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Article

The prognostic value of whole-blood PSMB5, CXCR4, POMP and RPL5 mRNA expression in patients with multiple myeloma treated with bortezomib

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Simple Summary: The mRNA expression of nine previously-described genes that may affect resistance to multiple myeloma (MM), viz. *ABCB1, CXCR4, MAF, MARCKS, POMP, PSMB5, RPL5, TXN* and *XBP1*, was compared between bortezomib-refractory and bortezomib-sensitive patients. *RPL5* was the only gene to be significantly down-regulated in MM patients compared with non-MM individuals, while *POMP* was significantly up-regulated in the bortezomib-refractory patients. Multivariate analysis found the best independent predictors of progression-free survival to be high *PSMB5* and *CXCR* expression and autologous stem cell transplantation, and that high expression of *POMP* and *RPL5* were associated with shorter survival.

Abstract: Proteasome inhibitors, like bortezomib, play a key role in the treatment of multiple myeloma (MM); however, most patients eventually relapse and eventually show multiple drug resistance, and the molecular mechanisms of this resistance remain unclear. The present study examines the expression of previously-described genes that may influence resistance to bortezomib treatment at the mRNA level (*ABCB1, CXCR4, MAF, MARCKS, POMP, PSMB5, RPL5, TXN* and *XBP1*). mRNA expression was determined in 73 MM patients treated with bortezomib-based regimens (30 bortzomib-sensitive and 43 bortezomib-refractory patients) and 11 healthy controls. *RPL5* was significantly down-regulated in multiple myeloma patients as compared with healthy controls. Moreover, POMP was significantly up-regulated in MM patients refractory to bortezomib-based treatment. In multivariate analysis, high expression of *PSMB5* and *CXCR* and autologous stem cell transplantation were independent predictors of progression-free survival, and high expression of *POMP* and *RPL5* was associated with shorter overall survival.

Keywords: ABCB1; bortezomib; CXCR4; gene expression; MAF; MARCKS; multiple myeloma; mRNA; POMP; PSMB5; refractory; RPL5; TXN; XBP1; sensitive

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1. Introduction

Multiple myeloma (MM, plasma cell myeloma) is a hematological malignancy characterized by accumulation of malignant plasma cells (PC) in the bone marrow (BM), often resulting in bone lesions, hypercalcemia, infections, anemia, and production of monoclonal immunoglobulin [1]. The disease occurs mainly in older patients and accounts for 15% of all hematologic malignancies, with an annual incidence of 4.5-6 cases per 100,000 [2], with an estimated 32,270 new cases and 12,830 deaths in the USA in 2020 [3]. Proteasome inhibitors (PI) play a key role in the treatment of MM [4-6]. Three PIs, bortezomib, carfilzomib and ixazomib, are currently approved for the treatment of MM and several others are undergoing clinical trials [7].

Bortezomib is the first-in-class selective and reversible inhibitor of the 26S proteasome. It demonstrates antiproliferative and antitumor activity, and its use has been a breakthrough in treating MM in the past 15 years [5]. It is a boronic acid-based compound, which inhibits β5 chymotrypsin-like (CT-L) and to a lesser extent, β1 caspase-like (C-L) of the proteasome; it has been approved for treatment both in front-line and in relapsed / refractory patients [7]. However the development of resistance and side effects can limit its use in MM [8]. Most patients show resistance to bortezomib after several courses of treatment and most of them demonstrate multiple drug resistance. In addition, approximately 20% of patients exhibit primary resistance, which determines lack of response to treatment [8,9].

Although resistance to PIs appears to be acquired through a number of different mechanisms, genetic abnormalities play a key role for most anti-myeloma drugs [8,10-12]. Single-point mutations and modification of gene expression in neoplastic cells refractory to PI have been reported in previous studies [11,13-17]. Several genes associated with bortezomib resistance have been identified in MM cells, including *POMP, XBP1, PSMB5, MARCKS, ABCB1, CXCR4, MAF, TXN, TJP1, RPL5, CDK5* and *CYP1A1* [18-25]; however, these genes have been examined individually, and usually only using commercially-available MM cell lines. The present study comprehensively examines the mRNA expression of nine previously-described genes that may affect resistance in patients with a clinically-detected loss of response to PI treatment: *ABCB1, CXCR4, MAF, MARCKS, POMP, PSMB5, RPL5, TXN* and *XBP1*. A better understanding of the genetic disorders involved in MM drug resistance can improve prognosis and prognostication, assist the development of new therapeutic options, and progress the treatment of this disease.

2. Results

The demographic, clinical, and laboratory characteristics of the MM patients enrolled for the study are presented in Table 1. Overall, 30 of the 73 patients were bortezomib sensitive, while the other 43 were refractory. No statistically-significant differences were observed between bortezomib-sensitive and bortezomib-refractory MM patients with regard to bone involvement at diagnosis (p = 0.96), calcium > 2.75 mmol/l at diagnosis (p = 0.89), creatinine > 2 mg/dl at diagnosis (p = 0.31) or Hb < 10 g/dl at diagnosis (p = 0.73) and ISS (p = 0.86). The only statistically-significant difference was observed in predominant paraprotein level (p = 0.02). In addition, light chain disease (LCD) was more common (36.7%) among the sensitive group than the refractory group (9.3%).

Table 1. The characteristics of the patients treated with bortezomib-based therapy and healthy donors. Data are presented as *frequency*, percentage (%) unless otherwise specified.

Variable	MM total	Refractory	Sensitive	Healthy donors	p
Number of patients	73	43	30	11	-

Gender N (%)	M: 43 (58.9) F: (41.1)	M: 25 (58.1) F: 18 (41.9)	M: 18 F: 12	M: 5 (45.5) F:6 (54.5)	0.69
Age + SD (range)	61.9 ± 10.8 (38.2-83.7)	62.2 ± 11.5 (38.2 – 83.7)	61.3 ± 9.7 (39.8 – 81.6)	63.0 ± 6.2 (52.6-74.4)	0.73
Bortezomib regimen:				-	0.18
VCD	58 (79.5)	32 (74.4)	26 (86.7)		
VMP	6 (8.2)	5 (11.6)	1 (3.3)		
VTD	4 (5.5)	2 (4.7)	2 (6.7)		
VD	4 (5.5)	4 (9.3)	0		
IsaVRD	1 (1.4)	0	1 (6.7)		
Paraprotein– N (%)				-	0.02
IgG	41 (56.2)	28 (65.1)	13 (43.3)		
IgA	17 (23.3)	11 (25.6)	6 (20.0)		
LCD	15 (20.5)	4 (9.3)	11 (36.7)		
Prior treatment	12 (16.4)	11 (25.6)	1 (3.3)		0.01
Bone involvement at diagnosis	40 (54.8)	23 (53.5)	17 (56.6)	-	0.96
Calcium >2.75 mmol/l at diagnosis	12 (16.4)	7 (16.3)	5 (16.7)	-	0.89
HB < 10g/dL at diagnosis	26 (35.6)	14 (32.6)	12 (40.0)	-	0.73
Creatinine > 2 mg/dL at diagnosis	10 (13.7)	4 (9.3)	6 (20.0)	-	0.31
International Staging System (ISS) at diagnosis	I: 22 (30.1) II: 17 (23.3) III: 32 (43.8)	I: 14 (32.6) II: 10 (23.3) III: 18 (41.9)	I: 8 (26.7) II: 7 (23.3) III: 14 (46.7)	-	0.86
CRP >5 mg/L	33 (45.2)	16 (37.2)	17 (56.7)	-	0.06
Beta2-microglobuline increased (> 3mg/L)	51 (69.9)	31 (72.1)	20 (66.7)	-	0.36
LDH >240U/L	9 (12.3)	5 (11.6)	4 (13.3)	-	0.85
Cytogenetics- (%)	N=41	N=24	N=17	-	
t(4;14)	9 (22.0)	7 (29.2)	2 (11.8)		0.26
t(14;16)	0	0	0		-
t(14;20)	0	0	0		-
del(17p)	6 (14.6)	4 (16.7)	2 (11.8)		1.00
amp(1q)	22 (53.7)	12 (50.0)	10 (58.8)		0.75
del(13q)	8 (19.5)	2 (8.3)	6 (35.3)		0.61
t(11; 14)	1 (2.4)	1 (4.2)	0		1.00
del(1p)	2 (4.9)	1 (4.2)	1 (5.9)		1.00
IGH rearrangements	19 (46.3)	12 (50.0)	7 (41.2)		0.71

Twelve patients had received at least one prior therapy before bortezomib-based regimen initiation and 11 of them had become refractory to bortezomib. It was found that 41 patients displayed IgG paraprotein, 17 demonstrated IgA, and 15 had LCD. Most of the patients (79.5%) had received a bortezomib, cyclophosphamide, and dexamethasone (VCD) regimen, six (8.2%) VMP (bortezomib, melphalan, and prednisone), four (5.5%) VTD (bortezomib, thalidomide, and dexamethasone), another four VD (bortezomib and dexamethasone), and one received IsaVRd (isatuximab, lenalidomide, bortezomib, and dexamethasone). Cytogenetics data was available in 41 patients (56.1%). Amp (1q) was the most common abnormality (53.7%), followed by IGH rearrangements (46.3%), t(4;14) (22.0%) and del(13q) (19.5%).

The expression of nine mRNAs (*ABCB1, CXCR4, MAF, MARCKS, POMP, PSMB5, RPL5, TXN* and *XBP1*) was determined in all 73 MM patients treated with bortezomib-based regimens and the 11 non-MM controls. Differential expression analysis indicated that RPL5 was significantly down-regulated in MM patients compared with controls (Table 2, Figure 1A). Moreover, POMP was significantly up-regulated in bortezomib-refractory MM patients (Table 3, Figure 1B). No statistically significant differences were found between the groups with regard to the expression of selected mRNAs and the quality of response to treatment (Table S1 and Table S2).

Table 2. mRNA expression in multiple myeloma patients (MM) and healthy donors. The higher ΔCt value represents the lower expression of gene at mRNA level.

mRNA	Δ Ct MM (N=73) mean ± SD	Δ Ct Healthy donors (N = 11) mean ±SD	FC	*p*-value	FWER
ABCB1	7.55 ± 0.99	7.12 ± 0.74	0.74	0.1075	0.6451
CXCR4	3.83 ± 0.82	3.56 ± 0.21	0.82	0.0209	0.1669
MAF	7.75 ± 1.08	7.20 ± 0.85	0.68	0.0737	0.5159
MARCKS	5.99 ± 0.83	5.63 ± 0.90	0.78	0.2346	1.0000
POMP	5.17 ± 0.67	5.12 ± 0.39	0.97	0.7541	1.0000
PSMB5	6.96 ± 0.78	6.80 ± 0.59	0.90	0.4341	1.0000
RPL5	2.73 ± 0.81	2.02 ± 0.46	0.61	0.0004	0.0033
TXN	3.43 ± 0.74	3.69 ± 0.66	1.20	0.2508	1.0000
XBP1	3.26 ± 0.92	3.21 ± 0.66	0.96	0.8036	1.0000

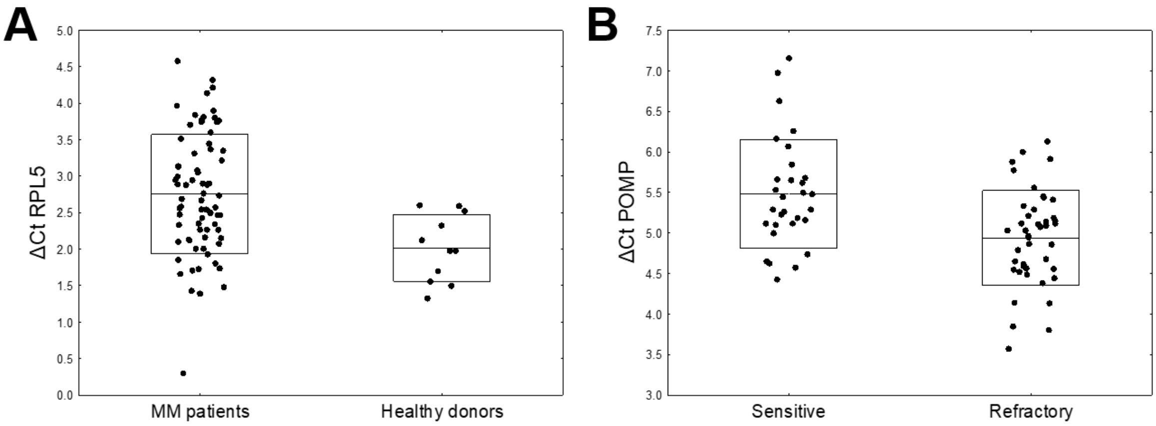

Figure 1. Dot plot representation of the ΔCt values of differentially expressed mRNA. The box plots depict the mean and SD. A higher ΔCt value represents the lower expression of the gene at the mRNA level: (A) ΔCt of *RPL5* in multiple myeloma patients healthy donors (p= 0.0033) and (B) ΔCt of *POMP* in sensitive and refractory to bortezomib MM patients (0.0062).

Table 3. mRNA expression in MM patients sensitive and refractory to bortezomib-based chemotherapy. The higher ΔCt value represents the lower expression of gene at mRNA level.

mRNA	Δ Ct Refractory (N=43) mean ± SD	Δ Ct Sensitive (N=30) mean ± SD	FC	p-value	FWER
ABCB1	7.58 ± 1.02	7.50 ± 0.98	0.95	0.7384	1.0000
CXCR4	3.75 ± 0.70	3.95 ± 0.96	1.15	0.3438	1.0000
MAF	7.70 ± 1.12	7.82 ± 1.03	1.09	0.6516	1.0000
MARCKS	5.79 ± 0.70	6.27 ± 0.92	1.40	0.0190	0.1522
POMP	4.94 ± 0.57	5.48 ± 0.67	1.45	0.0007	0.0062
PSMB5	6.84 ± 0.70	7.12 ± 0.87	1.22	0.1421	0.8523
RPL5	2.69 ± 0.87	2.78 ± 0.75	1.06	0.6622	1.0000
TXN	3.35 ± 0.72	3.55 ± 0.77	1.15	0.2676	1.0000
XBP1	3.08 ± 0.84	3.51 ± 0.97	1.35	0.0537	0.3759

To provide a unified assessment of the prognostic impact of selected mRNA expression level at diagnosis, twelve patients who had received prior treatment before the bortezomib-based regimen were excluded from the outcome analysis. Overall, data on progression free survival (PFS) was available for 49 patients and data on overall survival (OS) for 56 patients. The median PFS was 14.4

months and the median OS was 29.0 months. Univariate Cox proportional hazards regression analysis was conducted to determine the prognostic value of the quantified mRNA expression; the results indicated that in MM patients, higher expression of *CXCR4, MARCKS, POMP, PSMB5, TXN* and *XBP1* was significantly correlated with shorter PFS (Table 4, Figure 2). Univariate analysis found higher expression of POMP and RPL5 to be associated with shorter OS in MM patients (Figure 3). In addition, the only clinical variable which was related to PFS and OS was the use of autologous stem cell transplantation (ASCT) during the treatment schedule (Figure 4).

Table 4. Univariate Cox regression analyses for progression-free survival and overall survival.

Variables	**PFS**					**OS**				
	Coefficient	**p-value**	**HR**	**95% CI**		**Coefficient**	**p-value**	**HR**	**95% CI**	
				lower	**upper**				**lower**	**upper**
ABCB1 expression (high vs. low)	-0.248	0.2716	0.609	0.252	1.474	-0.226	0.2950	0.637	0.273	1.482
CXCR4 expression (high vs. low)	**0.571**	**0.0327**	**3.134**	**1.099**	**8.940**	0.272	0.2865	1.722	0.634	4.679
MAF expression (high vs. low)	0.261	0.1348	1.685	0.850	3.336	0.390	0.2968	2.183	0.504	9.464
MARCKS expression (high vs. low)	**0.594**	**0.0018**	**3.281**	**1.559**	**6.907**	-0.343	0.1115	0.504	0.217	1.172
POMP expression (high vs. low)	**0.409**	**0.0236**	**2.266**	**1.116**	**4.601**	**0.573**	**0.0108**	**3.144**	**1.303**	**7.585**
PSMB5 expression (high vs. low)	**0.476**	**0.0088**	**2.591**	**1.271**	**5.280**	0.348	0.1497	2.004	0.778	5.158
RPL5 expression (high vs. low)	-0.137	0.4206	0.760	0.389	1.483	**0.641**	**0.0035**	**3.607**	**1.526**	**8.524**
TXN expression (high vs. low)	**0.394**	**0.0290**	**2.198**	**1.084**	**4.456**	0.298	0.1683	1.813	0.778	4.228
XBP1 expression (high vs. low)	**0.479**	**0.0099**	**2.605**	**1.259**	**5.389**	0.270	0.2091	1.715	0.739	3.981
Age	0.006	0.7070	1.006	0.975	1.038	0.037	0.1281	1.038	0.989	1.089
ASCT										
No	**Reference**					**Reference**				
Yes	**-0.487**	**0.0089**	**0.378**	**0.182**	**0.783**	**-0.624**	**0.0157**	**0.287**	**0.104**	**0.790**
Bone involvement at diagnosis										
No	Reference					Reference				
Yes	0.303	0.1043	1.832	0.882	3.805	0.309	0.1932	1.856	0.731	4.709
Calcium >2,75 mmol/l at diagnosis										
No	Reference					Reference				
Yes	0.374	0.0929	2.112	0.883	5.052	-0.089	0.7501	0.837	0.281	2.495
CRP >5 mg/L										
No	Reference					Reference				
Yes	0.101	0.6100	1.224	0.563	2.663	-0.461	0.0637	0.398	0.150	1.054
HB < 10g/dL at diagnosis										
No	Reference					Reference				
Yes	0.092	0.6243	1.202	0.576	2.505	0.009	0.9698	1.018	0.409	2.530
ISS I	Reference					Reference				
ISS II	-0.682	0.0590	0.375	0.124	1.134	0.030	0.9389	1.828	0.460	7.267
ISS III	0.383	0.1594	1.089	0.509	2.326	0.544	0.0684	3.056	1.035	9.021
Creatinine > 2 mg/dL at diagnosis										
No	Reference					Reference				
Yes	-0.396	0.1952	0.453	0.136	1.502	-0.253	0.4984	0.603	0.140	2.606
LDH >240U/L										
No	Reference					Reference				
Yes	0.188	0.4221	1.457	0.581	3.651	0.411	0.1526	2.277	0.737	7.032
Gender										
F	Reference					Reference				
M	-0.287	0.1008	0.564	0.284	1.118	0.352	0.1583	2.022	0.760	5.376

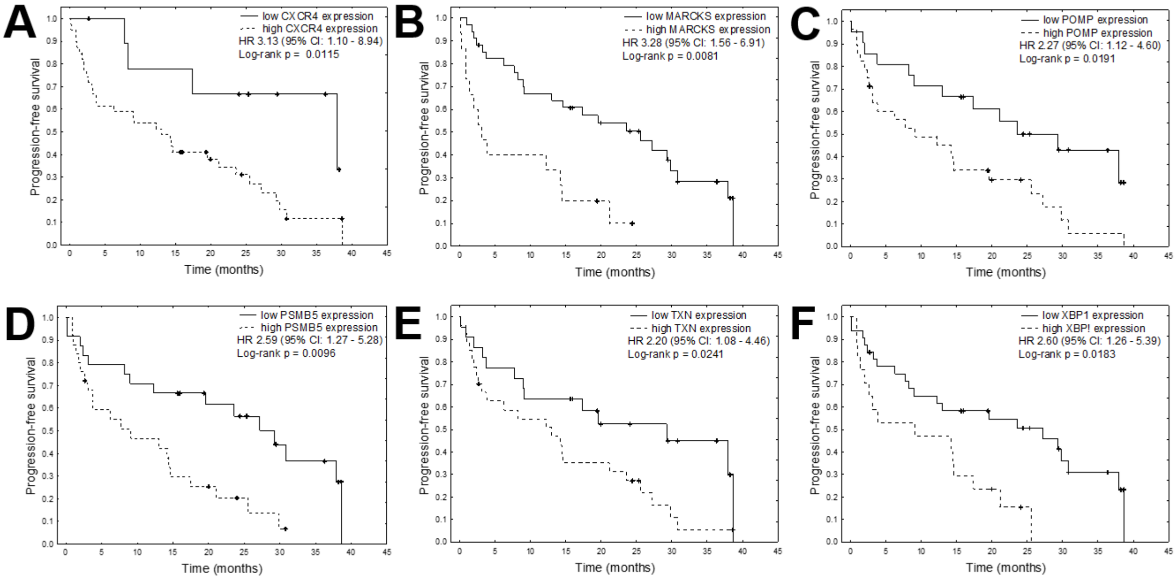

Figure 2. Kaplan-Meier plots for each of the significant mRNAs in the univariate analyses for PFS: **(A)** *CXCR4*, **(B)** *MARCKS*, **(C)** *POMP*, **(D)** *PSMB5*, **(E)** *TXN*, **(F)** *XBP1*.

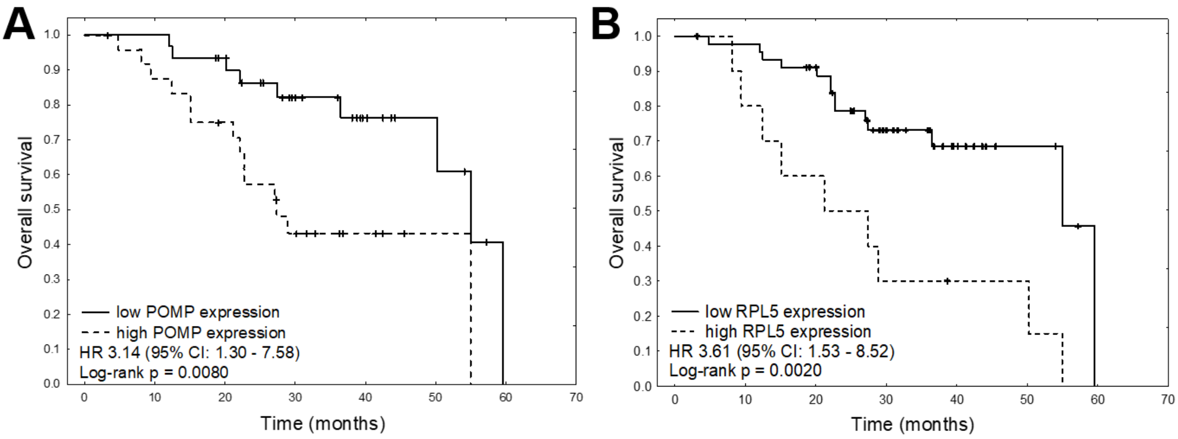

Figure 3. Kaplan-Meier plots for each of the significant mRNAs in the univariate analyses for OS: **(A)** *POMP*, **(B)** *RPL5*.

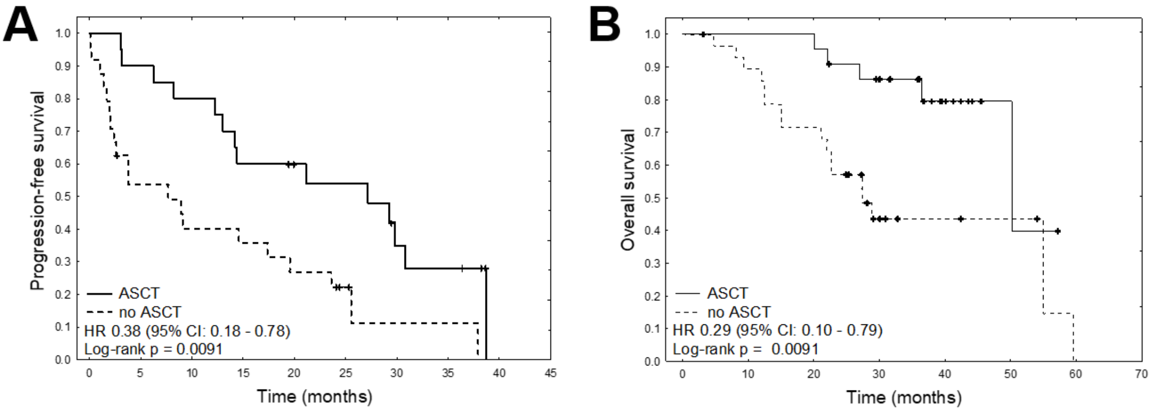

Figure 4. Kaplan-Meier plots for ASCT in the univariate analyses for **(A)** PFS and **(B)** OS.

To further investigate the prognostic factors, multivariate analyses were carried out using Cox's proportional hazards regression model with a stepwise selection procedure. As ASCT was the only significant clinical variable in our univariate analyses with proven prognostic significance, it was entered as covariate in the multivariable model. The results found high expression of *PSMB5* and *CXCR* and the presence of ASCT to be the best independent predictors of PFS (Table 5). Multivariate analysis of OS found high expression of *POMP* and *RPL5* to be associated with shorter survival.

Table 5. Final multivariate Cox regression analyses for PFS and OS of multiple myeloma patients.

Variables	**PFS**				
	Coefficient	**p-value**	**HR**	**95% CI**	
				lower	**upper**
PSMB5 expression (high vs. low)	0.386	0.0451	2.164	1.017	4.603
CXCR expression (high vs. low)	0.748	0.0073	4.465	1.496	13.320
ASCT					
No	Reference				
Yes	-0.612	0.0024	0.294	0.133	0.649
Variables	**OS**				
POMP expression (high vs. low)	0.523	0.0258	2.849	1.135	7.148
RPL5 expression (high vs. low)	0.664	0.0026	3.777	1.591	8.963

3. Discussion

The study comprehensively determined the mRNA expression of nine genes that may affect resistance in 73 MM patients treated with bortezomib-based regimens and 11 healthy volunteers: *ABCB1, CXCR4, MAF, MARCKS, POMP, PSMB5, RPL5, TXN* and *XBP1*. The genes were selected on the basis of previous laboratory and clinical studies investigating the bortezomib resistance in MM patients [19-21,26]. According to the differential expression analysis, *RPL5* gene was the only gene that was significantly down-regulated in MM patients compared with normal individuals; however, higher *RPL5* expression correlated with shorter survival in MM patients. *RPL5* has also been found to be deleted in 20-40% of MM patients, and it is the only recurrently-mutated ribosomal protein gene in MM [27,28].

In addition, RPL5 mRNA expression level was proposed as a clinical biomarker for response to bortezomib in MM patients: Hofman et al. reported significantly lower *RPL5* mRNA expression in patients with MM who initially responded to bortezomib and then relapsed, and both newly-diagnosed and relapsed patients with low *RPL5* expression had better PFS when bortezomib was used in their treatment. In addition, they reported an association between low *RPL5* mRNA levels and initial response to bortezomib in relapsed MM patients. *RPL5* expression has also been associated with shorter survival in newly-diagnosed patients [28].

In our study, *POMP* gene was significantly up-regulated in MM patients refractory to bortezomib-based treatment in comparison with bortezomib-sensitive patients. Higher expression of POMP was found to be associated with shorter survival: POMP protein expression is essential for the biogenesis of proteasome *de novo* and its increased expression facilitates acquired resistance to PI [17]. An increase in POMP protein expression has also been noted in V10R, RPMI 8226, OPM-2, ANBL-6 and KAS-6/1 MM cells resistant to bortezomib [17,29]. Similarly to the present study**,** POMP protein suppression via shRNAs restored cell sensitivity, while over-expression favoured resistance.

A protein-binding site for a suppressive factor, NRF2, has also been identified in the promoter region of the POMP protein. Although its increased expression should increase sensitivity to bortezomib, expression of POMP has been found to be increased in resistant cells, together with increased levels of POMP protein. The activation of both proteins varies according to cell line, and POMP appeared to have a greater effect on bortezomib sensitivity in the KAS-6/1 than OPM-2 line [30].

In the MM patients in the present study, univariate Cox proportional hazards regression analysis found the expression of six of the nine studies genes, viz. *PSMB5, CXCR4, MARCKS, POMP, TXN* and *XBP1*, to significantly correlate with PFS. In addition, the multivariate analysis found high expression of *PSMB5*, *CXCR* and *ASCT* to be the best independent predictors of PFS. Proteasome subunit β type 5 (PSMB5) is the target for bortezomib and other PI inhibitors that harbor chymotrypsin-like proteolytic aktivity [31]. Bortezomib occupies the PSMB5 substrate binding pocket, interfering with the catalytic N-terminal threonine residue. Apart from β5 point mutations, the most frequent change observed in the bortezomib-resistant cell lines was overexpression of the β5 subunit [22,32-34]**.** A recent study by Barrio et al. identfied somatic PSMB5 substitutions in an MM patient treated with bortezomib, suggesting that resistance acquired through PSMB5 point mutations is clinically relevant [23]. Recently, in KMS-18 and KMS-27 MM cells, the PSMB5 gene was found to harbor novel bortezomib resistance alleles which determine response to second-generation proteasome inhibitors in MM [35]. In addition, PSMB5 deletion resensitized drug-resistant, PSMB5-mutated cell lines to bortezomib, suggesting that PSMB5 mutation plays a role in drug resistance [36].

Our findings indicated that higher *CXCR4* expression correlated with shorter PFS. CXCR4 is a pleiotropic chemokine receptor which acts through its ligand (CXCL12) and influences proliferation, invasion, dissemination and drug resistance in MM [37,38]. The current therapeutic focus is on disrupting the interaction of MM cells with their protective tumor microenvironment, in which the CXCR4 axis plays an essential role [39]. In contrast to our present study, reduced expression of CXCR4, a single biomarker in the Bcl-XL/Myc model systém, has indicated poorer outcomes in MM patients treated with bortezomib [40]**.** In addition, low CXCR4 expression was associated with a worse outcome than high CXCR4 expression, and correlated with increased MM severity and aggressiveness in patients treated with bortezomib, either alone or in combination with other agents [20,40].

The univariate Cox proportional hazards regression analysis found the that higher expression of *MARCKS*, *TXN* and *XBP1* significantly correlated with shorter PFS in MM patients. Another marker of PI resistance is MARCKS. This protein is important in cell adhesion and metastatic spread [41] and is involved in resistance to apoptosis in prostate cancer cells [17]. Its expression is significantly elevated in many types of cancer [42]. Micallef et al. reported overexpression of MARCKS in nine of 18 (50%) studied MM cell lines [43]; in addition, in line with our present findings, Yang et al. reported increased MARCKS expression in bortezomib-refactory MM patients, as well as increased bortezomib sensitivity in bortezomib- resistant MM cells following inhibition of MARCKS phosphorylation [44]. Similar effects were achieved in an MM xenograft model [45].

A key role in bortezomib resistance is played by the increased expression of proteasomes and proteins involved in protection from oxidative stress, such as thioredoxin (TXN) [46]. Our findings indicate that higher expression of TXN correlates with shorter PFS. Previous studies have also found TXN to be overexpressed in primary myeloma cells isolated from bortezomib-resistant MM patients, and that overexpression of TXN correlated with poor overall survival in patients with MM [48]. In bortezomib-resistant myeloma cell lines, TXN inhibition overcomes adaptive bortezomib resistence [47]. In addition, higher TXN1 expression levels were found to correlate with myeloma cell survival and growth, and to protect MM cells against increased intrinsic oxidative stress [48]. Moreover, inhibition of TXN1 leads to apoptosis in drug-resistant MM.

Another gene whose high expression significantly correlated with shorter PFS in MM patients is *XBP1*, coding for X-box binding protein 1. The XBP1 protein is an important transcription factor necessary for differentiation of B cells into plasma cells, being responsible for the final maturation of plasmablasts to plasmocytes and the induction of immunoglobulin secretion [49]**.** XBP1 is also a particularly important regulator in the UPR mechanism. It is spliced into two isoforms. One isoform, XBPs1s, activates the genes necessary to reduce ER stress and UPR activation after penetration into the cell nucleus. XBP1 may have a significant impact on resistance to bortezomib in MM cells. Low expression of *XBPS1* has been associated with a lack of sensitivity to PI treatment [50]. Two point mutations in the *XBP1* gene have been identified to date [49,51]: the first, XBP1-L167I, is located within the splice site of the *XBP1* gene, and has been shown to prevent the XBP1 mRNA splicing process needed to form the active XBP1s protein, while the second, *XBP1s*-P326R, is located within the transactivation domain of the XBP1s molecule and has no effect on the splicing process. Cells displaying one of the described mutations lose their sensitivity to bortezomib, inducing disease resistance [52].

In conclusion, our results suggest that high expression of *PSMB5* and *CXCR* may serve as predictors of PFS in MM patients treated with bortezomib based regimens. In addition, high expression of *POMP* and *RPL5* can be useful to predict shorter survival of these patients. . However, further studies are needed to determine the role of this factors in effective strategy for improving anti-myeloma therapy.

4. Materials and Methods

4.1. Patients.

A total of 73 MM patients (43 men and 30 women) treated in our institution (Department of Hematology, Copernicus Memorial Hospital, Lodz, Poland) were included in the study. The mean age of the group was 61.9 ± 10.8 years (range: 38.2 to 83.7 years). Their demographic, clinical and laboratory details are shown in Table 1. All of the patients received bortezomib treatment as first-line treatment or in progression after previous therapy. No patients had received bortezomib-based therapy prior to the study. The participants were classified as either bortezomib-sensitive or bortezomib-refractory, as previously reported, according to their response to bortezomib-based therapy [12,53]. Response to treatment and relapse/progression events were classified according to the International Myeloma Working Group (IMWG) [54,55].

The bortezomib-sensitive patients demonstrated CR, VGPR or PR lasting longer than six months following discontinuation of bortezomib-based therapies [55-57]. In total, 30 patients were bortezomib refractory and 43 were bortezomib sensitive with no progression for at least six months of treatment discontinuation. The control group consisted of 11 healthy volunteers (six women and five men; mean age 61.9 ± 10.8 years; range: 38.2-83.7 years). The study was conducted according to good clinical and laboratory practice

All procedures were approved by the local ethical committee (The Ethical Committee of the Medical University of Lodz, No RNN/103/16/KE). All patients and controls enrolled in the study gave written informed consent for all examinations and procedures. The experimental protocol was conducted in accordance with the Declaration of Helsinki.

4.2. Blood collection

Venous blood samples were collected from MM patients, before treatment with bortezomib-based regimens, and non-MM individuals. Peripheral blood was taken from the 73 multiple myeloma patients and 11 healthy volunteers and collected to the PAXgene Blood RNA Tubes (Qiagen) and stored frozen at -80 °C.

4.3. The analysis of gene expression using real-time PCR

4.3.1. Isolation of total RNA

Frozen blood samples were thawed on ice and total RNA was isolated from 1.5 ml of blood using the QIAamp RNA Blood Mini Kit (Qiagen) according to the manufacturer's protocol. The final elution of total RNA was performed using 50 µl of RNase–free water. Total RNA quality was determined using the High Sensitivity RNA Screen Tape on a 2200 TapeStation bioanalyzer (Agilent). The degradation rate of RNA were determined using RNA integrity number (RIN). Only the samples with RIN>7 were further analyzed. The quantity of RNA was measured using NanoVue Plus Spectrophotometer (GE Healthcare). Directly after isolation, RNA was used for the reverse transcription process.

4.3.2. Reverse transcription reaction

The reverse transcription was performed using the High-Capacity cDNA Reverse Transcription Kit (ThermoFisher Scientific), according to the manufacturer's protocol. The total volume of reverse transcription mix was 20 μL per reaction, containing 2 μL RT buffer (10X), 0,8 μL dNTP mixture (100 mM of each dNTP), 2μL random primers (10X), 1 μL RNase inhibitor (20 U/μL), 1 μL MultiScribe Reverse Transcriptase (50 U/μL), and 10 μL RNA template, whereby the reagent mix was prepared on ice. The thermal profile of the reverse transcription program consisted of 10 min incubation at 25 °C, 120 min at 37 °C, 5 min reverse transcriptase inactivation at 85°C, and cooling down to 4 °C. Total amount of 100 ng of RNA was used as a sample input per 20 μl of reverse transcription reaction. All reactions were performed in a 96-well SureCycler 8800 thermal cycler (Agilent). The resulting cDNA was stored at -20 °C.

4.3.3. Selection of reference genes

A reference gene provides the internal control of the reaction and allow to determine the absolute and reliable value of the studied gene expression using real-time PCR. In order to normalize for variations in sample input for relative quantitation of gene expression, the selection of endogenous control genes was performed using the TaqMan™ Array Human Endogenous Control (Thermo Fisher Scientific). The analysis was performed for six total RNA samples isolated from whole blood of MM patients, according to the manufacturer'sprotocol.

The stability of mRNAs was measured by NormiRazor [58]. This is an integrative tool which implements existing normalization algorithms (geNorm, NormFinder and BestKeeper) in a parallel manner. Three reference genes were selected by NormiRazor and TaqMan™ probes were purchased from ThermoFisher Scientific company: ACTB (Assay ID: Hs99999903_m1), RPLP0 (Assay ID: Hs99999902_m1), MT-ATP6 (Assay ID: Hs02596862_g1) and their average expression was used as reference.

4.3.4. Real-time PCR

The expression of nine genes was analysed in all samples: *ABCB1, CXCR4, MAF, MARCKS, POMP, PSMB5, RPL5, TXN* and *XBP1*. The analysis was performed using commercially-available ready-to-use TaqMan® Assays (Thermo Fisher Scientific). These were preloaded with a probe labeled with 6-FAM™ dye (emission spectra at ~517nm) and forward and reverse primers for the amplification of the following genes: *ABCB1* (Assay ID: Hs00184500_m1), *CXCR4* (Assay ID: Hs00976734_m1), *MAF* (Assay ID: Hs00193519_m1), *MARCKS* (Assay ID: Hs00158993_m1), *POMP* (Assay ID: Hs01106088_m1), *PSMB5* (Assay ID: Hs00605652_m1, *RPL5* (Assay ID: Hs00851991_u1), *TXN* (Hs00828652_m1), *XBP1* (Assay ID: Hs00231936_ml).

The PCR mixture consisted of 10 μl of 2X TaqMan™ Genotyping Master Mix (Thermo Fisher Scientific), 1 μl of appropriate 20X TaqMan® Assay and 1 μl of cDNA template. The mixture was filled up with a distilled, DNase and RNase free water (Gibco) to a final volume of 20 μl. The analysis was carried out using the TOptical thermal cycler (Biometra). The reactions were performed under the following conditions: an initial denaturation step at 95 °C for 10 min, followed by 40 amplification cycles of denaturation (95 °C, 15 sec), a single annealing and extension step (60°C for 1 minute). Fluorescence signal detection was performed after each cycle. Gene expression analysis was performed for each sample in duplicates.. Absolute quantification analysis was performed using qPCR Soft 3.1.15.0 (Biometra).

4.4. Statistical analysis

4.4.1. Data preparation

The data was normalized based on the mean expression of three mRNAs in a given sample (ACTB, RPLP0, MT-ATP6); this has proved to be the most stable normalization factor (according to NormiRazor). The normalized Ct values were calculated as :

Normalized ΔCt = Ct mRNA – (mean Ct of ACTB, RPLP0 and MT-ATP6)

Normalized ΔCt values for all samples and with class assignments were provided as Table S3.

4.4.2. Analysis

Nominal variables were expressed as percentages and analyzed using the Chi-square test with appropriate corrections if needed: the Yates correction for continuity or Fisher's exact test.

For continuous variables, normally-distributed data was tested using a two-sided independent Student's t-test. Continuous variables were presented as mean ± standard deviation (SD) or medians with 25% to 75% values according to the data distribution. Survival analysis was conducted using a Kaplan-Meier estimate with univariate and multivariate Cox's proportional hazards models, as well as the log-rank test. Cutoff Finder was used to determine the optimal cutpoint for gene expression dichotomization based on the log-rank test minimum *P*-value approach [59].

All statistical analyses were conducted using Statistica Version 13.1 (TIBCO, Palo Alto, CA, USA) and R programming language (version 4.0.2). P values lower than 0.05 were considered statistically significant. To control the family-wise error rate (FWER), the significant genes were chosen at 5% using Holm's step-down method. FWER was used to insure a low probability of any false positives among differentially-expressed mRNA.

5. Conclusions

The present study examined the mRNA expression of nine genes with a possible influence on bortzomib sensitivity and refractoriness in MM, viz. *ABCB1, CXCR4, MAF, MARCKS, POMP, PSMB5, RPL5, TXN* and *XBP1*. Of these *RPL5* was down-regulated in MM patients as compared with normal individuals. *POMP* was significantly up-regulated in MM patients refractory to bortezomib-based treatment. Multivariate analysis found that high expression of *PSMB5* and *CXCR* and autologous stem cell transplantation were the best independent predictors of PFS, and that high expression of *POMP* and *RPL5* were associated with shorter survival. The clinical and biological importance of these findings need further investigation.

Supplementary Materials: The following supplementary tables are available on line Supplementary Table S1: *ABCB1, CXCR4, MAF, MARCKS, POMP, PSMB5, RPL5, TXN* and *XBP1* mRNA expression in MM patients with complete remission (CR) to bortezomib-based chemotherapy and those without. Supplementary Table S2: *ABCB1, CXCR4, MAF, MARCKS, POMP, PSMB5, RPL5, TXN* and *XBP1* mRNA expression in MM patients with at least very good partial response (VGPR), partial response, stable disease or disease progression (<VGPR) after bortezomib-based treatment. No difference was found between the two groups. Supplementary Table S3: Normalized ΔCt of mRNA expression for all samples and with class assignments.

Author Contributions: P.R., D.J. E.W. and I.D. performed the experiments; D.M., K.S. and W.F. performed statistical analyses; P.R., I.D., D.M. and T.R. designed the study and wrote the paper. P.R., I.D., D.J., D.M., A.K., M.S., M.M., K.S., W.F., J.S., P.S. and T.R. examined the available material, reviewed and revised the manuscript and provided their approval of the final version of the manuscript. All authors agree to be accountable for all aspects of the work.

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Abbreviations

ACTB – beta-actin gene
ASCT - autologous stem cell transplantation
BM - bone marrow
CXCR-4 - C-X-C chemokine receptor type 4
DLBCL - diffuse large B-cell lymphoma

ECM - extracellular matrix
IPO8 - Importin 8 gene
IsaRVD - isatuximab, lenalidomide, bortezomib, dexamethasone
MAF - musculoaponeurotic fibrosarcoma
MARCKS - myristoylated alanine-rich C-kinase substrate
MM - multiple myeloma
MT-ATP6 - mitochondrially Encoded ATP Synthase Membrane Subunit 6 gene
NRF2 - nuclear factor erythroid 2-related factor 2
NF-κB – nuclear factor kappa B
OS - overall survival
PC - plasma cells
POMP - proteasome maturation protein
PFS - progression free survival
PI - proteasome inhibitor
RPLP0 - Ribosomal Protein Lateral Stalk Subunit P0 gene
RPL5 - ribosomal protein L5
UPR - unfolded protein response
TXN - thioredoxin
VCD - bortezomib, cyclophosphamide, dexamethasone
VD -bortezomib and dexamethasone
VMP - bortezomib, melphalan and prednisone.
VTD -bortezomib, thalidomide, dexamethasone
XBP1 - X-box binding protein 1

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