjamini-corrected P = 1.03e-8) and cellular response to stimulus (Benjamini-corrected P = 1.97e-6). The abundance of all 8 proteins involved in the regulation of hydrolase activity linearly increased with the depth of response (the better response the higher abundance) (thyroxine-binding globulin, kallistatin, corticosteroid-binding globulin, phosphatidylinositol-glycan-specific phospholipase D, apolipoprotein C1 [ApoC1], heparin cofactor 2, alpha-2-macroglobulin, and fetuin B). The most highly correlated differential protein was thyroxine-binding globulin. The accumulation of this protein was 3.56-fold higher in the serum of the CR/VGPR group compared with the PD/SD group and 2.5-fold higher in the CR/VGPR group when compared with the PR group. Moreover, it increased proportionally to the depth of response to treatment. Among 23 proteins related to response to stimuli, some proteins were upregulated and some were downregulated. The accumulation levels of HLA class I histocompatibility antigen, inter-alpha-trypsin inhibitor heavy chain H4, complement factor H-related protein 5, sulfhydryl oxidase 1 (QSOX1), complement component C9, PDZ and LIM domain protein 1, thymosin beta-4, and CRP were upregulated in treatment nonresponders compared with the responders. Differential accumulation of CRP was also confirmed by the ELISA (FIGURE 4AB). On the other hand, the relative amount of adiponectin, gelsolin, C-X-C motif chemokine, attractin, and insulin-like growth factor-binding protein, as well as 3 proteins of the complement system component C7, C4B, and C4b-binding protein alpha, were downregulated in patients with worse response to treatment. The abundance of some differential proteins, which revealed linear upregulation or downregulation, is presented in FIGURE 3.

The PANTHER analysis revealed similar results: 42% of differentially expressed proteins were involved in immune system processes and response to stimulus and 29% of ANOVA-significant proteins were related to catalytic activity, mainly hydrolase activity. Additionally, in terms of physiological pathways, proteins involved in inflammation mediated by chemokines and cytokines were overrepresented. In comparison to Perseus, the PANTHER analysis revealed additional GO subclasses of overrepresented proteins in the categories of cellular process proteins, cell communication, and recognition proteins. This subclass includes, among others, platelet factor 4, ficolin 3, CD5 antigen-like, C-X-C motif chemokine, and attractin. These chemokines and signaling molecules are also involved in the response to stimulus processes.

DISCUSSION The achievement of deep disease reduction (VGPR and CR, especially with negative minimal residual disease) is correlated with prolonged PFS and OS to the first-line treatment.[5] A similar observation was also recently reported for patients who were on MM treatment due to refractory/relapsed disease.[15] Our analysis of PFS and OS in patients treated with a bortezomib-based regimen due to resistant disease (FIGURE 1) confirms this finding. Thus, the establishment of biomarkers predicting optimal response to treatment is crucial in the further development of an individualized approach. Such personalized treatment is becoming more important owing to the significant number of new drugs introduced for the treatment of MM. Proteomics is the perfect tool for identifying yet undiscovered biomarkers whose clinical significance can be later assessed by cheaper and more accessible laboratory techniques targeting specific, predefined proteins (eg, ELISA). We have recently shown the usefulness of the proteomic approach using bone marrow plasma cells.[8,16] However, due to the cost and inconvenience for patients, bone marrow is not an ideal source of biomarkers in daily practice.

To our knowledge, our study is the first analysis of pretreatment sera by modern proteomics in order to establish biomarkers that predict optimal

response to bortezomib-based chemotherapy in patients with refractory MM. Our data are of high quality, confirmed by a strong correlation between technical and biological replicates. Additionally, the scattering of variables in the PCA (FIGURE 2) confirms the clear separation between the analyzed groups. Validation studies conducted using ELISA to confirm the results of proteomic analysis in relation to CRP showed agreement between both methods (FIGURE 4). The role of elevated CRP production in MM, which reflects the activity of interleukin 6, as a prognostic factor is well known.[17] The appearance of CRP in our prognostic signature confirms the good experimental design and reaffirms the quality of our data.

The proteomic signature, differentiating 3 groups of different response levels, consists of 54 proteins. The majority of these proteins (51) differentiate the CR/VGPR group from the PD/SD group and underscore the potential role played by these proteins in the mechanism of resistance. The abundance of 27 proteins was linearly correlated with the depth of response: 11 of these 27 proteins increased while 16 decreased across the groups, starting with the CR/VGPR group through the PR group to the PD/SD group. These proteins deserve the highest attention due to their possible role in the biology of resistance to bortezomib. ApoC1 is among these proteins and has been positively correlated with the depth of response. This protein plays a central role in high-density lipoprotein and very low-density lipoprotein metabolism. Cholesterol metabolism has already been reported to be involved in apoptosis resistance in MM, but to date, no clear data on its impact on clinical strategy has been reported.[18] Interestingly, ApoC1 was described as a prognostic factor in breast cancer.[19] The mechanism of ApoC1 in terms of resistance to bortezomib is unknown; however, it has already been reported that this protein might be involved in the resistance of MM to VDD (bortezomib, liposomal doxorubicin, and dexamethasone) frontline treatment.[20,21] Another intriguing group of proteins identified from the proteomic signature is the complement components. Interestingly, some of these proteins were found to be linearly downregulated (C7 and C4) while some were upregulated (C9). It has already been described that component activity is affected in MM, and these proteins might be involved in more advanced bone destruction.[22,23] However, no clear relationship between complement activity and clinical outcomes has been shown.

Based on functional analysis, proteins were grouped according to the cellular processes that they are responsible for: regulation of hydrolase activity and cellular response to stimuli. These pathways were found in our previous studies performed on pretreatment plasma cells.[8,16] One of the most interesting proteins involved in this pathway is QSOX1, which was found to be upregulated in the PD/SD group. There was a linear trend in the abundance of QSOX1 in all analyzed groups (FIGURE 3). QSOX1 consists of 2 components: an N-terminal region that has a tandem pair of thioredoxin domains related to protein disulfide isomerase, and a C-terminal sulfhydryl oxidase-like retroviral element.[24] Notably, thioredoxin was found in our previous studies[8,16] to have a potential role in resistance to bortezomib-based chemotherapy. The extracellular location of QSOX1 suggests that it may be involved in remodeling of the extracellular matrix, particularly because QSOX1 can catalyze the formation of disulfide bridges, which are needed for the appropriate folding and stability of various matrix proteins.[25] QSOX1 generates hydrogen peroxide as a by-product of oxidation, and its cellular activity as a prooxidant results in the increased accumulation of reactive oxygen species, which are relevant to neoplastic processes.[26] QSOX1 has been reported to be overexpressed in diverse tumor types, such as neuroblastomas,[25] medulloblastomas,[27] pancreatic,[28] renal,[29] breast,[24] and prostate[26] cancer. Although there are a few publications related to QSOX1, none of them have so far been associated with MM. The ability to detect QSOX1 protein in body fluids (eg, plasma) makes it a promising target for study as a biomarker of resistance in MM.

In conclusions, the results demonstrate that label-free proteomic analysis is a useful method of identifying proteins differentially expressed in the sera of patients with MM. Among the 54 proteins differentiating patients from the 3 different groups of responses to PAD chemotherapy, the most interesting are ApoC1, complement components, and QSOX1. The latter consists of thioredoxin, confirming our previous finding of the role of this group of proteins involved in reactive oxygen species homeostasis in bortezomib-therapy resistance. Further studies are needed to analyze and validate the role of the proteins from our proteomic signature.

Contribution statement MŁ and DD conceived the idea for the study. MŁ, DD, and AJ contributed to the design of the research. MŁ, TK, ZR, TS, APC, BR, JC-R, AN, MJ, AJ, MK, and DD were involved in data collection. MŁ, DD, and TK analyzed the data. All authors edited and approved the final version of the manuscript.

Supplementary material online Supplementary material is available with the online version of the article at www.pamw.pl.

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