Novel panel of protein biomarkers to predict response to bortezomib-containing induction regimens in multiple myeloma patients

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ARTICLE INFO

ABSTRACT

Background: Multiple myeloma (MM) is a complex heterogeneous disease. Various risk stratification models have been recommended including cytogenetic and FISH analysis to identify high-risk patients who may benefit from novel treatments, but such facilities are not widely available. The International Scoring System (ISS) using beta-2-microglobulin and albumin remains a widely used prognostic scoring system in many clinical practices; however it is not useful in predicting response to treatment in MM. The aim of this study is to identify clinically useful biomarkers to predict response to treatment containing bortezomib.

Methods: 17 MM patient serum samples (9 responders/8 non-responders) were used for the discovery phase (label-free mass spectrometry) and an additional 20 MM patient serum samples were used for the ELISA-based validation phase (14 responders/6 non-responders).

Results: CLU and ANG mean levels were higher in the responders group, while Complement C1q had lower concentrations. The combination of all standard biomarkers (albumin, beta-2-microglobulin (ß2M), paraprotein and kappa/lambda (K/L) ratio) had an AUC value of 0.71 with 65% correct classification, while an overall combination of new candidate protein biomarkers with standard biomarkers had an AUC value of 0.89 with 85.3% correct classification.

Conclusions: A combination of new and standard biomarkers consisting of CLU, ANG, C1Q, albumin, ß2M, paraprotein and K/L ratio may have potential as a novel panel of biomarkers to predict MM response to treatment containing bortezomib.

General significance: Use of this biomarker panel could facilitate a more personalized therapy approach and to minimize unnecessary side effects from ineffective drugs.

1. Introduction

Multiple Myeloma (MM) is a plasma cell disorder characterized by bone marrow infiltration with clonal plasma cells, which secrete monoclonal immunoglobulin detectable in serum and/or urine. The development of novel targeted therapies has markedly improved the response rate and survival outcome, but MM remains incurable [1]. Bortezomib, which was the first proteasome inhibitor anticancer drug, is one of the many novel agents being used. It has numerous functionalities such as inducing apoptosis and growth arrest in the myeloma cell cycle, altering the bone marrow microenvironment, inhibiting nuclear factor kappa B and reversing chemoresistance in myeloma cells [2,3]. It also demonstrates an inhibitory effect on angiogenesis and DNA repair but also has no permanent impact on normal hematopoietic stem cells.

Following the APEX and SUMMIT trials, bortezomib monotherapy may overcome relapsed and refractory MM patients with poorer prognosis [4,5]. Bortezomib is now established as the backbone agent for combined induction therapy regimens [6–8].

Diagnostic criteria and frontline standards guidelines have been shown to also play an important role in deciding the outcome of MM disease and individualizing treatment [9,10]. Unfortunately, only a minority of centers has such extensive access to molecular and genetic sequencing studies and imaging facilities that will provide a better stratification of patients according to their disease burden [11].

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While novel agents have improved treatment outcomes, identification of biomarkers that will facilitate clinicians in determining which treatment is optimum for high-risk patients following initial diagnosis is a crucial area in the clinical management of MM. Quantitative measurement of plasma/serum and urinary protein concentrations plays a significant role in the monitoring of patients with MM. In 2005, an international consortium of researchers used serum β2M and serum albumin to create the International Staging System (ISS), which enabled clinicians to stage patients and predict their long-term prognosis, a process that has been refined with the addition of fluorescent in situ hybridization (FISH) data [12,13]. The serum free light chain assay also plays an important role in prognosis, with the International Myeloma Working Group identifying several uses for serum free light chain analysis in MM [14–16].

In view of the limited and restricted access to facilities such as advanced imaging and genetic testing, identifying the most specific and reliable prognostic biomarker remains a great challenge. We have focused specifically on identifying a panel of protein biomarkers that could be used to predict response following combined agent use in newly diagnosed MM patients. The ability to combine routine laboratory measurements, for example β2M, with newly discovered candidate biomarkers for predicting response to bortezomib therapy was an important consideration. In this investigation, all patients were naïve to bortezomib therapy at the time of sampling in order to facilitate the identification of predictive biomarker of treatment response. Some MM patients have de novo resistance to bortezomib therapy, with the identification of novel biomarkers of early resistance critical for their clinical management [17].

The availability of predictive biomarkers would be useful in avoiding ineffective treatments, and allow for the administration of alternative regimens which are continuing to be approved for the treatment of MM. Ineffective treatments can also be associated with undesired side effects, such as peripheral neuropathy and thrombocytopenia, which are important to avoid particularly if no benefit is associated with the specific treatment.

2. Material and methods

2.1. Patients' selection and sample collection

Thirty-seven newly diagnosed MM patients from 2011 to 2013 were selected for this study as shown in Table 1. They were naïve to bortezomib therapy. These patients were stratified to either responders or non-responders group according to the International Myeloma Working Group (IMWG) uniform response criteria for MM [16]. Responders were considered Complete Response (CR), Very Good Partial Response (VGPR) and Partial Response (PR) with non-responders considered Progressive Disease (PD) and Stable Disease (SD) as determined using the International Myeloma Working Group Uniform Response Criteria. Seventeen patient samples were used in the discovery phase and all samples were used in the validation phase.

Serum samples were obtained at diagnosis or prior to commencing therapy. The participating subjects gave written informed consent in accordance with the Declaration of Helsinki that was approved by local ethics committees. These samples were collected according to standard phlebotomy procedures. 10 ml of blood was collected into additive free (serum) blood tubes and was allowed to clot for 30 min to 1 h at room temperature. The serum was denuded by pipette from the clot and aliquoted into a clean tube. The tubes were centrifuged at $400 \times g$ for 30 min at 4 °C. Serum was aliquoted in cryovial tubes, labeled and stored at -80 °C until time of analysis. The time from sample procurement to storage at -80 °C was < 3 h.

2.2. Serum protein sample fractionation and enzymatic digestion

The Proteome Purity™ 12 Human Serum Protein Immunodepletion Resin from R & D Systems, United Kingdom was selected to remove high abundance protein. It depletes the 12 most abundant proteins (alpha 1- Acid Glycoprotein, alpha 1-Antitrypsin, alpha 2-Macroglobulin, Albumin, Apolipoprotein A-I, Apolipoprotein A-II, Fibrinogen, Haptoglobin, IgA, IgG, IgM, Transferrin) from the serum as described by the manufacturer's protocol [18].

100 μL of the Blue Nanotrap particles (RB4VSA) from CeresNano was added to each prepared immunodepleted sample that contained Tris-HCl buffer. These particles were resuspended into the prepared samples and allowed to incubate for 30 min at room temperature. The suspension was centrifuged at $16,800 \times g$ (Hettich Mikro 200R, United Kingdom) for 10 min at room temperature and the supernatant was carefully removed from each sample and transferred to individually labeled clean microcentrifuge tubes. The pelleted particles were resuspended in 500 μL of LC-MS grade water. Once more, the particles were centrifuged at $16,800 \times g$ for 10 min at room temperature. The supernatant was removed and discarded. A total of 3 washes with the Ultra High Purity Water were performed leaving a pellet particle to be resuspended in an eluent.

100 μL of freshly made protein elution buffer (70% acetonitrile, ACN from Sigma-Aldrich/10% ammonium hydroxide from Sigma-Aldrich) was added to the particles before vortexing and then sonicating using the water-bath method (Precision ultrasonic cleaning, DP201–00, Ultrawave, UK) for 2 min. These samples were centrifuged (Hettich Mikro 200R, United Kingdom) at $16,800 \times g$ for 7 min at room temperature and the eluted proteins obtained were carefully removed and transferred to a clean microcentrifuge tube. The protein elution step was repeated for a total of 200 μL. The combined eluent, collected in the microcentrifuge tubes, was vacuum-dried at room temperature. Samples were then digested with trypsin overnight according to standard procedures [19].

2.3. Nano HPLC and mass spectrometry analysis

The nano LC-MS/MS analysis of responders versus non-responders patient samples was carried out using an Ultimate 3000 nanoLC system (Dionex) coupled to an LTQ XL Orbitrap mass spectrometrer (Thermo Fisher Scientific, Dublin, Ireland) in the Proteomics Facility of the National Institute for Cellular Biotechnology, Dublin City University. Peptide mixtures (5 μl volume) were loaded onto a C18 trap column (C18 PepMap, 300 μm id × 5 mm, 5 μm particle size, 100 Å pore size; Dionex). Desalting was achieved at a flow rate of 25 μl/min in 0.1% TFA for 3 min. The trap column was switched on-line with an analytical PepMap C18 column (75 μm id × 500 mm, 3 μm particle and 100 Å pore size; Dionex). Peptides generated from the digestion were eluted with the following binary gradients: solvent A (2% ACN and 0.1% formic acid in LC-MS grade water) and 0–25% solvent B (80% ACN and 0.08% formic acid in LC-MS grade water) for 160 min and 25–50% solvent B for a further 20 min. The column flow rate was set to 350 nl/min. Data was acquired with Xcalibur software, version 2.0.7 (Thermo Fisher Scientific). The MS apparatus was operated in data-dependent mode. Survey MS scans were acquired in the Orbitrap in the 400–1200 m/z range with the resolution set to a value of 30,000 at m/z 400 and lock mass set to 445.120025 m/z. CID fragmentation was carried out in the linear ion trap with the three most intense ions per scan. Within 40 s, a dynamic exclusion window was applied. Normalized collision energy of 35%, an isolation window of 2 m/z and one microscan were used to collect suitable tandem mass spectra.

2.4. Quantitative protein profiling by label-free LC-MS/MS analysis

Processing of the raw data generated from LC-MS/MS analysis was carried out with Progenesis QI for Proteomics label-free LC-MS software (version 3.1; Non-Linear Dynamics, Newcastle upon Tyne, UK). Data alignment was based on the LC retention time of each sample, allowing for any drift in retention time given and adjusted retention time for all runs in the analysis. A reference run was established with the sample run that yielded most features (i.e. peptide ions). The retention times of

Table 1
Patient clinical details.
  Patient details used in the study with 23 responders and 14 non-responders.

Code	Gender	Age	ISS	Immunofixation	Predicted response	Bortezomib	IMIDs	Overall survival (mo)
1	Female	65	3	IgG Lambda	Responder	Y		17.00
2	Male	54	1	Kappa Light Chain	Responder	Y	Y	27.00
3	Female	61	1	IgG Kappa	Responder	Y		57.00
4	Female	60	2	Kappa Light Chain	Responder	Y	Y	19.00
5	Male	51	1	Lambda Light Chain	Responder	Y	Y	28.00
6	Male	70	2	IgG Kappa	Responder	Y	Y	28.00
7	Male	69	1	IgA Kappa	Responder	Y	Y	50.00
8	Male	56	3	Kappa Light Chain	Responder	Y	Y	17.00
9	Female	75	2	IgA Lambda	Responder	Y		53.00
10	Male	58	2	IgA Kappa	Responder	Y	Y	27.00
11	Female	78	3	Lambda Light Chain	Responder	Y	Y	31.00
12	Female	64	2	IgA Lambda	Responder	Y		31.00
13	Male	76	2	Kappa Light Chain	Responder	Y	Y	62.00
14	Female	64	2	IgA Kappa	Responder	Y		70.00
15	Male	80	3	IgG Kappa	Responder	Y	Y	30.00
16	Male	67	2	IgG Kappa	Responder	Y		48.00
17	Female	54	1	IgG Kappa	Responder	Y		16.00
18	Male	64	2	IgG Kappa	Responder	Y	Y	50.00
19	Male	44	1	IgG Kappa	Responder	Y	Y	52.00
20	Male	64	2	IgG Lambda	Responder	Y	Y	23.00
21	Male	68	2	IgG Lambda	Responder	Y	Y	33.00
22	Male	77	3	IgA Kappa	Responder	Y	Y	9.00
23	Male	78	3	IgA Kappa	Responder	Y	Y	14.00
24	Male	66	3	IgG Kappa	Non-responder	Y	Y	17.00
25	Female	55	1	IgG lambda	Non-responder	Y	Y	3.00
26	Female	75	2	IgG Lambda	Non-responder	Y	Y	9.00
27	Male	83	2	IgG Lambda	Non-responder	Y		7.00
28	Female	58	2	IgA Kappa	Non-responder	Y		81.00
29	Male	76	2	IgG Kappa	Non-responder	Y		3.00
30	Male	65	2	IgA Kappa	Non-responder	Y		4.00
31	Male	87	2	IgA Kappa	Non-responder	Y	Y	47.00
32	Male	76	3	IgA Lambda	Non-responder	Y		14.00
33	Female	83	2	IgA Lambda	Non-responder	Y	Y	35.00
34	Male	67	2	IgG Kappa	Non-responder	Y		17.00
35	Male	67	3	IgG Lambda	Non-responder	Y		31.00
36	Female	45	1	IgG Kappa	Non-responder	Y	Y	20.00
37	Female	58	1	IgG Lambda	Non-responder	Y		78.00

all of the other runs were aligned to this reference run and peak intensities were then normalized. Prior to exporting the MS/MS output files to Proteome Discoverer 1.4 (Thermo Fisher Scientific) for protein identification, a number of criteria were employed to filter the data including: (i) peptide features with ANOVA < 0.05 between experimental groups, (ii) mass peaks (features) with charge states from + 2, + 3, and (iii) greater than one isotope per peptide. The exported MS/MS file from Progenesis label-free LC-MS software was searched using a dual algorithm search with both MASCOT and SEQUEST in Proteome Discoverer 1.4 against the human Uni-SwissProt database (taxonomy: *Homo sapiens)*. The following search parameters were used for protein identification: (i) MS/MS mass tolerance set at 0.6 Da, (ii) peptide mass tolerance set to 20 ppm, (iii) carbamidomethylation set as a fixed modification, (iv) up to two missed cleavages were allowed and (v) methionine oxidation set as a variable modification. The following criteria were applied to assign positively identified proteins: (i) an ANOVA score between experimental groups of ≤ 0.05, (ii) proteins with ≥ 1 peptides matched and (iii) a MASCOT score > 40.

2.5. Enzyme-linked immunosorbent assays (ELISA)

ELISA kits were used to verify the different levels for selected proteins identified from the discovery phase using label free mass-spectrometry analysis. Four potential biomarkers were chosen and were validated using raw unfractionated serum samples from the same cohort of patients. Four commercially available kits for these four proteins; angiogenin (ANG) [Abcam, UK - ab99970], clusterin (CLU) [R & D system, UK - DCLU00], C-C Motif Chemokine 18 (CCL18)

[Abcam, UK - ab100620], and Complement C1q [Abcam, UK - ab170246] were used. Duplicate and triplicate serum samples were used during this analysis. Each of these ELISA assays was performed according to their individual manufacturer's protocol and guidelines. The concentration of each protein in the serum samples was measured by comparing the optical density (OD) using a microplate reader (Bio-Tek and Luminex). Standard curves were calculated for each ELISA kit.

2.6. Statistical analysis

Proteins differentially expressed in responder and non-responder groups underwent statistical analyses. Multivariate logistic regression (LR) and receiver operating characteristic (ROC) curve analysis were selected to interpret the various protein combinations. The ROC plots were obtained by plotting all sensitivity values (true positive fraction) on the y-axis against their equivalent (1-specificity) values (false positive fraction) for all available thresholds on the x-axis (MedCalc for Windows 8.1.1.0, Medcalc Software, Mariakerke, Belgium). The probability of correctly predicting a given model was calculated from the ROC curve by determining the area under curve (AUC) [20,21]. In our study, we considered AUC values ranging from $0.5 \rightarrow 0.7$ as poor, $0.7 \rightarrow 0.8$ as average, $0.8 \rightarrow 0.9$ as good and > 0.9 as outstanding. Proteins and any combination of proteins providing an AUC value > 0.8 was deemed to be effective for the discrimination of responders from non-responders [22].
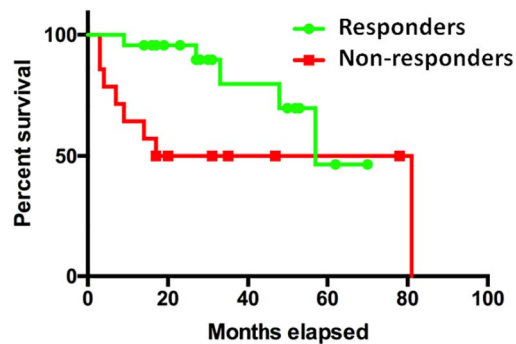
Fig. 1. Survival data.
Overall survival for responders and non-responders patients following induction therapy containing bortezomib.

3. Results

3.1. Sample group

The study group consists of 14 females and 23 males; mean age of patients was 66.5 ± 10.5 years (range 44–87 years). As per International Myeloma Working Group (IMWG) uniform response criteria, 14 patients were identified as non-responders and 23 patients were responders to induction therapy containing bortezomib (Fig. 1 and Table 1). Age was closely matched for both groups with responders mean age of 65 ± 9.5 years (range 44–80 years) and non-responders mean age of 68.7 ± 11.9 years (range 46–87 years). As ISS classification remains important in providing prognostic outcome of MM disease, patients were also classified to each respective stages; I ($n = 11$ cases), II ($n = 17$ cases) and III ($n = 9$ cases).

3.2. Proteomic profiling and identification

16 proteins showing differential expression with significant *p*-values and fold changes were identified using Progenesis QI for Proteomics. Four proteins (ANG, CLU, CCL18 & C1Q) were selected for further validation based on the availability of commercial ELISA kits to evaluate the abundance of these proteins in the validation cohort (Table 2). ANG, CLU, CCL18 & C1Q also had the following criteria: *p*-value ≤ 0.05, protein fold change > 1.5 and proteins with ≥ 2 peptides matched.

3.3. ELISA data and ROC curve analysis

Standard markers such as albumin, ß2M, paraprotein and K/L ratio that are routinely used in clinical practice were measured and compared with the new candidate biomarkers. CLU, ANG, C1Q and CCL18 levels were compared between the responder and non-responder patients (Table 3). A similar analytical approach was applied to this cohort of patient as described in our previous study with thalidomide-based therapy samples [23].

CLU showed higher levels in the responders compared to non-responders with a mean of 291.123 ± 243.13 ng/mL and 181.760 ± 208.096 ng/mL respectively; corresponding with the discovery phase pattern observed by LC-MS analysis. The ROC curve from this ELISA data was found to have an AUC of 0.651 ($p = 0.129$). ANG levels were also significantly higher in the responders compared to non-responders group with a mean of 14.383 ± 0.436 ng/mL and 13.839 ± 0.635 ng/mL respectively. ROC curve analysis for ANG had an AUC value of 0.748 ($p = 0.012$). No significant difference in the mean and AUC for CCL18 protein was observed.

C1Q was found to have higher levels in the non-responders compared to responders group that was also observed in the discovery phase. Mean levels for non-responders were 249.170 ± 190.604 µg/L and 149.675 ± 138.883 µg/L in the responders group. An AUC of 0.722 with a *p*-value of 0.032 was calculated from the ROC curve.

Albumin and K/L ratio mean abundance levels were higher in the responders group as shown in Table 3. ß2M and paraprotein mean levels were higher in the non-responders group. Paraprotein was the only protein to have a significant *p*-value (0.027) and an associated AUC value of 0.719.

In clinical settings, patients would generally have several different markers measured to assess their disease burden and progression. To identify a predictive panel of protein biomarkers, the standard biomarkers consisting of albumin, ß2M, paraprotein and K/L ratio were assessed initially. A combination of proteins that are used alone in the ISS (albumin, ß2M) had an AUC of 0.66 with 67.6% correct classification. By combining albumin and ß2M with paraprotein and K/L ratio, the AUC increased to 0.708 with 64.9% correct classification. Based upon this analysis, further combinations of proteins were performed to identify a better predictive model.

Four new combinations of biomarkers from this study were found to be successful in predicting response to bortezomib therapy (Table 4). These combinations consist of either all four new candidate biomarkers (ANG, CLU, C1Q and CCL18) with an AUC = 0.850 and 76.5% correct classification, or three new candidate biomarkers without CCL18, AUC = 0.802 and 76.5% correct classification. The other two combinations include all four standard biomarkers that are routinely used in

Table 2
Label-free mass spectrometry data.
List of statistically significant discovered proteins using LC-MS analysis. Proteins (in bold) were selected for further validation using ELISAs.

Accession	Peptide count	Confidence score	Anova (p)	Max fold change	Highest mean condition	Lowest mean condition	Description
P10909	**3**	**139.15**	**0.003**	**1.87**	**Responders**	**Non-responders**	**Clusterin**
P01024	3	100.75	0.01	1.85	Responders	Non-responders	Complement C3
P08603	10	369.28	0.02	2.75	Responders	Non-responders	Complement factor H
P01009	3	71.37	0.02	2.09	Responders	Non-responders	Alpha-1-antitrypsin
P55774	**2**	**106.68**	**0.02**	**2.26**	**Responders**	**Non-responders**	**C-C motif chemokine 18**
P10720	2	103.88	0.02	2.53	Responders	Non-responders	Platelet factor 4 variant
P02790	2	118.53	0.03	1.58	Non-responders	Responders	Hemopexin
Q14624	3	125.97	0.03	3.62	Responders	Non-responders	Inter-alpha-trypsin inhibitor heavy chain H4
P03950	**3**	**92.77**	**0.03**	**1.86**	**Responders**	**Non-responders**	**Angiogenin**
P02647	1	49.62	0.03	1.56	Responders	Non-responders	Apolipoprotein A-
Q03591	6	208.05	0.03	1.93	Responders	Non-responders	Complement factor H-related protein 1
P00751	1	68.05	0.04	1.73	Responders	Non-responders	Complement factor B
P01031	1	71.55	0.04	4.15	Responders	Non-responders	Complement C5
P00747	1	46.69	0.04	2.13	Responders	Non-responders	Plasminogen
P02745	**2**	**64.53**	**0.05**	**2.96**	**Non-responders**	**Responders**	**Complement C1q subcomponent subunit A**
Q9BXR6	1	63.61	0.05	2.32	Responders	Non-responders	Complement factor H-related protein 5

Table 3

ELISA Data.

Mean, SD, Area under the curve (AUC) and *p*-value for each of the new and standard proteins found in the two groups of patients compared.

Proteins		n	Mean	Std. deviation	AUC	*p*-value
Candidate biomarkers						
Clusterin (ng/mL)	Responders	23	291.12	243.13	0.651	0.129
	Non-responders	14	181.76	208.10		
Complement C1q (μg/mL)	Responders	21	149.68	138.88	0.722	0.032
	Non-responders	13	249.17	190.60		
Angiogenin (ng/mL)	Responders	23	14.38	0.44	0.748	0.012
	Non-responders	14	13.84	0.64		
C-C motif ligand 18 (ng/mL)	Responders	23	7.00	0.40	0.549	0.633
	Non-responders	13	6.96	0.41		
Standard biomarkers						
Albumin (g/L)	Responders	23	33.04	7.11	0.672	0.082
	Non-responders	14	28.21	7.68		
Beta 2 Microglobulin (mg/L)	Responders	23	3.45	2.24	0.565	0.511
	Non-responders	14	4.39	3.16		
Paraprotein (g/L)	Responders	23	16.13	19.12	0.719	0.027
	Non-responders	14	29.07	18.34		
Kappa/Lambda ratio	Responders	23	176.33	425.43	0.512	0.908
	Non-responders	14	102.09	257.69		

most clinical settings with either 3 new candidate biomarkers (ANG, CLU and C1Q) with an AUC = 0.890 and 85.3% correct classification or 4 new candidate biomarkers including CCL18, AUC = 0.905 and 82.4% correct classification.

4. Discussion

Numerous clinical trials have shown a dramatic improvement in the clinical outcome for MM patients over the last decade but all patients will still relapse following treatment. Over the last decade, the US Food and Drug Administration (FDA) have approved the use of bortezomib as a front-line treatment for MM. Despite the developments of novel biological agents such as first and second-generation proteasome inhibitors (bortezomib and carfilzomib), many MM patients will develop drug resistance and in some cases have *de novo* resistance to these novel agents [17,24,25]. Furthermore, bortezomib has a range of adverse reactions such as thrombocytopenia, neutropenia, anemia, infections, gastrointestinal discomfort, musculoskeletal pain and commonly peripheral neuropathy that may affect a patient's subsequent treatment [26,27]. The ability to avoid side effects from ineffective treatments is a crucial area where biomarkers can play a significant role.

Biomarkers have been effective in individualizing treatments in various malignancies including breast, lung and nasopharyngeal cancers [28–30]. In MM, there are still limited published studies on biomarkers that could be used to predict treatment (e.g. bortezomib) response. Recently, Cereblon (CRBN) has been found to be a target for immunomodulatory agents (IMiDs) and its lack of expression shows a high correlation with resistance to these agents [31,32]. Employing CRBN as a biomarker in MM, accurately predicts response rate and survival in patients treated with IMiDs.

Albumin, ß2M, paraprotein and kappa/lambda ratio are the standard biomarkers currently used in the clinical setting. Despite that,

these panels of blood tests are not specific to predict response to any form of therapy [12]. In this study, a number of proteins were found to be significantly changed in abundance between responders and non-responders to treatment containing bortezomib. C1Q is a hexamer of three unique protein subunits (A, B, and C) and plays a key role in apoptotic cell, immunological and inflammatory processes. It is a 460 kDa protein composed of 18 polypeptide chains of (6 A-chains, 6 B-chains and 6 C-chains) [33]. Its role has been thought to be involved with advanced glycation end (AGEs) products through its binding properties in human serum [34], and studies have shown that these proteins have the ability to induce precipitation of soluble gamma globulin complexes [35]. C1Q receptors are directed to the heavy polypeptide chains of IgG and IgM [36]. It has been suggested that some myeloma IgG proteins undergo unusual glycosylation processes during disease progression [37]. These glycosylation studies together with our current findings suggest that there is an association between the increased levels of C1Q in the non-responders group.

ANG, a 14 kDa angiogenic ribonuclease, is an actin-binding protein on the surface of endothelial cells, promoting cell invasion and migration [38]. Various studies have shown that immunomodulatory drugs such as thalidomide have an inhibitory effect on angiogenesis in cancer, thus reinforcing the importance of ANG's role in MM disease progression [39–41]. A study has recently shown that myeloma patients who were treated with bortezomib and had responded well, displayed a significant decrease in microvessel density, which suggests that bortezomib may have an anti-angiogenic effect [42].

CLU, a ubiquitous extracellular protein is expressed in a range of diseases that arise from protein misfolding and deposition of highly structured protein [43]. CLU exists in several isoforms; secretory and nuclear CLU. Its exact mechanism of action remains unknown but it is thought to play a role in promoting cancer cell survival through the activation of Akt and NFκB pathways [44–47]. Studies have shown that

Table 4

Logistic regression analysis data.

List of different protein combinations used to establish the best model that can be used as a predictive panel for response to induction therapy containing bortezomib regime.

Protein combinations	AUC	Hosmer & Lemeshow test (*p* value)	Correct classification (%)
International Scoring System: albumin and ß2M	0.660	0.702	67.600
Standard biomarkers: albumin, ß2M, paraprotein and K/L Ratio	0.708	0.750	64.900
Candidate biomarkers: Angiogenin, Clusterin, Complement C1q	0.802	0.760	76.500
Candidate biomarkers: Angiogenin, Clusterin, Complement C1q, CCL18	0.850	0.636	76.500
4 standard and 3 candidate biomarkers (Angiogenin, Clusterin and Complement C1q)	0.890	0.495	85.300
4 standard and 4 candidate biomarkers (Angiogenin, Clusterin, Complement C1q, CCL18)	0.905	0.238	82.400

bortezomib plays an important role with transcription nuclear factor kB (NFκB) by blocking the degradation of its inhibitor, IkB [48].

CCL18, also known as macrophage inflammatory protein 4, is a chemotactic cytokine that has no known receptor. Yuan and co-workers recently found using survival and multivariate analysis that CCL18 was an independent favorable prognostic factor in patients with colorectal cancer. Similarly in this investigation, CCL18 levels were found to be significantly increased in the responder group [49].

By combining these new candidate biomarkers (ANG, CLU, C1Q) with standard biomarkers (Albumin, ß2M, Paraprotein and K/L ratio), we have developed a novel panel of biomarkers to predict response to bortezomib treatment in MM. These ELISA kits are widely available and inexpensive in comparison to other prognostic methods. This novel biomarker panel may assist clinicians in choosing a more personalized and efficacious treatment, whilst minimising unnecessary side effects. Further prospective multi-centre randomized studies will be useful to determine the efficacy of this panel of biomarkers. MM is not one disease and therefore for any potential biomarker to be useful in monitoring or predicting response to treatment, it will most likely be as part of a panel of disease relevant biomolecules with direct connection to the phenotypic machinery associated with MM. Most biomarkers current used in MM management are either diagnostic or prognostic. This study has demonstrated that by combining biomarkers that are currently used in the management of MM, with additional candidate protein biomarkers, it is possible to develop a sensitive test that predicts response to bortezomib treatment, an established component of anti-myeloma therapy.

In this study, a combination of fraction methods was employed to reduce the dynamic range of proteins under investigation and facilitate the detection/quantification of more low abundant molecules by high abundant protein removal/depletion. However, high abundant proteins sequestering low abundant cargo proteins can help to increase their abundance and protect them from elimination by the body. A drawback of compressing the dynamic range by protein removal/depletion is the potential loss of information on the abundance levels for these proteins under different conditions (response to treatment) and the valuable proteins that can be found attached to these molecules [50].

The ISS predicts survival of newly diagnosed MM patients by using a combination of two routine biomarkers, ß2M and albumin, and uses this information to separate patients into three stages with a distinct prognosis. However this approach is not specific to any individual treatment. Therefore, the focus of this investigation was to combine routine biomarkers, such as those used by the ISS, with novel biomarkers discovered using proteomics analysis of MM patient samples. Ultimately the aim is to develop a predictive model using easily quantifiable and accessible platforms, directly associated with specific therapeutic options, such as bortezomib. Such theranostics are intended to give clinicians the means to implement a treatment plan customized for each patient. Prospective studies will be planned to validate the proposed risk stratification panel in this study and will also take into account the revised International Staging System (R-ISS), to improve risk stratification by evaluating the presence of chromosomal abnormalities detected by iFISH (t(4;14), t(14;16) and del17p) and elevated serum lactate dehydrogenase in addition to ISS.

Conflict of interest statement

We wish to confirm that there are no known conflicts of interest associated with this publication and there has been no significant financial support for this work that could have influenced its outcome.

Transparency document

The http://dx.doi.org/10.1016/j.bbacli.2017.05.003 associated with this article can be found, in online version.

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