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Evaluation of serum glucose-regulated protein 78 (GRP78) as a biomarker of treatment response to bortezomib-based induction regimen in multiple myeloma: A cross-sectional pilot study

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GRP78 overexpression in myeloma cells has been associated with bortezomib resistance in multiple myeloma (MM). However, serum GRP78 as a maker of bortezomib-based treatment response remains unexplored. The objective of the study was to evaluate serum GRP78 levels in MM patients who underwent a bortezomib-based induction regimen. This cross-sectional study included adult MM patients (n=30) who completed at least four cycles of bortezomib-based induction therapy. Healthy volunteers (n=30) and newly diagnosed MM patients (n=19) were also recruited to identify the disease-associated change in GRP78 levels. Serum GRP78 was estimated by ELISA. Surface and intracellular expression of GRP78 in bone marrow plasma cells was evaluated in ten MM patients by flow cytometry. Among 30 MM patients [median (range): 52 (38-68) years; 20 males] who completed at least four cycles of bortezomib-based induction therapy, 20 were responders and 10 were non-responders. Serum GRP78 levels were not significantly different between responders [median (IQR): 5.2 (3.1, 8.0) μg/ml] and non-responders [median (IQR): 4.3 (0.1, 7.1) μg/ml] (p=0.4). Although non-significant (p=0.3), median serum GRP78 was higher in newly diagnosed patients when compared to healthy volunteers. Bone marrow plasma cells ranged from 0.2 to 57.8% in the analyzed samples. Intracellular GRP78 expression in bone marrow plasma cells was higher (1.6 to 5 times) when compared to surface expression. To conclude, serum GRP78 levels vary widely in different MM patient groups but did not correlate with response to a bortezomib-based induction regimen.

Key words: bortezomib, GRP78, glucose-regulated protein, multiple myeloma, resistance, responders

Over the last two decades, with the advent of novel therapeutic agents, overall survival has increased for multiple myeloma (MM) patients. Bortezomib, a first-in-class proteasome inhibitor, is a key component of the induction therapy of myeloma patients [1, 2]. Despite an overall improvement in the response rate, 10–30% of patients do not achieve optimal response during bortezomib-based treatment, and most initial responders relapse into progressive disease [3]. Various mechanisms have been studied for bortezomib resistance like point mutations in proteasome subunit beta, upregulation of survival pathways, including Glucose-regulated protein (GRP78) [3–5]. GRP78, an endoplasmic reticulum (ER) chaperone protein, is well known for its physiological role in protein folding and assembly [6]. Under cellular stress such as glucose depletion, hypoxia, and oxidative stress, it has been shown to be upregulated and promote cell survival pathways.

Overexpression of GRP78 has been reported in many solid tumors and hematological malignancies including MM and has been associated with tumor aggressiveness, invasion, and metastasis and correlated with poor overall survival [7, 8]. Notably, GRP78 overexpression has been associated with chemoresistance in cancer. Suppression of this protein by various means such as natural products like fucoidan, kaempferol, small molecule drugs like metformin, bacterial toxin as well as gene silencing by small interference RNA technique have been known to increase the sensitivity to other anti-cancer agents [9, 10]. Studies have demonstrated the upregulation of GRP78 in bortezomib-resistant cell lines, quiescent cells surviving bortezomib treatment

and bone marrow (BM) plasma cells of relapsed/refractory MM patients [8, 11–13]. GRP78, conventionally present in ER, has been found to be translocated to the cell surface in various tumors and relapsed/refractory myeloma cells [14, 15]. The secretory nature of the protein has been demonstrated before as it was detected in the plasma of healthy volunteers [16]. Hence, it was hypothesized that GRP78 levels might get altered in the serum of MM patients undergoing bortezomib-based regimen and can indicate treatment response. This pilot study aimed to evaluate serum GRP78 as a biomarker of responsiveness to bortezomib-based induction regimen in multiple myeloma patients. Serum GRP78 was also compared between newly diagnosed MM patients and healthy volunteers to identify disease-specific alterations in GRP78 levels. Bone marrow samples of a few MM patients were also explored to characterize GRP78 cellular localization in plasma cells.

Patients and methods

This cross-sectional study was conducted after institute's ethics committee approval and conformed to the Declaration of Helsinki. All participants provided written informed consent.

Study population selection and sample collection. Two groups of MM patients of age ≥18 years, were enrolled: newly diagnosed treatment naïve patients and those who had completed at least 4 cycles of bortezomib-based induction regimen. Patients diagnosed with other cancers or autoimmune disorders, and pregnant females were excluded from the study. Apparently healthy volunteers of age >18 years were also recruited. Patient demographic details, treatment details, and laboratory parameters were captured in study specific case record form and 5 ml of venous blood was collected at the time of enrolment. The serum was separated and stored in aliquots at –80 °C until further analysis. To characterize the cellular distribution of GRP78, left-over BM samples were obtained from another ten MM patients who routinely underwent bone marrow examination for disease monitoring.

Treatment regimen and response assessment. The bortezomib-based induction regimens prescribed were either bortezomib, lenalidomide, and dexamethasone (VRD) or bortezomib, cyclophosphamide, and dexamethasone (VCD). The response was assessed according to International Myeloma Working Group uniform response criteria as stringent Complete Response (sCR), Complete response (CR), Very Good Partial Response (VGPR), Partial Response (PR), Stable Disease (SD), and Progressive Disease (PD) [17]. For this study purpose, patients with sCR, CR, VGPR, and PR were categorized as responders and those with SD and PD as non-responders [18].

Serum GRP78 estimation. Serum GRP78 levels were measured using a commercially available ELISA kit

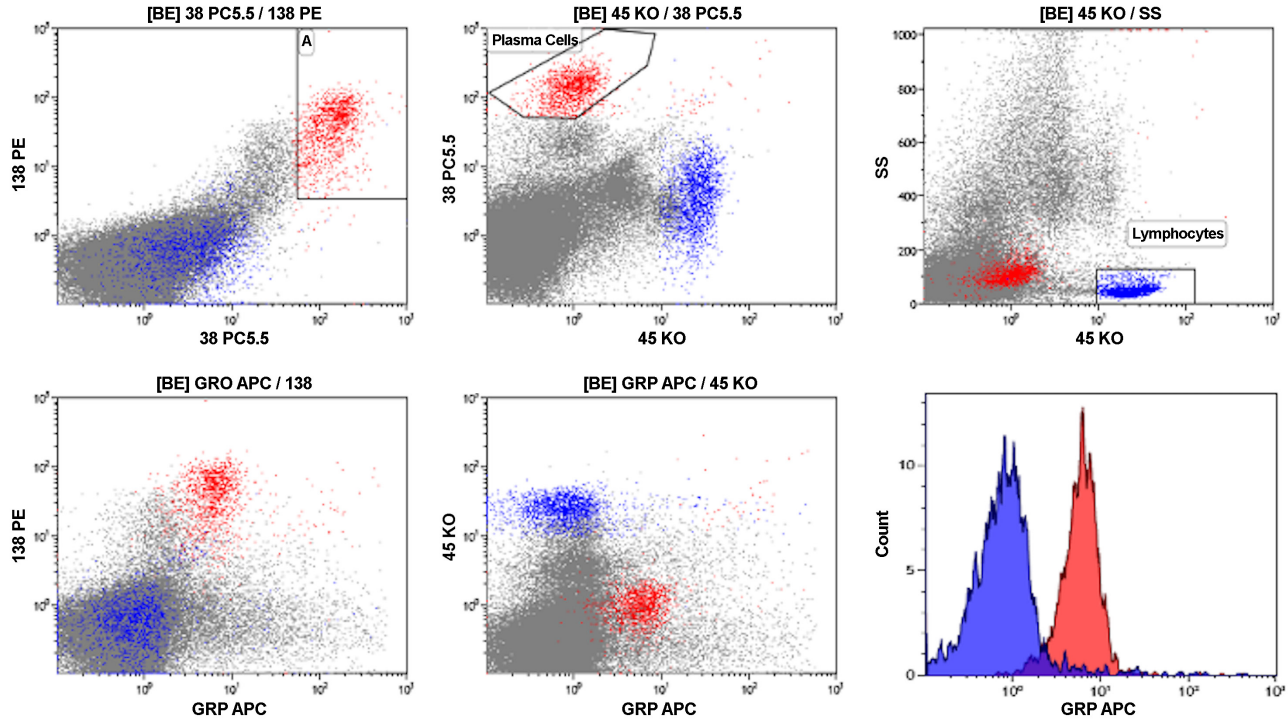

Figure 1. Representative flow cytometry image of GRP78 expression in bone marrow plasma cells and B-lymphocytes. Abbreviations: APC-allophycocyanin; GRP-Glucose regulated protein 78; PE-Phycoerythrin; PC5.5-peridinin-chlorophyll proteins cyanine 5.5; KO-krome orange

(ADI-900-214 - Enzo Life Sciences), as per the manufacturer's protocol [https://www.enzolifesciences.com/ADI-900-214/grp78-bip-elisa-kit/]. The reported sensitivity of the assay was 8.4 ng/ml.

Bone marrow plasma cells GRP78 expression by flow cytometry. Expression of both surface and intracellular GRP78 was carried out in nine out of ten samples and in one sample, the intracellular expression could not be performed because of an inadequate amount of sample. 150 µl of bone marrow aspirate, each containing 5×10^5 cells, was pipetted into two disposable polystyrene tubes. One was processed for surface GRP78 expression and the other for intracellular GRP78 expression after the addition of permeabilization buffer (eBiosciences; catalog no. 00-8333-56). Flow cytometry was performed using a six-color panel of antibodies: Allophycocyanin (APC) conjugated Human Immunoglobulin Binding Protein (BIP) Antibody (Assay Pro; catalog no. 30226-05161), CD138 PE (BD Biosciences; catalog no. 552026), CD38 PerCP-Cy™5.5 (BD Biosciences; catalog no. 561106), CD45 KO (Beckman Coulter; catalog no. B36294), CD56 PC 7 (Beckman Coulter; catalog no. A21692), and CD19 BB515 (BD Biosciences; catalog no. 564456). The data acquisition was done by flow cytometer (Beckmann Coulter Gallios, USA) and the analysis was done by Kaluza software version 2.1. Plasma cells were gated as events with a bright expression of CD38, CD138, and a dim expression of CD45, and the malignant variant of plasma cells was identified from plasma cells with a bright expression of CD56 and a dim expression of CD19 (Figure 1). The expression of GRP78 is represented as the median fluorescent intensity (MFI) in the gated plasma cells as MFI-1 and in malignant plasma cells as MFI-2. MFI of GRP78 in B cells (CD 45+ and CD19+) (MFI-3) was taken as an internal control. The ratio of MFI of plasma cells to MFI of B cells was calculated as the ratio of MFI i.e., RFI. RFI >1.0 corresponds to higher expression in plasma cells when compared to B cells. The ratio of median Fluorescence Intensity-1 (RFI-1) was calculated as the ratio of MFI-1 upon MFI-3 and RFI-2 was calculated as the ratio of MFI-2 upon MFI-3.

Statistical analysis. Data are represented as median with interquartile range (IQR). Mann Whitney-U test was applied to compare the difference in serum GRP78 levels between bortezomib responders and non-responders as well as healthy volunteers and newly diagnosed MM patients, following two-tailed distributions. The Chi-square test was applied for the comparison of categorical variables; and wherever required, Fisher's Exact test was used. A post-hoc analysis for progression-free survival was performed for newly diagnosed MM patients based on their median baseline serum GRP78 levels (low and high expression) and the log-rank test was used for the analysis. A p-value less than 0.05 was considered significant. Statistical analyses were performed in STATA software version 15.1 (Stata-corp LLC, TX, USA). BM-GRP78 expression is presented as descriptive data.

Results

A total of fifty-nine MM patients were enrolled of which, nineteen were newly diagnosed, drug naïve patients. Among thirty patients who had completed at least 4 cycles of induction therapy with a bortezomib-based regimen, twenty were responders and ten were non-responders (Supplementary Figures 1A–1C). Baseline clinical and laboratory characteristics between responders were similar in most of the parameters except serum M protein which was higher (p=0.04) and serum albumin which was lower (p=0.04) in non-responders (Table 1). Half of them were on the VRD regimen and half on the VCD regimen in both responders and non-responders. Among responders, 10% were in sCR, 35% in CR, 35% in VGPR, and 20% in PR. Among non-responders, 50% were in SD and 50% in PD. Thirty healthy volunteers were also enrolled with median age of 29 years (IQR: 28.33) and the male : female ratio of 17 : 13. BM expression of GRP78 was studied in another ten MM patients and their clinical and laboratory profiles are given in Table 2.

Serum GRP78 levels. The median concentration for responders was 5.2 µg/ml (IQR: 3.1; 8.0 µg/ml) and the median concentration of non-responders was 4.3 µg/ml (IQR: 0.1; 7.1 µg/ml). There was wide interindividual variation and the difference between responders and non-responders was not statistically significant (p=0.4). The median GRP78 concentration for healthy volunteers was 7.4 µg/ml (IQR: 3.6; 11.4 µg/ml) for newly diagnosed MM patients, was 8.5 µg/ml (IQR: 4.7; 13.0 µg/ml) and the difference between the two groups was not statistically significant (p=0.3). GRP78 concentrations were not significantly different in newly diagnosed patients as compared to responders (p=0.09) and non-responders (p=0.05) (Figure 2). The distribution of serum GRP78 levels in the three groups (newly diagnosed MM patients, responders, and non-responders) stratified by clinical and laboratory parameters are described in Table 3. There was no significant association between serum GRP78 levels and any of the analyzed parameters.

Correlation of serum GRP78 levels with progression-free survival in newly diagnosed MM patients. Newly diagnosed patients were categorized into high and low GRP78 expression based on their median serum GRP78 levels. Though the median progression-free survival was longer i.e., 3.2 years in the low GRP78 expression (median <8.5 mcg/ml) group than in the high GRP78 expression group (1.9 years); the difference was not found to be statistically significant (p=0.7, Figure 3).

Bone marrow GRP78 expression. The bone marrow plasma cells (CD138+ CD38+ and CD45–) ranged from 0.2 to 57.8% of the total cells acquired. Out of 10 samples analyzed, eight had CD56+ CD19– expression and their percentage ranged between 0.1 to 49.3%. GRP78 MFI and RFI are given in Table 3. Intracellular expression was found to be higher (1.6–5 times) when compared to surface expression in eight out of nine samples. Six out of ten CD138+ CD38+

Table 1. Clinical and laboratory characteristics of participants.

Characteristics	N	Newly diagnosed	N	Responders	N	Non-responders
Age (years)	19	60 (52, 64)	20	57 (46.5, 64)	10	47.5 (43, 52)
Male : female	19	10 : 9	20	14 : 6	10	6 : 4
Anemia at diagnosis, n (%)	19	09 (47.4)	20	13 (65)	10	07 (70)
Hypercalcemia at diagnosis, n (%)	19	04 (21)	20	02 (10.5)	10	01 (10)
Serum creatinine at diagnosis, n (%)	19					
≥2 mg/dl		05 (26.3)		04 (20)		03 (30)
<2 mg/dl		14 (73.7)	20	16 (80)	10	07 (70)
Bony lytic lesions at diagnosis, n (%)	19	17 (89.5)	20	19 (95)	10	10 (100)
ISS staging, n (%)	19		20		10	
I		04 (21)		3 (15)		2 (20)
II		08 (42.1)		7 (35)		4 (40)
III		07 (36.8)		10 (50)		4 (40)
DSS staging, n(%)	19		20		10	
I		0 (0)		0 (0)		0 (0)
II		04 (21.1)		04 (20)		01 (10)
IIIA		11 (57.9)		13 (65)		07 (70)
IIIB		04 (21.1)		03 (15)		02 (20)
Myeloma subtype, n (%)	19		20		10	
IgG κ		08 (42.1)		11 (55)		07(70)
IgG λ		01 (5.3)		02 (10)		02 (20)
IgA κ		02 (10.5)		02 (10)		01 (10)
IgA λ		03 (15.8)		0 (0)		0 (0)
κ light chain only		03 (15.8)		3 (15)		0 (0)
λ light chain only		01 (5.3)		1 (5)		0 (0)
Negative		01 (5.3)		01 (5)		0 (0)
Comorbidities, n (%)	19		20		10	
Diabetes mellitus		03 (15.7)		05 (25)		01 (10)
Hypertension		08 (42.1)		07 (35)		02 (20)
Coronary artery disease		0 (0)		01 (5)		01 (10)
Serum creatinine (mg/dl)[#]	19	1.0 (0.9,1.7)	20	1.2 (0.9, 2.0)	10	0.9 (0.8, 1.3)
Serum calcium (g/dl)[#]	19	9.7 (9.0, 10.1)	20	9.4 (8.6, 9.9)	10	9.3 (8.7, 9.8)
Serum LDH (U/l)[#]	17	207.8 (168, 242)	17	215 (156, 314)	10	254 (202, 281)
Serum M protein (g/dl)[#]	19	1.7 (0.3, 2.7)	20	3.4 (0, 4.8)	10	5.6 (3.6, 6.6)*
Serum albumin (g/dl)[#]	19	3.9 (3.5,4.3)	19	3.6 (3.4, 4.2)	08	3.2 (2.9, 3.7)*
Serum β2microglobulin (mg/l)[#]	19	4.7 (3.2, 7.6)	19	5.2 (3.6, 10.1)	10	4.0 (2.5, 10.1)
Bone marrow plasma cell (%)[#]	18	32.5 (12, 45)	18	35 (12, 75)	09	55 (20, 80)

Notes: [#]values represent median (IQR); *p<0.05 as compared to responders

Table 2. Clinical and laboratory profile of patients evaluated for GRP78 expression in bone marrow plasma cells.

Patient ID	Age (year)	Gender	Durie salmon staging (DSS)	Treatment regimen	Disease status	Bone marrow plasma cell (%)	Serum M protein (g/dl)
BM1	70	M	IIIB	Treatment naive	ND	70	0
BM2	56	F	IIIA	VD	PD	30	NA
BM3	50	F	IA	T	PD	80	4.2
BM4	61	M	IIIB	Nil	Relapse	50	1.5
BM5	63	M	IIIA	VCD	CR	2	0
BM6	65	M	IIIA	VRD	PD	70	4.2
BM7	62	M	IIIB	nil	Relapse	50	2.1
BM8	43	F	IIIB	nil	PD	NA	NA
BM9	41	M	IIIB	Treatment naive	ND	35	0
BM10	45	M	IIIA	Treatment naive	ND	12	0.4

Abbreviations: M-male; F-female; ND-newly diagnosed; PD-progressive disease; T-Thalidomide; CR-complete response; VCD-Bortezomib+Cyclophosphamide+Dexamethasone; VRD-Bortezomib+Lenalidomide+Dexamethasone; VD-Bortezomib+Dexamethasone; NA-not available; nil-previously treated with multiple regimens; not on any regimen at the time of bone marrow sample collection.

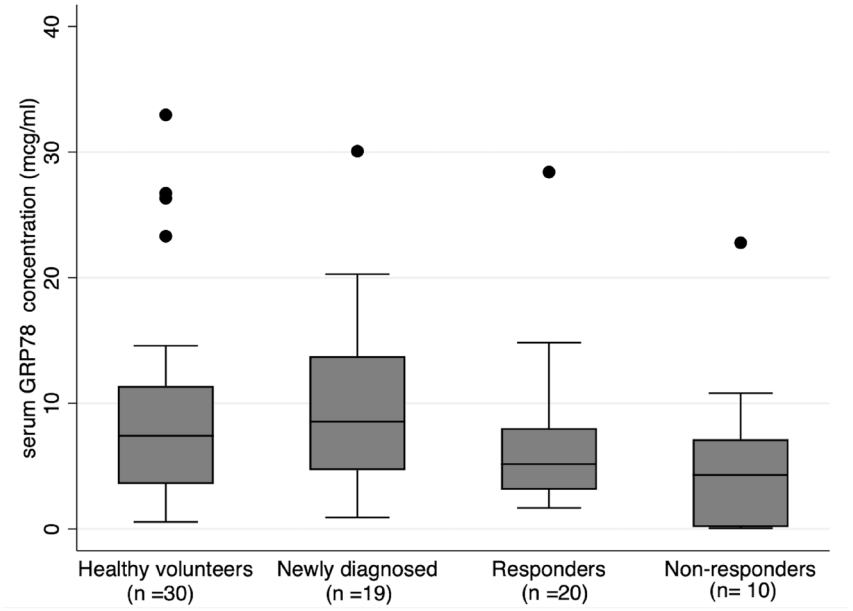

Figure 2. Serum GRP78 concentrations in the study population.

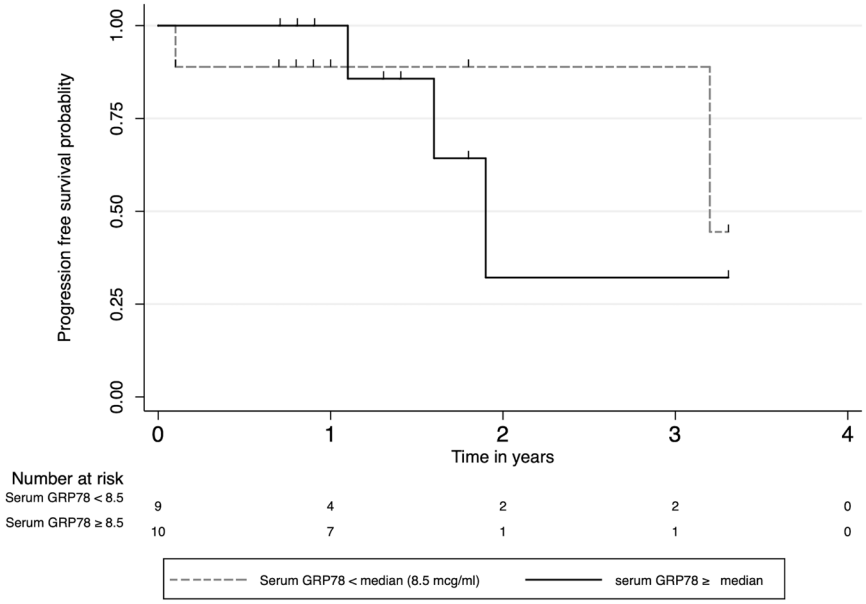

Figure 3. Kaplan-Meier plot of progression-free survival in newly diagnosed MM patients (n=19).

and CD45-plasma cells had higher surface GRP78 expression than CD45+ and CD19+ B cells with RFI-1 values ranging from 1.3 to 11.5. Seven out of nine CD138+ CD38+ and CD45-plasma cells had higher intracellular GRP78 expression than B cells with RFI-1 values ranging from 1.2 to 6.1.

Discussion

GRP78 expression has been shown to be upregulated in resistant MM cell lines as well as in primary BM cells of refractory/resistant MM patients and has been implicated

in bortezomib resistance [8, 19]. However, BM GRP78 expression cannot be routinely employed as a biomarker of bortezomib resistance due to the invasive nature of the procedure and logistic challenges. Serum GRP78, on the other hand, has not been explored as a marker of bortezomib response in MM patients. In this study, we examined whether serum GRP78 differed between responders and non-responders to bortezomib-based induction therapy. The baseline clinical characteristics of patients were found to be consistent with previous studies [18, 20]. Notable exceptions were higher serum M-protein and lower serum albumin in non-responders than responders and such a difference has not been reported before [21].

In our study, GRP78 was found to be present in the serum of healthy volunteers which is consistent with earlier reports [16, 22]. Although non-significant, median serum GRP78 levels in newly diagnosed MM patients showed a higher trend than in healthy volunteers. It could be attributed to a diseased state and associated cellular stress in myeloma cells due to excessive immunoglobulin production [23]. Similar findings have been reported in other cancers. Higher plasma GRP78 level was noted in patients with endometrial cancer as compared to the healthy control women [22]. Also, serum GRP78 was found to be high in the late stages of lung cancer compared to the early stages and has been linked to poor survival [24]. Contrary to our hypothesis, we observed a lower trend of median serum GRP78 levels in non-responders when compared to responders. Steiner et al. reported a higher trend, although not significant, in the bone marrow and peripheral blood plasma in relapsed/refractory MM patients when compared to newly diagnosed MM patients [12].

To interpret the results of serum GRP78 concentration, it is important to know the factors that may contribute to its variability. We, therefore, tested whether the serum GRP78 concentrations varied with clinical and laboratory parameters. An exploratory regression analysis was performed for the adjustment of covariates like age, gender, disease staging, myeloma subtype, and associated comorbidities like hypertension, diabetes, etc. These factors were not found to be significantly associated with serum GRP78 concentrations (data not shown). We noticed a trend of improved progression-free survival in newly diagnosed patients with lower serum median GRP78 levels which corroborates with earlier findings noted in other cancers. However, it contradicts the findings of Nincovic et al., which show that a lower

Table 3. Association of serum GRP78 levels with clinical and laboratory parameters.

Patients	Newly diagnosed		Responders		Non-responders	
	N (19)	GRP78 (μg/ml)	N (20)	GRP78 (μg/ml)	N (10)	GRP78 (μg/ml)
Age						
<median	09	5.3 (4.7, 11.7)	09	5.6 (3.5, 8.8)	05	6.5 (2.1, 7.1)
≥median	10	10.1 (6.6, 14.0)	11	4.8 (2.9, 7.7)	05	0.4 (0.1, 6.9)
Gender						
Female	09	10.9 (9.6)	06	6.6 (5.5, 8.8)	04	4.4 (5.2)
Male	10	10.0 (5.1, 11.7)	14	3.8 (2.9, 7.9)	06	4.5 (3.6, 7.1)
ISS staging						
I	04	9.4 (5.7, 12.7)	03	5.6 (3.5, 7.9)	02	7.0 (6.9, 7.1)
II	08	11.6 (5.9, 16.4)	07	3.2 (2.9, 5.4)	04	1.2 (0.2, 6.4)
III	07	4.7 (1.7, 13.8)	08	6.6 (4.1, 8.8)	04	3.3 (0.1, 14.6)
DSS staging						
II	04	7.5 (5.9, 10.5)	04	5.7 (3.3, 11.4)	01	7.1 (NA)
III A	11	11.7 (5.3, 16.0)	13	5.4 (3.2, 7.7)	07	2.1 (0.2, 10.8)
III B	04	2.9 (1.0, 8.1)	03	4.1 (1.7, 8.8)	02	3.3 (0.1, 6.5)
Hemoglobin						
≥10 g/dl	11	11.4 (5.1, 13.7)	08	6.6 (4.2, 9.1)	05	7.1 (6.9, 10.8)
<10 g/dl	08	7.0 (2.9, 15.9)	12	4.8 (2.5, 6.6)	05	0.1 (0.07, 2.1)
Serum creatinine						
<2 mg/dl	15	11.7 (5.3, 14.0)	16	5.2 (3.1, 8.0)	07	6.9 (3.6, 10.8)
≥2 mg/dl	05	4.7 (1.1, 8.5)	04	5.9 (2.9, 8.2)	03	0.08 (0.06, 6.5)
Serum calcium						
<11 mg/dl	16	11.6 (5.9, 13.9)	17	4.8 (3.0, 7.9)	09	6.5 (3.6,7.1)
≥11 mg/dl	04	5.0 (2.9, 8.4)	02	7.2 (5.6, 8.8)	01	7.9 (NA)
β2 microglobulin						
≤3.5 mg/l	03	5.3 (3.9, 11.4)	04	4.5 (2.6, 4.5)	04	4.5 (1.2, 7.0)
>3.5 mg/l	17	11.6 (5.2, 13.8)	16	5.2 (3.1, 8.5)	06	3.3 (0.1, 10.8)
Serum LDH						
≤ULN	14	9.4 (4.6, 13.3)	11	5.3 (3.0, 7.9)	07	6.5 (0.4, 10.8)
>ULN	04	11.2 (4.7, 15.0)	06	4.5 (3.2, 14.8)	03	0.1 (0.07, 7.1)

Abbreviation: NA-not applicable; ULN- upper limit of normal value

BM GRP78 expression has been associated with a shorter progression-free survival [6, 15, 25].

Another factor that may contribute to variability in serum GRP78 concentration is the cellular distribution of the protein. We explored bone marrow GRP78 expression and its cellular localization in ten MM patients. Surface GRP78 expression, quantified by median fluorescence intensity in the plasma cells, was observed in all the samples as noted in previous studies [8, 11]; but, the intensity highly varied across the samples. The intracellular expression was found to be higher when compared to surface expression conforming to the fact that GRP78 is basically an ER protein. Of the seven relapsed/refractory MM patients, two patients who were on bortezomib-based therapy and not responding to the treatment had higher intracellular GRP78 expression. As expected, and shown before, the GRP78 expression was found to be higher in plasma cells (CD138+ CD38+) when compared to B lymphocytes (CD45+ CD19+) [23]. Further, a comparison among plasma cells of newly diagnosed/ responders/ relapsed refractory MM patients against those of healthy volunteers needs to be done to understand the role of GRP78 in MM.

To the best of our knowledge, serum concentrations of GRP78 in various phenotypes of MM have not been reported previously. We found that the serum GRP78 concentrations vary widely among different categories. The limitations of the study such as cross-sectional design and limited sample size are expected considering the exploratory nature of the study. This study, importantly, provides the concentrations of serum GRP78 and its variance in various phenotypes of MM which will be very helpful in the design and sample size calculations of future studies. Evaluation of serum and bone marrow GRP78 concentrations in a prospective cohort study, with appropriate sample size, adjusting for covariates, will aid in demonstrating its clinical utility.

Table 4. Bone marrow GRP78 expression by flow cytometry.

	N	Surface GRP78 expression [median (range)]	N	Intracellular GRP78 expression [median (range)]
MFI-1 a.u.	10	2.8 (0.8 to 12.6)	9	9.4 (3.2 to 28.5)
MFI-2 a.u.	8	2.7 (0.8 to 10.2)	7	9.2 (3.1 to 15.2)
RFI-1	10	1.4 (0.4 to 11.5)	9	1.3 (0.5 to 6.1)
RFI-2	8	1.5 (0.4 to 11.5)	7	1.3 (0.5 to 2.3)

Abbreviations: a.u.-arbitrary units; MFI-1-median fluorescence intensity of GRP78 in CD138+ CD38+ and CD45– plasma cells; MFI-2-median fluorescence intensity of GRP78 in CD56+ CD19– plasma cells; RFI-1-ratio of median fluorescence intensity of CD138+ CD38+ CD45– plasma cells (MFI-1) to CD19+ CD45+ B lymphocytes (MFI-3); RFI-2-ratio of median fluorescence intensity of CD56+ CD19- plasma cells (MFI-1) to CD19+ CD45+ B lymphocytes

Supplementary information is available in the online version of the paper.

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Evaluation of serum glucose-regulated protein 78 (GRP78) as a biomarker of treatment response to bortezomib-based induction regimen in multiple myeloma: A cross-sectional pilot study

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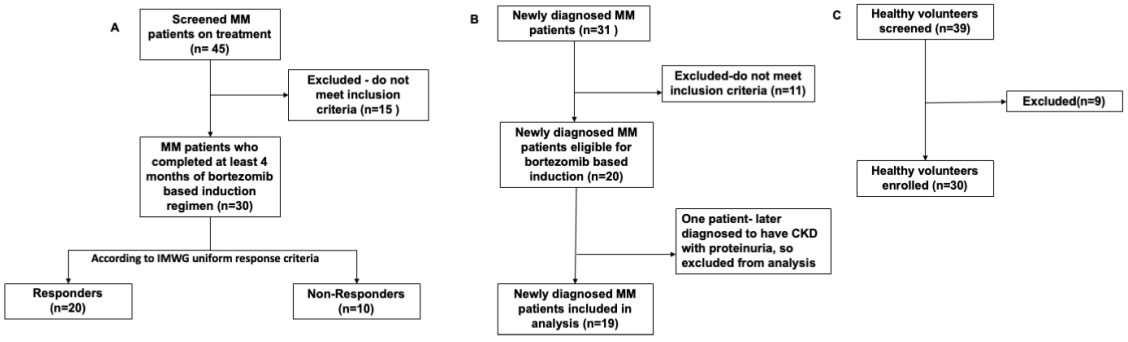
Supplementary Information



Supplementary Figure S1. Patients' flowchart. Multiple myeloma patients on treatment. B) Newly diagnosed multiple myeloma patients. C) Healthyvolunteers.