


Research Paper

High numbers of CD163+ tumor-associated macrophages correlate with poor prognosis in multiple myeloma patients receiving bortezomib-based regimens

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Abstract

The prognostic significance of tumor-associated macrophages (TAMs) in multiple myeloma (MM) in the era of novel drugs remains unclear. CD163 expression was detected by immunohistochemistry to determine the number of TAMs in 198 MM patients receiving bortezomib-based regimens and the data were used to evaluate its relevance with clinical characteristics, treatment response, and prognosis. Patients with high levels of infiltrated CD163+ TAMs (>55/HPF) at diagnosis tended to have more adverse clinical characteristics. Patients with high CD163+ TAM content (>55/HPF) at diagnosis had worse progression-free survival (PFS) ($P<0.001$) and overall survival (OS) ($P<0.001$), and achieved lower complete remission (CR)/near-CR rate ($P<0.001$), than patients with low CD163+ TAM levels. Multivariate analysis revealed that CD163+ TAM content was an independent adverse prognostic factor for PFS and OS. Our data indicated that CD163+ TAM content at diagnosis is a powerful predictor of prognosis for MM in the era of novel drugs, and this discovery offers new insight into potential therapeutic strategies.

Key words: Tumor-associated macrophages; multiple myeloma; CD163; Prognosis

Introduction

Multiple myeloma (MM) is a fatal haematological malignancy that mainly affects elderly people, and is characterized by the proliferation of malignant plasma cells in the bone marrow, resulting in osteolytic lesions, anemia, renal impairment and hypercalcemia [1]. Clinically, the major clinical prognostic system is applied in MM: Revised International Staging System (R-ISS), which divides patients into three groups based on serum albumin, β_2 -microglobulin, lactate dehydrogenase levels, and cytogenetics [2]. The interaction between MM cells

and the different cell components of the tumor microenvironment play a significant role in mediating drug resistance and maintaining minimal residual disease, ultimately influencing the clinical behavior and prognosis [3, 4]. Therefore, the R-ISS cannot provide complete prognostic information because of a lack of tumor-microenvironment information.

Tumor-associated macrophages (TAMs), which are important constituents of the inflammatory infiltrate in various malignancies, are M2-type macrophages, and are characterized with enhanced

CD163 expression [5, 6]. Recently, Chen et al. explored the prognostic value of TAM in a cohort of patients and found that the pretreatment of CD163+ macrophage count in tumor tissue can serve as a valuable biomarker of prognostic factors [7]. However, only 30 patients (12.5%) received bortezomib-based or lenalidomide-based therapy and nearly a quarter of the patients received VAD or MP regimens, which are now considered inappropriate for MM patients. In recent years, the outcome of MM has remarkably improved due to the introduction of bortezomib in frontline regimens [8]. Thus, in the era of novel agents, whether TAM content can retain their prognostic value or not remains to be investigated.

In this retrospective study, we explored the correlation between CD163+ macrophage count in tumor tissues and the clinical features (such as response rate and survival) in MM patients treated with upfront bortezomib-based therapy.

Methods

Eligibility criteria

This retrospective study included 198 patients with symptomatic MM, who were treated in Sun Yat-Sen University Cancer Center from July 2009 to December 2017. These patients were identified using the hospital discharge registry system and their electronic medical records. This study was approved by the institutional review board and ethics committees of the Sun Yat-Sen University Cancer Center. All patients signed written informed consent for tissue samples and medical information. This study was conducted in agreement with the guidelines of the Declaration of Helsinki. The inclusion criteria for this study were as follows: (1) newly diagnosed with symptomatic MM based on the diagnostic criteria of the World Health Organization; (2) had either measurable monoclonal protein (M protein) in blood or urine; (3) previously untreated; (4) no previous or concomitant tumour; (5) available pathology specimens acquired before the initial treatment; (6) complete clinical information and long-term follow-up data were available. All patients were staged according to the International Staging System (ISS) staging.

Treatment and response evaluation

All patients received bortezomib-based regimens such as PAD (bortezomib, doxorubicin, and dexamethasone) [9], VCD (bortezomib, cyclophosphamide, and dexamethasone), [10] and VTD (bortezomib, thalidomide, and dexamethasone)[11]. Patients underwent at least four cycles of treatment, followed by autologous stem cell transplantation (if eligible) or maintenance with bortezomib. Treatment

responses were evaluated after each cycle according to the International Myeloma Working Group criteria [12].

Immunohistochemistry (IHC)

Representative formalin-fixed paraffin-embedded tissues obtained from bone marrow biopsy were submitted for IHC to identify and quantify TAMs in MM. Immunohistochemical staining of CD163 was performed on selected cases by using a CD163 antibody (clone KP1, sc-20060; Santa Cruz) incubated at a 1:50 dilution. Four-micrometer-thick sections embedded with bone marrow paraffin were cut, placed on slides, deparaffinized in xylene, and hydrated in a graded alcohol series. The antigen retrieval procedure was performed by immersing the slides in 10-Mm sodium citrate buffer (pH 8.0), and then heating the slides at 100 °C for 10 min. IHC was performed using a modified avidinbiotin peroxidase complex amplification and detection system.

For gathering the counts of CD163+ TAMs, three slides from each sample and five representative regions (the plasma cell-rich area, approximately 1 mm×1 mm) from each slide were inspected at low-power magnification (×100) under a light microscope (Olympus, BX51, Japan). Afterward, the number of CD163+ TAMs was assessed at high-power magnification (×400) from these fields per case. Only CD163+ cells that displayed macrophagic morphology were counted. The mean number per high-power field was calculated. Quantification was performed in duplicate by two different pathologists, and the data were then averaged and defined as the CD163+ TAM content of each case. Cases that varied significantly between the observers were re-evaluated to arrive at a consensus.

Statistical analysis

The CD163+ TAM counts were presented as median (min, max), whereas categorical data were expressed as *n* (percentages). Differences between the two groups were tested using the Mann-Whitney U test. The receiver operating characteristics (ROC) curve analysis was employed to determine the cutoff value for CD163+ TAMs in the prediction of survival. Kaplan-Meier method was used to calculate the probability of survival, and survival curves were tested using the log-rank test. Univariate and multivariate analyses were performed using the Cox proportional hazards model. Two-sided P values < 0.05 were considered statistically significant. Statistical analysis was performed using the SPSS software (SPSS Standard version 19.0, SPSS Inc., Chicago, IL, USA).

Table 1. Correlation of TAMs with clinical characteristics in MM patients.

Characteristics	No	Median TAMs count (/hpf), (range)	P value
No. of cases	198		
Age			0.752
≤ 65	116	39 (5-126)	
>65	82	40 (5-160)	
Gender			0.822
male	140	40 (5-160)	
female	58	38(8-126)	
ECOG PS			0.010
0-1	114	38 (5-126)	
≥ 2	84	47 (5-160)	
ISS stage			0.122
I	54	38 (5-86)	
II	92	44 (5-126)	
III	52	40 (8-160)	
Type of myeloma			0.564
IgG	92	39(5-117)	
IgA	64	43 (5-160)	
Light chain	42	41 (5-126)	
Serum LDH			<0.001
≤ULN	156	38 (5-160)	
> ULN	42	56 (25-126)	
Genetic abnormalities			0.519
yes ^a	88	42 (5-160)	
no	110	38 (5-126)	
Response after treatment			<0.001
CR/nCR	76	31 (5-126)	
No CR/nCR	122	57(20-160)	

^a Patients with abnormalities of 13q14, 1q21, 14q32 and 17p13

Abbreviations: ECOG, Eastern Cooperative Oncology Group;ISS, international staging system;PS, performance status; LDH, lactate dehydrogenase; CR/nCR,Complete Remission/Near-complete Remission.

Results

Patient characteristics and correlation with CD163+ TAM content.

In total, 198 patients (140 male, 58 female; median age 59 years) met the inclusion criteria. Their clinical characteristics are listed in Table 1. More than half (58.6%) of the patients were <65 years old. A total of 114 patients (57.6%) showed favorable performance

status (ECOG PS 0-1). Good balances of distribution were found in ISS stage with stages I (27.3%), II (46.5%), and III (26.3%). The types of monoclonal protein were as follows: IgG for 92 patients, IgA for 64 patients, and light chain disease for 42 patients. Elevated LDH levels were observed for 42 cases (21.2%). A total of 88 patients (44.4%) presented genetic abnormalities. CD163 expression was detected in the cytomembrane of the macrophages in all 198 cases. Myeloma cells were not stained with the CD163-antibody. Most cases showed CD163+ TAMs infiltrating massively in the tumor tissue (Fig. 1). The mean number of CD163+ TAM content of the whole group was 47.2/high-power field (HPF) with a median of 40/HPF (range, 5-160/HPF). Table 1 shows that the CD163+ TAM content was significantly higher in patients with poor PS, elevated LDH levels, or no complete remission (CR) after treatment. However, no significant correlation was found between CD163+ TAM and age, gender, type of myeloma, and genetic abnormalities. To identify an optimal cutoff point for the survival outcomes, the ROC curve analysis was selected. The most discriminative cutoff levels of CD163+ TAM content was 55/HPF with an area under the curve value of 0.721 (Fig. 2). Based on the ROC analysis result, we used the CD163+ TAM content >55/HPF as the cutoff value in the present study. Based on this cutoff value, 120 patients (60.6 %) were categorized into the low-TAM group (≤55/HPF), and 78 patients (39.4 %) were categorized into the high-TAM group (>55/HPF).

Treatment modalities and response

For the primary treatment modalities, 64 cases (32.3%) received chemotherapy followed by autologous stem cell transplantation (ASCT), and 134

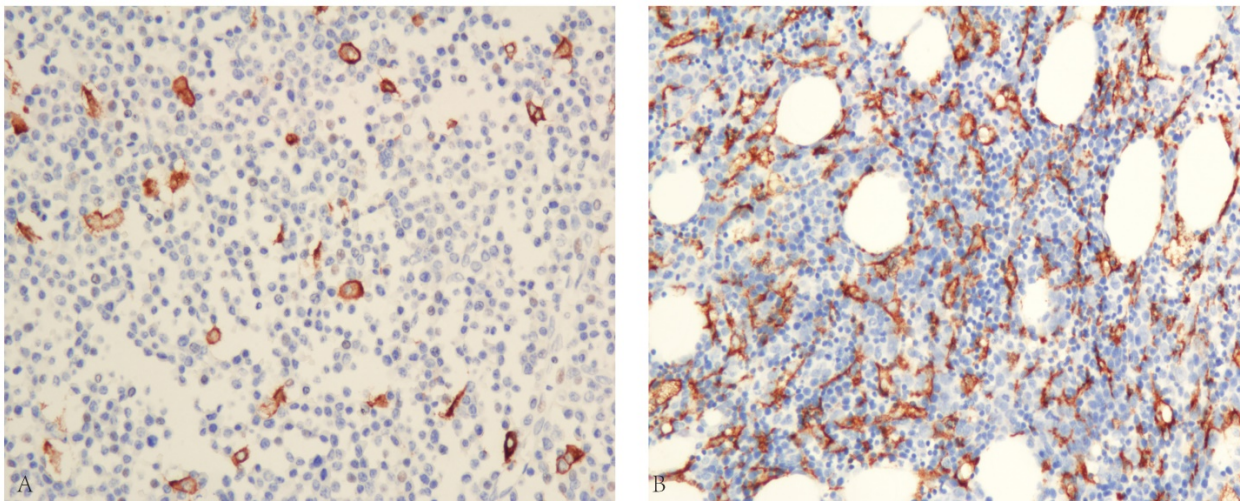


Figure 1. (A) Representation of low TAMs content in immunostained specimens by the LSAB method(×200) (B) Representation of high TAMs content in immunostained specimens by the LSAB method (×200)

cases (67.7%) received chemotherapy alone. The treatment details and responses are listed in Table 2. No significant difference was found in the treatment modalities between the low-TAM and high-TAM group ($P=0.757$). After the initial treatment involving ASCT, 76 of the 198 treated patients (38.4%) achieved CR/nCR. The CR/nCR rate in the low-TAM group was significantly higher than that in the high-TAM group (55.0% vs. 12.8%, $P<0.001$). The overall response rate in the low-TAM group was significantly higher than that in the high-TAM group (85.0% vs 56.4%, $P<0.001$).

ROC Curve

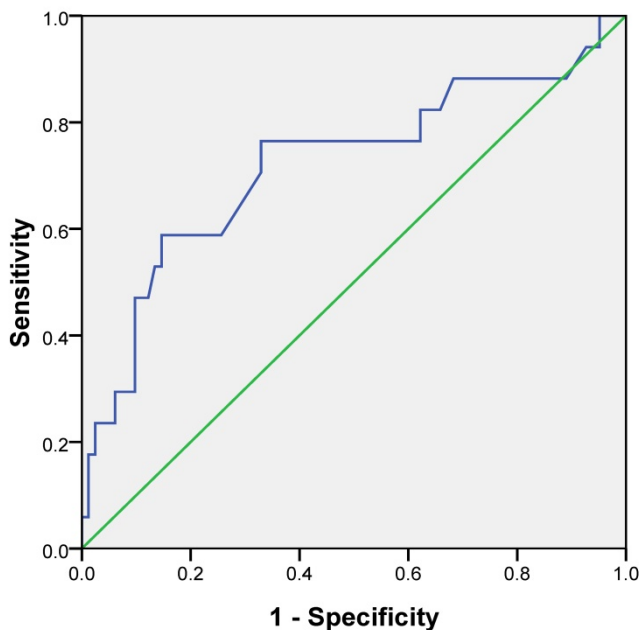


Figure 2. ROC curve analysis for the optimal cut-off point of TAMs content. The most discriminative cut-off value of TAMs content was 55/hpf with an AUC value of 0.721. The sensitivity and specificity were 70.6% and 67.1%, respectively.

Table 2. Primary treatment and response in patients with multiple myeloma.

Treatment	Low-TAM group, n (%)	High-TAM group, n (%)	P value
Treatment modalities			0.757
CT followed by ASCT	40 (33.3)	24 (30.8)	
CT alone	80 (66.7)	54 (69.2)	
Treatment response			
ORR (\geq PR)	102 (85.0)	44 (56.4)	<0.001
CR/nCR	66 (55.0)	10 (12.8)	<0.001
VGPR	16 (13.3)	16 (20.5)	0.236
PR	20 (16.7)	18 (23.1)	0.274

Abbreviations: CT, Chemotherapy; ASCT, Autologous Stem Cell Transplantation; CR/nCR, Complete Remission/Near-complete Remission; VGPR, Very Good Partial Response; ORR, Overall Response Rate; PR, Partial Response

Survival and prognostic factors

At a median follow-up of 28 months (3–106), the 3-year PFS and OS rates for all 198 patients were 41.3 (95%CI 32.3–50.3%) and 82.0% (95%CI 74.6–89.4%), respectively. Patients in the low-TAM group had

significantly better survival than those in the high-TAM group (3-year PFS rate: 60.3% vs 15.6%, $P<0.001$, Figure 3A; 3-year OS rate: 94.8% vs 59.7%, $P<0.001$, Figure 3B). In the patients who received chemotherapy alone ($n=134$, 67.7%), high level of TAM content was associated with shorter PFS and OS ($P<0.001$ and $P<0.001$, respectively) and was also related to inferior PFS and OS ($P<0.001$ and $P=0.018$, respectively) in the patients who received chemotherapy followed by ASCT ($n=64$, 32.3%). Table 3 displays the results of the univariate and multivariate analyses of the potential predictors of PFS and OS. Multivariate analysis by using forward conditional Cox regression model revealed that TAMs content >55 /HPF at diagnosis and certain cytogenetic abnormalities were two adverse factors for PFS and OS.

Discussion

Macrophages are the main components of the tumor microenvironments and are found in different types of cancer. Furthermore, TAMs have a phenotype very that is similar to that of M2-type macrophages, and actively suppress adaptive immunity and promote tumor initiation, growth, and angiogenesis [13–15]. The role of TAMs on influencing the biological behavior of tumor has been extensively studied in hematologic malignancies, including classic Hodgkin's lymphoma (CHL), non-Hodgkin lymphoma (NHL), and multiple myeloma [16–18]. Chen et al. evaluated the content of CD163+ TAMs in 240 cases of MM by IHC, and concluded that a high level of diametrically polarized TAMs is a novel independent poor prognostic factor [7]. Notably, most patients were not treated with bortezomib-based regimens in this study. A lot of progress has been made in MM treatment with the extensive use of novel drugs. New drugs such as bortezomib may overcome some prognostic factors established in the conventional chemotherapy period. However, few studies have looked into the prognostic significance of TAMs in the tumor microenvironment of MM in the era of new drugs.

In this study, we found that CD163+ TAM content was correlated with clinical characteristics, such as poor ECOG PS, elevated serum LDH, and no CR after bortezomib-based chemotherapy, indicating that TAMs might promote the development and progression of myeloma. According to the Cox regression model that included the levels of CD163+ TAM content, ISS stage, elevated serum LDH, fluorescence in situ hybridization (FISH), and ECOG PS score (>2), it was concluded that CD163+ TAM content and FISH abnormalities were independent prognostic factors for both PFS and OS.

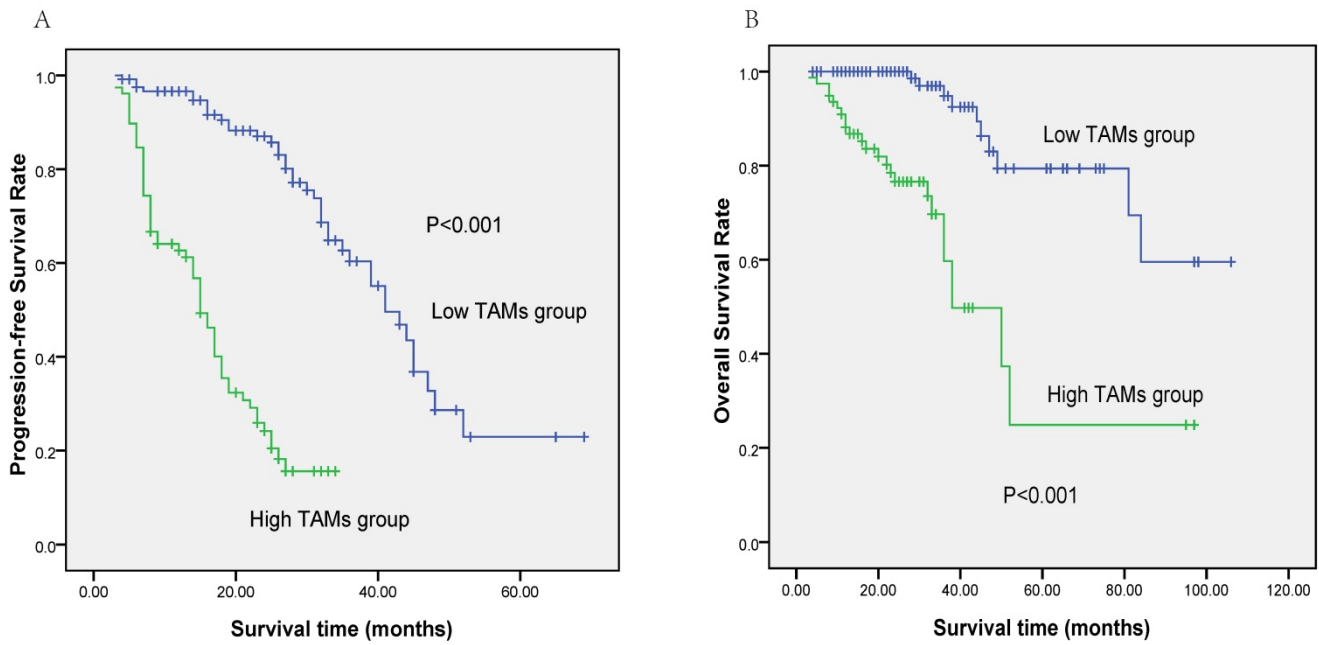


Figure 3. Survival outcome of patients based on the TAMs content. (A) Progression-free survival (PFS) of patients according to TAMs content ($\leq 55/hpf$ vs. $>55/hpf$). (B) Overall survival (OS) of patients according to TAMs content ($\leq 55/hpf$ vs. $>55/hpf$).

Table 3. Univariate and multivariate analysis of factors associated with Progression-Free Survival and Overall Survival of all patients.

Parameters	PFS			OS		
	Univariate analysis P value	Multivariate analysis HR (95%CI)	P value	Univariate analysis P value	Multivariate analysis HR (95%CI)	P value
Age ≥ 65 years	0.272			0.682		
Gender, male	0.431			0.141		
ECOG PS score (>2)	0.043			0.177		
ISS Stage	0.026			0.042		
LDH $>$ ULN	0.042			0.049		
FISH ^a	0.001	2.911 (1.894-4.473)	<0.001	<0.001	6.820(2.781-16.725)	<0.001
TAMs content $>55/hpf$	<0.001	8.301 (5.019-13.727)	<0.001	<0.001	8.370(3.677-19.054)	<0.001

Abbreviations: OS, overall survival; PFS, progression-free survival; HR, hazard ratio; CI, confidence intervals; ECOG PS, Eastern Cooperative Oncology Group performance status; ISS, International Staging System; LDH, lactate dehydrogenase; FISH, interphase fluorescence in situ hybridization;

^aPatients with abnormalities of 13q14, 1q21, 14q32 and 17p13 compared with no FISH abnormalities.

In the present study, the expression of CD163+ TAMs in MM patients who received bortezomib-based regimens was studied. On the basis of the ROC curve analyses, 55/HPF was an optimal cutoff value for distinguishing between different outcomes. Patients with low level of CD163+ TAM content ($\leq 55/HPF$) tended to have higher CR/nCR rates (55.0 %) than those with high level of CD163+ TAM content ($>55/HPF$) (12.8%) ($P<0.001$). This finding suggested that TAMs may be involved in the development of MM resistance to bortezomib-based chemotherapy, although its specific mechanism remains unclear. Zheng et al. found that TAMs can directly come in contact with myeloma cells and attenuate the activation of caspase-dependent apoptotic signaling, and therefore protect these cells from spontaneous and melphalan-induced apoptosis, which implies a crucial role of TAMs in the survival and chemotherapy-resistant mechanism of the

myeloma cell [4]. The 3-year PFS and OS rate in the low-TAM group was longer than those in the high-TAM group (PFS, 60.3% vs. 15.6%, $P<0.001$; OS, 94.8% vs. 59.7%, $P<0.001$). Multivariate analysis including ISS stage, LDH level, and FISH in the molecular cytogenetics also indicated that CD163+ TAM content was an independent prognostic factor for PFS and OS. These results are similar to those of some previous studies [7, 18]. Anderson et al. found that macrophage-derived soluble CD163 was also a prognostic marker in patients with multiple myeloma, implying that TAM, as one of the important components in tumor microenvironment, may influence the clinical course and long-term survival of patients [19]. A common limitation was found among all other studies. First, the data on cytogenetic and FISH markers were not adequate to be included in the analysis. Second, the majority of the patients was treated with conventional chemotherapy regimens in

all other studies. In other words, we first reported that the CD163+ macrophage count in tumor tissue was an independent adverse prognostic factor regardless of the present prognosis evaluation system in the era of novel drug.

Infiltrating macrophages exhibited the M1/M2 polarization phenotype, as a result of responding to the products of malignant cells and adapting to a range of activation states [20, 21]. M1-type macrophages, which are stimulated by interferon- γ and toll-like receptor ligands M1 macrophages, can secrete diverse cytokines and iNOS and participate in killing pathogens and tumor cells in Th1 immune response [22]. By contrast, M2-type macrophages, which are activated by interleukin-4, can secrete interleukin-6 (IL-6) and interleukin-10 (IL-10) and have the tendency to promote tumor growth [23]. CD68 is a general marker of macrophages and CD163 expression was more specific to M2 macrophages than CD68. In this study, we evaluated the number of CD163+ macrophages in tumor tissue that might mainly contain M2 macrophages, which constitute the predominant source of IL-10. In addition, we have demonstrated that increased serum levels of interleukin-10 correlate with negative prognostic factors in multiple myeloma in another study [24]. Xu et al. have reported that M2-type macrophages can enhance the invasive activity of tumor cells and promote cancer stem cell-like properties through the secretion of IL-6 and IL-8 by paracrine secretion [25]. Additional experimental researches focusing on the functions of tumor-associated macrophages are needed to illuminate the mechanism of inducing drug resistance and promoting the development of myeloma.

In conclusion, our study demonstrated the close relationship of CD163+ TAM content with several clinical features of MM, including poor ECOG PS and elevated LDH levels. The high level of CD163+ TAM content is still an independent poor prognostic factor for myeloma in the era of novel drug. These results imply the role of TAMs in the pathogenesis of MM and provide a new insight into potential therapeutic strategies to further improve their therapeutic effect. Further prospective study should be performed with larger sample sizes to validate the prognostic value of TAMs in MM patients receiving bortezomib-based regimens.

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Ethical approval

All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards.

Competing Interests

The authors have declared that no competing interest exists.

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