



Article

Diagnostic, prognostic value of PACAP in multiple myeloma

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Abstract:

Pituitary adenylate cyclase-activating polypeptide (PACAP) is a multifunctional neuropeptide with well-known anti-inflammatory, antioxidant, antitumor, and immunomodulatory effects. PACAP regulates the production of various proinflammatory factors and may influence the complex cytokine network of the bone marrow microenvironment altered by plasma cells affecting the progression of age-related multiple myeloma (MM) and the development of various end-organ damage.

The aim of our study was to investigate the changes in PACAP-38 levels in patients with MM to explore its predictive value as a potential biomarker in this disease. We compared the plasma PACAP-38 levels of MM patients with healthy individuals by ELISA method and examined its relationship with various MM-related clinical and laboratory parameters.

Lower PACAP-38 levels were measured in treated MM patients compared with healthy controls, but this difference disappeared if the patient achieved any response better than partial response. In addition, lower peptide levels were found in elderly patients. Significantly higher PACAP-38 levels were seen in patients with lower stage, lower plasma cell fraction in bone marrow, lower tumor markers in serum, lower urinary protein levels, and in patients after lenalidomide therapy. Higher PACAP-38 levels in newly diagnosed MM patients predicted longer survival and higher probability of response to treatment.

Our findings confirm the hypothesis that PACAP plays an important role in the pathomechanism of MM. Furthermore, our result suggest that PACAP might be used as a valuable, non-invasive, alternative biomarker in diagnosis, and may be used for prognosis prediction and response monitoring.

Keywords: PACAP; multiple myeloma; ELISA; diagnostic and prognostic factor

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1. Introduction

Multiple myeloma (MM) is a monoclonal proliferation of plasma cells that primarily affects the elderly population and accounts for 1% of all cancers and 10% of all hematologic malignancies. The average age of patients is 66–70 years, that contributes significantly to the fact that treatment options at this age are limited due to the comorbidities and performance status of patients. For this reason, and because the disease can relapse up to 10 years after remission, MM is still considered an incurable disease [1]. The Global Cancer Observatory estimates that there were 176,404 new cases and 117,077 deaths worldwide in 2020, which means that despite improving mortality statistics, the incidence

and prevalence of MM is increasing [2]. The disease develops due to the genetic alteration of healthy plasma cells resting in specific niches of the bone marrow. This occurs through the development of monoclonal gammopathy of unknown significance (MGUS) and smouldering myeloma (SMM) leading to an active MM that requires treatment [3]. Given the affected population, ageing is also thought to play an important pathologic role in the development of the disease [4]. After induction therapy, autologous hematopoietic stem cell transplantation (ASCT) provides the longest progression-free survival (PFS) in the treatment setting. However, published data regarding the effect of ASCT in prolonging overall survival (OS) are contradictory [5, 6, 7]. Despite the initial positive results, relapses occur in a significant proportion of patients. It is crucial to intervene in the progression of the disease before serious organ complications occur, because the survival of MM patients is mainly determined by the degree of end-organ damage. Thus, it will become increasingly important in the future to identify new, reliable biomarkers of malignancy that can be easily and rapidly used in the clinical setting to diagnose disease, determine prognosis and monitor response to therapy.

Pituitary adenylate cyclase-activating polypeptide (PACAP) has well known immunomodulatory, anti-inflammatory and antioxidant effects, therefore, it could be one of the most promising markers in the future [8, 9]. The peptide was discovered in 1989 [10] and belongs to the vasoactive intestinal peptide (VIP)/secretin/glucagon peptide family [11]. It exists in two biologically active forms (PACAP-27 and -38) of which PACAP-38 is the dominant form [12]. Its receptors are the specific PAC1 and the VPAC1 and VPAC2 receptors shared with VIP [11, 13]. The concentration of PACAP in the plasma is relatively stable in healthy individuals. Previous studies have shown that its concentration does not depend on gender and is not affected by the female menstrual cycle in the young population between 20 and 40 years [14, 15].

In recent years, significant alterations of this peptide have been detected in human tissues under various physiological and pathological conditions (e.g., neurological disorders: Parkinson's disease, posttraumatic stress disorder; cardiac disorders: cardiomyopathies, acute myocardial infarction; kidney disorders: nephrotic syndrome, nephrectomy) in addition to various malignant disorders. These results suggest a potential role of PACAP in the diagnosis, prognosis, and clinical therapy of certain diseases [8, 16, 17, 18, 19].

The beneficial effects of PACAP on renal function in MM patients and its successful use as an anti-tumor agent have been demonstrated in several in vivo and in vitro studies. It has been described to be protective in proximal tubule cells and to affect signaling processes involved in osteolytic bone destruction [20, 21, 22]. Similarly to dexamethasone, PACAP also inhibits the growth of plasma cells [23]. The peptide is involved in the regulation of the production of several proinflammatory mediators (e.g., TNF- α , IL-6, MIP-1 α) and may affect the complex cytokine network of the bone marrow microenvironment, which is altered by the MM cells influencing the course and progression of the disease and the development of various end-organ damages [20, 24, 25]. Expression of PAC1 receptor mRNA has been detected in human bone marrow stromal cells, MM cells and proximal tubule cells [20, 26]. In a clinical trial, Li and colleagues administered PACAP-38 as a continuous infusion to an 81-year-old patient with active MM. The results were encouraging, as the patient's free lambda light chain excretion was reduced after starting therapy [16].

It is also known that PACAP may play an important role in hematopoiesis and in the development of cells from the mesodermal maturation lineage. The peptide promotes hematopoietic stem cell population via the PAC1 receptor by increasing the number of cells in the S phase of the cell cycle by enhancing the mRNA expression of cyclin D1 and Ki67 proteins [26]. Besides, PAC1 receptor expression is higher on CD34⁺ stem cells and decreases or disappears when the cell is mature [26]. It is suggested that PACAP detectable in bone marrow is of neuronal origin, and sympathetic innervation may be responsible for PACAP-regulated hematopoiesis in bone marrow [26].

These results suggest a beneficial antitumor and renoprotective effect of PACAP in MM and open the possibility of using this peptide in clinical practice. Our aim was to investigate the changes in PACAP levels in patients with MM to explore the role of this peptide as a potential biomarker in this disease. We correlated the detectable changes in PACAP levels with other markers used in clinical practice, such as clinical scores, and laboratory parameters, to gain a more detailed understanding of the pathomechanisms underlying the abnormalities detectable in this disease.

2. Results

2.1. Plasma PACAP-38 levels in MM patients and healthy controls

We found significantly lower endogenous plasma PACAP-38 levels in MM patients (n=66, mean: 208.4 +/- 103.8 pg/mL) compared to healthy controls (C) (n=10, mean: 311.7 +/- 82.19 pg/mL) (p=0.0012) [Fig. 1.].

Out of our patients, 8 were newly diagnosed MM (NDMM), untreated patients with clear medical histories who had died by the end of our investigation, allowing us to monitor the diagnostic and prognostic value of PACAP-38 levels. There was also a significant difference in PACAP-38 values when only these NDMM patients (n=8, mean: 217.4 +/- 84.89 pg/mL) were compared with the control group (p=0.0314).

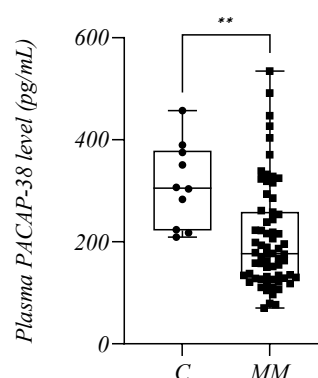


Figure 1. Plasma PACAP-38 levels in multiple myeloma (MM) patients and healthy controls (C). The box plot diagram represents the interquartile range and median values. Whiskers indicate the most extreme observations. The individual values are presented with black dots (control group, n=10), squares (MM patients, n=66). Mann-Whitney U test was used for statistical analysis. **p ≤ 0.01.

2.2. Endogenous PACAP-38 levels in relation to demographic and clinical parameters

No significant difference was found between the patient and control groups, regarding either gender or age (p < 0.05). In our study, we performed correlation analyses between plasma PACAP-38 values and controls' age, patients' age, OS, and PFS. We found a significant weak negative correlation between patients' age and PACAP-38 levels (n=66, p=0.0379, r=-0.2561, Spearman) [Fig. 2.] and there was no significant correlation with controls' age (n=10, p=0.8567, r=-0.06577, Pearson).

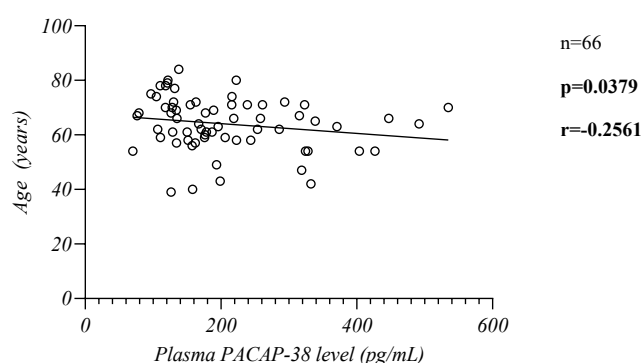


Figure 2. Correlation between endogenous PACAP-38 levels and patients' age. Spearman rank correlation test was used for the analysis.

When we examined survival of NDMM patients, we found a strong positive correlation between plasma PACAP-38 levels and OS ($n=8$, $p=0.0131$, $r=0.8383$, Spearman), and PFS ($n=8$, $p=0.0190$, $r=0.8301$, Spearman). We also found a significant moderate positive correlation between PACAP-38 and PFS, when we examined the survival of patients who had undergone ASCT and then went into remission but had progressed by the end of our study ($n=13$, $p=0.0203$, $r=0.6445$, Spearman) [Fig. 3].

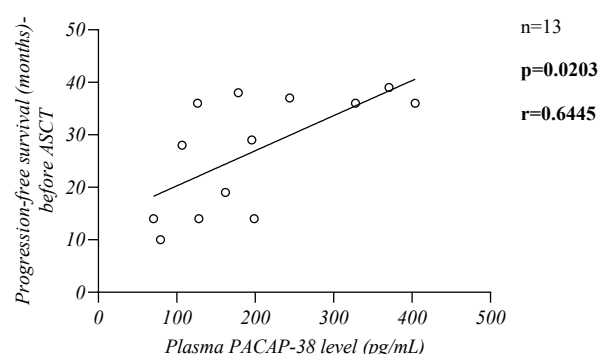


Figure 3. Correlation between endogenous PACAP-38 levels and progression-free survival in patients who undergone autologous stem cell transplantation (ASCT). Spearman rank correlation test was used for the analysis.

Furthermore, we examined the comorbidities of our patients. No significant differences were found between the hypertensive ($n=28$) and non-hypertensive group ($n=35$) ($p=0.3459$) and the diabetic ($n=11$) and non-diabetic group ($n=52$) ($p=0.0743$) [Table 1.]. We also determined the performance status of our patients according to the Eastern Cooperative Oncology Group (ECOG) system and formed four groups: 0 (fully active, able to carry on all pre-disease performance without restriction; $n=16$), 1 (restricted in physically strenuous activity but ambulatory and able to carry out work of a light or sedentary nature, e.g., light house work, office work; $n=26$), 2 (ambulatory and capable of all selfcare but unable to carry out any work activities; up and about more than 50% of waking hours; $n=14$), 3 (capable of only limited selfcare; confined to bed or chair more than 50% of waking hours; $n=1$). No significant difference was found between the tested groups ($p>0.05$) [Table 1.].

Table 1. Plasma PACAP-38 levels in relation to comorbidities and performance status. Mann-Whitney U and Kruskal-Wallis test with Dunn's comparison analysis were used for the statistical analysis. ECOG: Eastern Cooperative Oncology Group, SD: standard deviation.

Tested parameters	Patient groups	Number of patients (n)	PACAP-38 level: Mean +/- SD (pg/mL)	Significance (p)
Hypertension	hypertensive	28	183.3 +/- 71.41	0.3459
	non-hypertensive	35	218.0 +/- 115.0	
Diabetes mellitus	diabetic	11	265.9 +/- 139.9	0.0743
	non-diabetic	52	185.7 +/- 80.36	
ECOG performance status	0	16	240.0 +/- 81.74	p(0 vs 1)=0.7593 p(0 vs 2)>0.9999 p(1 vs 2)=0.3661
	1	26	174.6 +/- 83.69	
	2	14	237.1 +/- 116.0	
	3	1	338.8 +/- 0.00	

2.3. Endogenous PACAP-38 levels in relation to current disease status

2.3.1. Plasma PACAP-38 levels in patients with active disease and in remission

Patients with MM were divided into two groups according to their response to therapy. The active disease cohort (AD) included all individuals with progressive disease, and patients relapsed after a previous remission (n=17, mean: 137.9 +/- 30.13 pg/mL). Patients in remission (R) included individuals who achieved a partial response (PR), very good partial response (VGPR), complete remission (CR), or minimal residual disease negativity (MRD neg.) during treatment (n=41, mean: 216.6 +/- 108.3 pg/mL). Our study showed that the active disease cohort had significantly lower PACAP-38 levels compared to the R cohort (p=0.0137). In addition, plasma PACAP-38 levels of patients in groups AD and R were significantly lower compared to the control group (p(C vs AD)<0.0001, p(C vs R)=0.0170) [Fig. 4.].

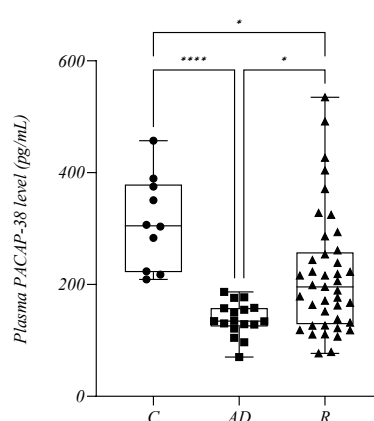


Figure 4. Plasma PACAP-38 levels in control group (C), in patients with active disease (AD) and in remission (R). The box plot diagram represents the interquartile range and median values. Whiskers indicate the most extreme observations. The individual values are presented with black dots (control group, n=10), squares (patients with AD, n=17), triangles (patients in R, n=41). Kruskal-Wallis with Dunn's multiple comparison test was used for statistical analysis. *p ≤ 0.05, ****p ≤ 0.0001.

2.3.2. Endogenous PACAP-38 levels in relation to the depth of therapeutic response

We also performed a detailed analysis regarding the depth of therapeutic response. The AD group included the same patients from the previous analysis, patients in remission were further divided into four groups based on their response to treatment as indicated: PR (partial response; $n=10$, mean: 159.4 ± 77.42 pg/mL), VGPR (very good partial response; $n=13$, mean: 194.9 ± 65.37 pg/mL), CR (complete remission; $n=11$, mean: 231.8 ± 111.0 pg/mL), MRD neg. (minimal residual disease negativity; $n=7$, mean: 314.6 ± 148.6 pg/mL). Significant differences were also observed in this analysis. We found that as the depth of response increased, the patients' PACAP-38 levels steadily increased. The significance shown in the previous figure between the AD and R groups persisted when the AD and MRD neg. groups were examined ($p=0.0048$), with a similar, but not significant tendency between the AD and CR groups ($p=0.0815$). In addition, the MRD neg. patients presented significantly higher PACAP-38 levels than PR patients ($p=0.0350$) [Fig. 5.]. The significant difference we previously showed between MM patients and controls [Fig. 1. and Fig. 4.] gradually disappeared as PACAP-38 values increased with the depth of the therapeutic response [Fig. 5.]. Thus, we did not detect significant differences in PACAP-38 levels between patients with VGPR ($p=0.2083$), CR and healthy controls ($p>0.9999$) and between the MRD neg. group and healthy controls ($p>0.9999$). PACAP-38 concentrations in MRD neg. patients were almost identical to PACAP-38 concentrations measured in the healthy control group (control mean: 311.7 ± 82.19 pg/mL) [Fig. 5.].

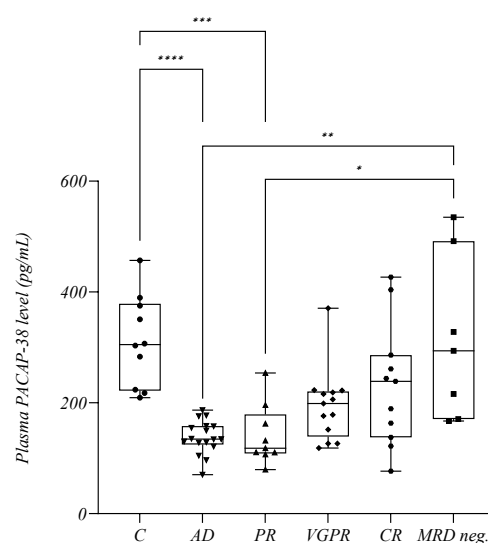


Figure 5. Plasma PACAP-38 levels in relation to the depth of therapeutic response. The box plot diagram represents the interquartile range and median values. Whiskers indicate the most extreme observations. The individual values are presented with black dots (control group (C), $n=10$), downwards triangles (patients with active disease (AD), $n=17$), upwards triangles (patients in partial response (PR), $n=10$), rhombuses (patients in very good partial response (VGPR), $n=13$), hexagons (patients in complete remission (CR), $n=11$), squares (patients with minimal residual disease negativity (MRD neg.), $n=7$). For statistical analysis, the Kruskal-Wallis test with Dunn's multiple comparison was used. * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$, **** $p \leq 0.0001$.

We also examined the extent to which baseline PACAP-38 levels in untreated NDMM patients predicted whether the patient would achieve CR. We divided these patients into two groups: at least CR ($n=3$, mean: 316.4 ± 20.18 pg/mL) and worse than CR ($n=5$, mean: 158.0 ± 25.59 pg/mL). Despite the small number of elements, our preliminary results showed higher PACAP-38 values in patients who reached CR. However, we found a significant difference between patients with relapsed/refractory (RRMM) ($n=4$, mean:

153.2 $\pm$ 26.81 pg/mL) and non-relapsed/refractory MM (nRRMM) (n=4, mean: 281.6 $\pm$ 71.47 pg/mL) (p=0.0302).

2.4. Changes in endogenous PACAP-38 levels in relation to plasma cell infiltration in bone marrow

Our next goal was to examine the alteration of plasma PACAP-38 levels in relation to plasma cell infiltration in the bone marrow, which was assessed by histological analysis [Fig. 6. (a)] and flow cytometry (FCM) results [Fig. 6. (b)] to better estimate the proportion of clonal plasma cells. In both analyses, we found a significant moderate negative correlation between the percentage of plasma cells in the bone marrow and plasma PACAP-38 levels (histological results: n=41, p=0.0020, r=-0.4681; FCM: n=32, p=0.0030, r=-0.5078).

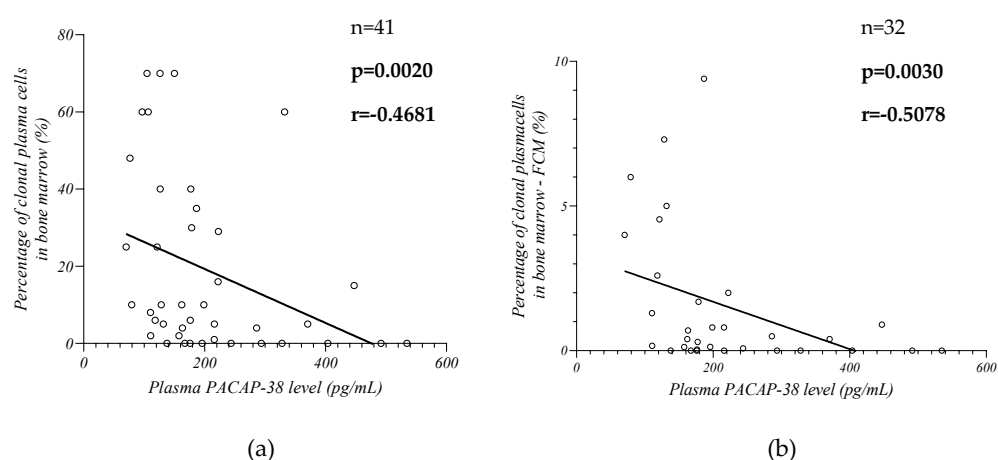


Figure 6. Correlation between endogenous PACAP-38 levels and percentage of clonal plasma cells in bone marrow with histological (a) and flow cytometry (FCM) (b) examination. Spearman rank correlation test was used for the analysis.

When we divided our patients eligible for ASCT into two groups according to the percentage of bone marrow plasma cells below (n=11, mean: 252.4 $\pm$ 115.7 pg/mL) or above 5% (n=17, mean: 168.0 $\pm$ 80.32 pg/mL), we could detect a statistically significant difference. Those with a plasma cell count below 5% had significantly higher PACAP-38 levels than the group above 5% of clonal plasma cells in bone marrow (p=0.0417).

2.5. Examination of endogenous PACAP-38 levels in relation to end-organ damages

2.5.1. Plasma PACAP-38 levels in relation to bone lesion and serum calcium levels

No significant relationship was found either between serum calcium and PACAP-38 levels (n=43, p=0.3657, r=0.1414, Spearman), or between patients with (n=35) and without (n=26) bone lesions. However, the low p-value may indicate a trend towards lower plasma PACAP-38 levels in patients with bone lesions (p=0.0626) [Table 2.].

2.5.2. Plasma PACAP-38 levels in relation to kidney disease

No significant association was found between the plasma PACAP-38 levels and MM-related renal disease either in the study of laboratory parameters (carbamide (n=65, p=0.4363, r=-0.09823, Spearman), creatinine (n=65, p=0.9126, r=0.01388, Spearman), glomerular filtration rate (GFR) (n=65, p=0.9381, r=0.009821, Spearman) indicative of end-organ damage, or when patients were divided into groups with (n=43) and without renal impairment (n=22) (p=0.6448) [Table 2.]. We also formed six groups according to the stage of chronic kidney disease (CKD): normal (normal or increased GFR (above 90 mL/min/1.73 m² body surface) without other evidence of kidney damage; n=22), CKD1

(normal or increased GFR (above 90 mL/min/1.73 m² body surface) with other evidence of kidney damage; n=4), CKD2 (slight decrease in GFR (60-89 mL/min/1.73 m² body surface) with other evidence of kidney damage; n=13), CKD3 (moderate decrease in GFR (59-30 mL/min/1.73 m² body surface) with or without other evidence of kidney damage; n=19), CKD4 (severe decrease in GFR (29-15 mL/min/1.73 m² body surface) with or without other evidence of kidney damage; n=3), CKD5 (established renal failure (below 15 mL/min/1.73 m² body surface); n=4), but we found no significant differences between the tested groups ($p > 0.9999$) [Table 2.].

2.5.3. Plasma PACAP-38 levels in relation to MM-associated anemia

In the case of anemia associated with MM, we examined the connection between plasma PACAP-38 levels and laboratory values indicative of anemia (hemoglobin (Hgb), hematocrit (Htc)). We formed two groups based on whether the patient was anemic or not according to the definition of MM-related anemia (Hgb below 100 g/L). In the present study, we found no significant correlation or association in either case (Hgb (n=64, $p=0.8737$, $r=-0.02027$, Spearman), Htc (n=64, $p=0.7210$, $r=-0.04552$, Spearman); between anemic (n=12) and non-anemic (n=52) groups ($p=0.6898$)) [Table 2.].

2.5.4. Plasma PACAP-38 levels in relation to extramedullary disease, plasma cell leukemia and other end-organ damage

We found no significant difference between patients with extramedullary disease (EMD) or plasma cell leukemia (PCL) (n=18) as a result of the evolutionary process of MM disease and patients who were unaffected (n=48) in this regard ($p=0.3488$). In addition, there was no significant difference between patients with MM-related Amyloid light-chain (AL) amyloidosis (renal or cardiac involvement) (n=4) and non-affected individuals (n=62) ($p=0.1728$) [Table 2.].

Table 2. Plasma PACAP-38 levels in relation to end-organ damages. Mann-Whitney U test and Kruskal-Wallis test with Dunn's comparison analysis were used for the statistical analysis. AL: Amyloid light-chain, CKD: chronic kidney disease, EMD: extramedullary disease, PCL: plasma cell leukemia, SD: standard deviation.

Tested parameters	Patient groups	Number of patients (n)	PACAP-38 level: Mean +/- SD (pg/mL)	Significance (p)
Bone lesions	affected	35	171.1 +/- 67.64	0.0626
	non-affected	26	209.9 +/- 81.52	
Renal impairment	affected	43	207.4 +/- 97.40	0.6448
	non-affected	22	209.9 +/- 119.8	
	normal	22	209.9 +/- 119.8	
Chronic kidney disease	CKD1	4	192.5 +/- 52.53	$p(\text{between all subgroups}) > 0.9999$
	CKD2	13	212.7 +/- 108.6	
	CKD3	19	219.1 +/- 105.4	
	CKD4	3	153.9 +/- 21.74	
	CKD5	4	189.5 +/- 106.7	
Anemia	anemic	12	194.5 +/- 93.06	0.6898
	non-anemic	52	199.8 +/- 89.86	
EMD or PCL	affected	18	233.9 +/- 130.5	0.3488
	non-affected	48	198.8 +/- 91.63	
AL amyloidosis	affected	4	254.3 +/- 87.36	0.1728
	non-affected	62	205.0 +/- 104.7	

2.6. Plasma PACAP-38 levels in relation to staging and risk classifications used for MM

Based on the recommendations of the International Myeloma Working Group (IMWG) for the staging and risk classification of MM, endogenous PACAP-38 levels were examined according to the International Staging System (ISS: Stage I: serum beta-2 microglobulin (B2M) < 3.5 mg/L; serum albumin $\geq$ 3.5 g/dL; Stage II: serum B2M < 3.5 mg/L; serum albumin < 3.5 g/dL; or B2M 3.5 to 5.5 mg/L, irrespective of serum albumin; Stage III: serum B2M > 5.5 mg/L), Revised International Staging System (R-ISS: Stage I: serum B2M < 3.5 mg/L, serum albumin $\geq$ 3.5 g/dL, standard-risk chromosomal abnormalities (CA) by FISH (fluorescence in situ hybridization), normal lactate dehydrogenase (LDH); Stage II: not R-ISS Stage I or III; Stage III: serum B2M $\geq$ 5.5 mg/L and either high-risk CA by FISH or high LDH) and cytogenetic risk classification (high: FISH: 17p-, 1q+, t(14;16), t(14;20) or GEP (gene expression profiling): high-risk signature, estimated 5-year survival: <50%; intermediate: FISH: t(4;14), cytogenetic deletion 13 or hypodiploid or Plasma Cell Labeling Index > 3%, estimated 5-year survival: 50-75%; standard: all other cases, including t(11;16), t(6;14) and hyperdiploid, estimated 5-year survival: >75%). For the analysis, only the PACAP-38 levels of those patients who were diagnosed with MM disease within 1 year of sample collection were examined. For R-ISS and cytogenetic risk scores, we found no significant difference between the tested groups ($p > 0.05$), but there was a decreasing trend between Stage I and II ($p=0.1547$) [Table 3.]. For ISS, we formed three groups in relation to the stages of MM. As shown in the figure, PACAP-38 levels decreased progressively in the higher stages (Stage I: $n=4$, mean: 305.8 ± 147.1 pg/mL, Stage II: $n=13$, mean: 175.9 ± 72.33 pg/mL, Stage III: $n=14$, mean: 143.9 ± 38.59 pg/mL). Significant differences were found between PACAP-38 levels of Stage I and Stage II ($p=0.0108$) and between Stage I and Stage III patients ($p=0.0014$) [Fig. 7.].

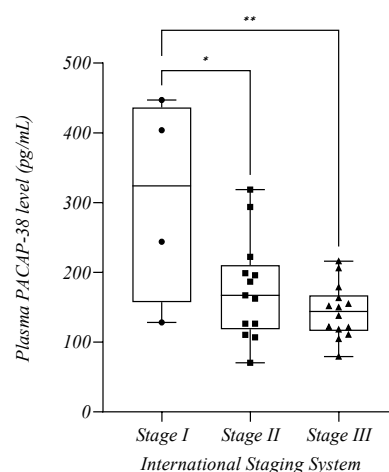


Figure 7. Plasma PACAP-38 levels in relation to stages of the International Staging System. The box plot diagram represents the interquartile range and median values. Whiskers indicate the most extreme observations. The individual values are presented with black dots (Stage I, $n=4$), squares (Stage II, $n=13$) and triangles (Stage III, $n=14$). One-way ANOVA with Tukey's post hoc test was used for the statistical analysis. * $p \leq 0.05$, ** $p \leq 0.01$.

Table 3. Plasma PACAP-38 levels in relation to stage and risk stratification. One-way ANOVA with Tukey's post hoc test and unpaired t test with Welch's correction were used for statistical analysis. R-ISS: Revised International Staging System, SD: standard deviation.

Tested parameters	Patient groups	Number of patients (n)	PACAP-38 level: Mean +/- SD (pg/mL)	Significance (p)
R-ISS	Stage I	4	289.2 +/- 162.8	p(Stage I vs II)= 0.1547 p(Stage I vs III)= 0.8807 p(Stage II vs III)= 0.3914
	Stage II	14	183.9 +/- 74.56	
	Stage III	4	256.6 +/- 85.95	
Cytogenetic risk	standard	14	233.0 +/- 103.5	p(standard vs high)= 0.4283
	inter-mediate	1	106.8 +/- 0.00	
	high	12	201.4 +/- 96.60	

2.7. Endogenous PACAP-38 values in relation to laboratory parameters

In our study, we also investigated the relationship between various laboratory parameters important in MM and plasma PACAP-38 levels. Correlation tests showed a significant, strong negative correlation between B2M (above 5.5 mg/l, n=9, p=0.0255, r=-0.7500), and moderate negative correlation between Bence-Jones (BJ) protein (n=19, p=0.0180, r=-0.5359) and PACAP-38 levels. Besides, we found a negative trend for PACAP-38 and erythrocyte sedimentation rate (ESR) (n=25, p=0.0833, r=-0.3531), urinary total protein (UTP) (n=23, p=0.0522, r=-0.4191) and serum kappa/lambda ratio (KLR) above 1.65 (n=18, p=0.1144, r=-0.3849), but there was a positive trend with KLR below 0.26 (n=8, p=0.1323, r=0.5952). In correlation tests, no significant relation was found between serum LDH, M-protein, albumin, total protein (STP) and PACAP-38 levels [Table 4. (a)]. However, when two groups were formed according to the LDH serum concentrations (225 U/L was chosen as the cutoff between abnormal (n=43) and normal (n=7) LDH values), a substantial decrease in plasma PACAP-38 was observed in patients with higher LDH values (p=0.0339) [Table 4. (b)]. A significant difference was also found when UTP was considered and two groups (above (n=15) and below (n=8) 0.2 g/L protein excretion) were compared (p=0.0282) [Table 4. (b)], confirming the trend of the previous correlation analysis, that revealed lower PACAP-38 values in patients with higher UTP [Table 4. (a)].

Table 4. (a) Plasma PACAP-38 levels in relation to laboratory parameters. Spearman correlation analysis was used for the analysis. B2M: beta-2 microglobulin, BJ: Bence-Jones, ESR: erythrocyte sedimentation rate, LDH: lactate-dehydrogenase, k: kappa light chain, l: lambda light chain, KLR: kappa/lambda ratio, sFLC: serum free light-chains assay, STP: serum total protein, UTP: urinary total protein.

Tested parameters	Number of patients (n)	Correlation coefficient (r)	Significance (p)
B2M (all values, mg/L)	37	-0.09627	0.5709
B2M (>5,5 mg/L)	9	-0.7500	0.0255
LDH (U/L)	50	-0.1472	0.3076
STP (g/L)	52	0.06989	0.6225
Albumin (g/L)	53	-0.09439	0.5014
M-protein (g/L)	38	-0.04738	0.7657
sFLC: KLR – overproduction: l (<0.26)	8	0.5952	0.1323
sFLC: KLR – normal range (0.26-1.65)	22	0.1249	0.5796
sFLC: KLR – overproduction: k (>1.65)	18	-0.3849	0.1144
UTP (g/L)	23	-0.4191	0.0522
BJ Protein (g/L)	19	-0.5359	0.0180
ESR (mm/h)	25	-0.3531	0.0833

Table 4. (b) Plasma PACAP-38 levels in relation to laboratory parameters. Mann-Whitney U test was used for the statistical analysis. LDH: lactate-dehydrogenase, SD: standard deviation, UTP: urinary total protein.

Tested parameters	Patient groups	Number of patients (n)	PACAP-38 level: Mean +/- SD (pg/mL)	Significance (p)
LDH	below 225 U/L	7	249.4 +/- 64.19	0.0339
	above 225 U/L	43	187.2 +/- 84.92	
UTP	below 0.2 g/L	8	344.7 +/- 185.9	0.0282
	above 0.2 g/L	15	193.1 +/- 78.89	

2.8. Plasma PACAP-38 levels in relation to the therapy of MM

2.8.1. Plasma PACAP-38 levels in relation to combination treatment

We also analyzed the PACAP-38 values of our patients in relation to combination treatment and we formed three groups: VTD (bortezomib-thalidomide-dexamethasone) (n=18, mean: 172.9 +/- 72.09 pg/mL), RVd (lenalidomide-bortezomib-dexamethasone) (n=11, mean: 258.1 +/- 107.4 pg/mL), and VCD therapies (bortezomib-cyclophosphamide-dexamethasone) (n=6, mean: 146.3 +/- 44.34 pg/mL). PACAP-38 values of patients treated with RVd were much higher than those of the other groups (p=0.0307) [Fig. 8.].

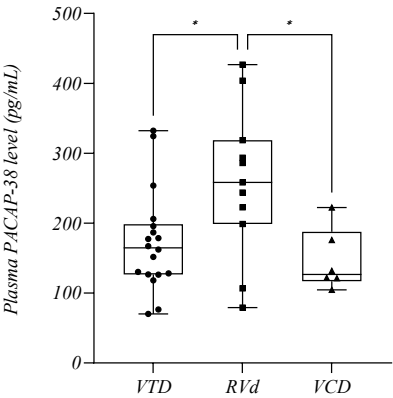


Figure 8. Plasma PACAP-38 levels in relation to the therapeutic combinations. The box plot diagram represents the interquartile range and median values. Whiskers indicate the most extreme observations. The individual values are presented with black dots (bortezomib-thalidomide-dexamethasone (VTD), n=18), squares (lenalidomide-bortezomib-dexamethasone (RVd), n=11) and triangles (bortezomib-cyclophosphamide-dexamethasone (VCD), n=6). One-way ANOVA with Tukey's post hoc test was used for the statistical analysis. *p ≤ 0.05.

The PACAP-38 values of our patients were also analyzed with respect to the therapeutic agents associated with the treatments, and the obtained results were shown in the following table [Table 5.]. We found a significant difference between the lenalidomide-treated (n=18) and the untreated group (n=40) presenting higher PACAP-38 values in the treated cohort (p=0.0062). However, the alkylating agents-treated group (n=10) presenting lower PACAP-38 values than the untreated group (n=48) (p=0.0200). The PACAP-38 values of patients treated with daratumumab (n=4) were also higher, but probably due to the small number of elements, no significant difference could be detected (p=0.0773) [Table 5.].

Table 5. Plasma PACAP-38 levels in relation to therapeutic agents. Mann-Whitney U test was used for the statistical analysis. IMiDs: immunomodulatory agents, SD: standard deviation.

Tested parameters	Patient groups	Number of patients (n)	PACAP-38 level: Mean +/- SD (pg/mL)	Significance (p)
IMiDs	treated	37	199.0 +/- 87.54	0.0558
	not treated	21	154.3 +/- 40.80	
Lenalidomide	treated	18	228.6 +/- 95.63	0.0062
	not treated	40	161.7 +/- 55.43	
Thalidomide	treated	14	164.9 +/- 38.48	0.3083
	not treated	44	198.6 +/- 82.59	
Proteasome inhibitor	treated	44	185.8 +/- 74.11	0.4443
	not treated	14	198.7 +/- 66.63	
Alkylating agents	treated	10	141.3 +/- 38.16	0.0200
	not treated	48	192.8 +/- 71.95	
Steroid	treated	45	181.0 +/- 66.77	0.1582
	not treated	13	225.6 +/- 104.8	
Daratumumab	treated	4	289.6 +/- 150.7	0.0773
	not treated	54	174.3 +/- 64.45	

2.8.2. Plasma PACAP-38 levels in relation to stem cell mobilization

Several plasma samples were collected from patients admitted to the clinic for stem cell mobilization and subsequent peripheral stem cell collection (PBSC). First, samples were collected before the start of conditioning treatment; and second, during stem cell collections. A total of 15 patients with MM were observed throughout the study; in 12 of these patients, collection had to be performed in two sessions (PBSC1 and PBSC2), and in another 2 patients in three sessions (PBSC1, PBSC2 and PBSC3). The samples collected at different times are therefore: before conditioning (BC; n=15, mean: 121.0 +/- 90.79 pg/mL), first peripheral stem cell collection (PBSC1; n=15, mean: 261.6 +/- 150.5 pg/mL), second peripheral stem cell collection (PBSC2; n=12, mean: 276.8 +/- 155.8 pg/mL) and third peripheral stem cell collection (PBSC3; n=2, mean: 694.1 +/- 177.3 pg/mL). We observed that the endogenous PACAP-38 levels were significantly elevated throughout the process (p(BC vs PBSC1)=0.0464, p(BC vs PBSC2)=0.0096) [Fig. 9].

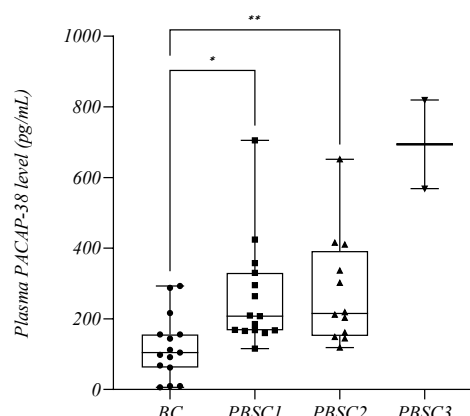


Figure 9. Plasma PACAP-38 levels in relation to the peripheral stem cell mobilization and collection. The box plot diagram represents the interquartile range and median values. Whiskers indicate the most extreme observations. The individual values are presented with black dots (before conditioning (BC), n=15), squares (peripheral blood stem cell collection (PBSC)1, n=15), upwards triangles (PBSC2, n=12) and downwards triangles (PBSC3, n=2). Mixed-effects analysis with Geisser-Greenhouse correction and Holm-Sidak multiple comparison test, with individual variances computed for each comparison were used for statistical analysis. * $p \leq 0.05$, ** $p \leq 0.01$.

3. Discussion

The purpose of this study was to detect alterations of PACAP levels in patients with MM to investigate the role of this peptide as a potential biomarker. We correlated the PACAP-38 levels with other markers used in clinical practice to gain a more detailed understanding of the pathomechanisms underlying the abnormalities seen in this disease.

For a long time, the presence of disease-related end-organ damage was a diagnostic cornerstone of the definition of MM [27]. However, in 2014, the criteria were modified by the IMWG to include three new biomarkers (SMM with more than 60% plasma cells, with more than 100 for free light chain ratios, and/or more than one focal lesion on MRI scans) that were previously considered a group of extremely high-risk abnormalities [28, 29]. The latter modification was necessary because a randomized trial showed that treatment of patients with high-risk SMM can improve their survival [30]. This fact represents a paradigm shift in the approach of the disease, as these changes allow us to intervene in the development and progression of MM before end-organ damage sets in. Traditional biomarkers such as LDH or B2M are useful for estimating tumor mass and, to a limited extent, predicting disease prognosis. However, they cannot account for the clonal heterogeneity that becomes increasingly characteristic of MM cells as the disease progresses. Recently, a number of new biomarkers have been discovered, their use is becoming more widespread and allow us to identify the genetic abnormalities underlying MM disease [31]. However, tumorigenesis in MM is highly dependent on the local tissue microenvironment and its alterations. Some studies report that despite successful antitumor induction, the inflammatory environment formed by tumor cells persists and provides a perfect basis for more pronounced infiltration of residual MM cells. The best therapeutic response can be expected from a therapeutic approach that simultaneously targets the destruction of abnormal plasma cells and the complex bone marrow microenvironment [32]. With the development of diagnostic and proteomic techniques, it is possible to screen for several proteins in MM that may reveal altered factor expression in the bone marrow-specific microenvironment [33], which are necessary for the diagnosis and the prognostic evaluation of MM to enable early diagnosis, the use of personalized biology-based treatments and appropriate monitoring of therapeutic response [34].

Our research suggests that PACAP may be one of these promising markers in the future. PACAP is a multifunctional neuropeptide with proven anti-inflammatory, antioxidant and immunomodulatory effects. The level of endogenous PACAP varies considerably in different diseases, so changes in PACAP levels serve as diagnostic and prognostic markers and may also be helpful in planning clinical therapies for certain diseases [12]. In malignancies, a decrease in the tissue PACAP level was described in colon carcinoma [35], non-small cell lung cancer [35], renal tumors [36], papillary thyroid carcinoma [37], pituitary adenoma [38], pancreatic ductal adenocarcinoma [39], and insulinoma [40], while an increase was observed in neuroblastoma [41, 42], ductal and lobular breast carcinoma [43], cervical cancer [44] and prostate tumor [36], but no significant difference was found in bladder and testicular tumors compared to healthy tissue [36]. The antitumor activity of the peptide has been described in MM and PACAP has also been shown to be protective in the development of osteolytic bone destruction [23] and disease-related renal injury [20, 21]. PACAP has also been described to affect signaling pathways directly involved in MM cell survival and disease progression [21]. In addition, expression of its receptors on MM cells, bone marrow stromal cells and proximal tubule cells has been described, showing that PACAP also affects the abnormal expression of disease-associated factors in the bone marrow microenvironment [21, 26]. However, PACAP has already been shown to contribute to hematopoiesis by affecting various cell cycle regulatory factors via the PAC1 receptor [26].

In our study, we demonstrated a significant decrease in PACAP levels in patients with MM compared with age- and gender-matched healthy controls, which may open the possibility in the future to use PACAP as a potential biomarker for the diagnosis of MM.

We found a significant negative correlation between patients' age and PACAP levels. The results of several *in vivo* experiments suggest that dysregulation of PACAP plays an important role in ageing and the level of this neuropeptide progressively decreases with age. PACAP knockout mice show signs of premature ageing even at a young age. Age-related degenerations due to increased neuronal vulnerability, apoptosis, oxidative stress, and inflammatory processes are observed much earlier in these animals than in wild-type counterparts [45]. PACAP deficiency leads to earlier retinal lesions, corneal keratinization and blurring [46]; senile systemic amyloidosis [47]; and early cartilage degeneration [48]. Although the significant correlation between PACAP levels and age has not been described in either healthy or patient populations, but recently our research group has demonstrated significantly lower PACAP levels in the population of Parkinson's disease patients older than 50 years. In this case, however, an increase in younger patients due to deep brain stimulation could not be excluded [14, 17]. Thus, our study is currently the first in clinical research to demonstrate a significant decrease in plasma PACAP levels in correlation with age of MM patients. While the probability of developing cancer at a young age being 1 in 29, this ratio increases to 1 in 3 at age 70 [49]. So ageing is an important risk factor for most cancers, especially MM [50], which is confirmed by the fact that the incidence of MGUS increases steadily with age [51]. The main risk factors for the development of this disease are age-related immunological changes at the level of terminally differentiated plasma cells, which may lead to stochastic, genetic, epigenetic, and cellular processes that ultimately result in clonal selection and expansion of MM cells. In these processes, PACAP may play a key role by reducing oxidative stress, preventing DNA damage and the development of structural and numerical chromosomal aberrations characteristic of the disease. However, the evolution of manifest MM from asymptomatic forms (MGUS, SMM) is an extremely slow process, and prolonged progression leads to clonal heterogeneity of tumor cells, which also significantly compromises the efficacy of therapy and limits clinical options [4]. So, treating elderly, polymorbid patients with poor performance status is a major challenge [52, 53].

We also found a significant positive correlation between PACAP, OS and PFS. However, the great number of RRMM patients among NDMM patients may have biased this

association between PACAP and PFS. Therefore, we collected follow-up data from patients with few months earlier diagnosis who were about to undergo their first ASCT at the time of our sample collection. In this case, we also found a significant positive association between PFS and PACAP, supporting the potential prognostic role of PACAP in MM [54, 55].

No significant association was found between hypertension, diabetes, ECOG performance status and PACAP levels, suggesting that the most common comorbidities of our patients and their current performance did not significantly affect the results of our study.

In our cross-sectional study, some patients had active disease and others were in remission, therefore, we could assess their current disease status. It was found that patients with active disease had significantly lower PACAP levels. Further grouping of patients in remission according to the depth of response to therapy showed that PACAP values increased steadily with the depth of response until the PACAP values of MRD-negative patients (which is the best prognostic factor [56, 57]) reached the PACAP values of healthy controls. Among NDMM patients, those with lower PACAP values reached CR less frequently, and there were also more RRMM patients. These results confirm our hypothesis that PACAP may be a promising biomarker for evaluating and monitoring therapeutic response.

The significant negative correlation we found between PACAP and the percentage of clonal plasma cells may also be helpful in assessing therapeutic response. This result is consistent with previous studies by Li and Arimura. It has been nearly 20 years since they described that PACAP has antineoplastic effects and directly inhibits plasma cell proliferation. The peptide decreases the levels of IL-6 and TNF- α , leading to the inhibition of p38-MAPK and NF- κ B signaling pathways, which play important roles in plasma cell proliferation, migration and survival [16, 20, 21, 22, 23]. In addition, apoptosis and cell cycle dysregulation are also pathogenic factors in MM, which could be affected by PACAP through the regulation of cyclin D1, Ki67 and various antiapoptotic proteins (e.g., Bcl-2) [26]. Previous research and the results of our study suggest that PACAP restores cell cycle control in MM and, like dexamethasone, can induce apoptosis in MM cells. PACAP could also affect the activation of MYC via the PI3K-mTOR pathway and the function of DNA repair systems by affecting cyclin D [16, 20, 21, 22, 23, 26]. This finding also has prognostic significance, as it is known that a high proportion of plasma cells is associated with shorter life expectancy OS, and in our study, low levels of PACAP were associated with a high proportion of plasma cells [58, 59]. In addition, literature data suggest that patients with a plasma cell count below 5% before transplantation can expect a better therapeutic response and longer survival after ASCT [60]. In our study, we showed that patients before ASCT with plasma cell percentage below 5% had higher PACAP levels, which is also consistent with our previous findings and supports a possible prognostic role of the peptide.

No significant difference was found for the end-organ damage test. Thus, it is unlikely that the presence of organ damage had a significant effect on our studies. However, during the investigation of bone lesion, the low p value already indicates a trend for a difference between the two groups of studied patients, suggesting that PACAP levels may be lower in patients with bone lesions. This result may be explained by the association of PACAP and MIP-1 α , which plays an important role in the development of osteolytic bone destruction by osteoclast activation and may affect patient survival [61, 62, 63, 64, 65]. It has been described that PACAP can inhibit the expression of MIP-1 α already at the mRNA level, suggesting that PACAP may play an important role in the development MM-induced bone destruction [66, 67, 68].

We also tested PACAP values for the IMWG-recommended risk and staging classification systems used in our patients, and only in ISS have we found significant differences. Significant decrease in PACAP levels was observed in higher ISS stages, which may indicate a potentially valuable prognostic biomarker role of PACAP. For R-ISS, there was already a trend toward decline between Stages I and II, but no significant difference was observed between the two groups. This result is probably due to the fact that ISS uses B2M

and albumin to assess stage, whereas R-ISS also considers cytogenetic risk and LDH. In our study, cytogenetic risk was not associated with PACAP levels, so we suggest that the significance of ISS is biased by the inclusion of genetic abnormalities in plasma cells [69, 70].

For B2M, the laboratory parameter that most strongly influences ISS, a significant negative correlation with PACAP was described at elevated levels, with a very strong r value. In the case of LDH, an indicator of tumor mass, we have shown that lower PACAP values are associated with higher LDH values. Elevated values of both laboratory parameters serve as poor prognostic markers [71, 72, 73]. When we examined various serum and urine proteins that indicate disease progression of MM, we found significant changes between BJ protein, UTP and PACAP. Increased levels of both protein levels were observed in correlation with lower PACAP levels. Our results on BJ protein confirm previous data in the literature, as Li et al. reported in their case study that continuous PACAP infusion reduced the patient's free lambda light chain excretion [16]. The amount of BJ protein refers to two things, one is the extent of renal damage and the other is the tumor mass [74, 75, 76, 77, 78]. When these proteins were examined, patients showed renal damage, irrespective of UTP. Renal function fell within the normal GFR range in only one patient. Considering this information, we suggest that this result is not related to the renal function of the patients, but to the amount of paraproteins produced by the MM cells. The most accurate method currently available to detect paraproteins in serum is the serum free light-chain (sFLC) assay, in which the KLR was measured. A negative trend between PACAP and KLR above 1.65 and a positive trend below 0.26 was observed. This result is confirmed by the correlation with the amount of light chains in urine. In our study, there was also a negative trend between PACAP and ESR. The altered albumin/globulin ratio indicated by ESR also suggests that PACAP must affect the amount of paraproteins produced by plasma cells. Besides, ESR is an independent marker of MM, which elevated level is associated with poor prognosis. The opposite trend to PACAP also confirms the potential role of this neuropeptide as a biomarker [79, 80].

In the last part of our study, we examined the relationship between PACAP and therapeutic procedures in MM. In the case of combination treatments, the highest PACAP levels were measured for the RVd protocol. There have been several studies comparing the therapeutic protocols and there is evidence to suggest that RVd may be associated with better outcomes compared to VTD. RVd is associated with a higher overall response rate, a longer time to disease progression, and a longer PFS to VTD. RVd also showing improved efficacy and tolerability compared to VTD [81, 82]. It is known that the RVd protocol is the most effective among currently used antimyeloma therapies, it is listed in the first line in the EHA-ESMO 2021 guideline, because this protocol increases OS and PFS the most [5, 83]. The RVd protocol differs from the others in that it contains lenalidomide, which also has immunomodulatory effects. When therapeutic agents were examined, lenalidomide was the only agent that showed a significant correlation with an increase in PACAP levels, while in the case of other agents PACAP levels decreased. The effects of immunomodulators (IMiDs) are highly variable. They increase the expression of tumor suppressor genes, arrest the cell cycle, inhibit angiogenesis, reduce the levels of adhesion molecules, inhibit the adhesion of MM cells to bone marrow stromal cells, suppress osteoclast formation and function, and decrease the expression of proinflammatory cytokines. IMiDs also affect the immune system's ability to recognize tumor cells [5, 84, 85]. Although thalidomide, lenalidomide, and pomalidomide have similar biological activity, the latter two are more effective [86]. Bone marrow stromal cells are of particular importance in supporting MM cells. MM cells modulate the bone marrow microenvironment by inducing the production of cytokines that promote plasma cell survival and proliferation, and this inflammatory environment persists after remission [32]. Regarding the study of these cytokines, it is already known that both PACAP and lenalidomide have effects that lower the levels of these factors (IL-6 and TNF- α) thus attenuating the processes in the microenvironment that contribute to the survival of MM cells [20, 84, 85]. A similarly promising

and effective agent is daratumumab, which also showed an increasing trend in PACAP, but presumably due to the small number of elements, no significant difference was detected. The explanation for the decrease in PACAP with other therapeutic agents, especially alkylating agents (cyclophosphamide, melphalan), requires further investigation. There is no extensive literature on this topic yet, but it is known that low-dose PACAP treatment protects against cyclophosphamide-induced thymic atrophy. Cyclophosphamide also increases PAC1 receptor expression. These results suggest that alkylating agents may lead to a decrease in endogenous PACAP levels [87].

For the therapies used in MM, we also examined how PACAP levels behaved during stem cell mobilization and collection. This showed that PACAP levels gradually increased during the process, which is likely related to G-CSF injection [83]. The exact role of PACAP in hematopoiesis is still unclear, but previous studies suggest that PACAP may contribute to the regulation of hematopoiesis in the bone marrow through its receptors and affects the function and maturation of CD34⁺ hematopoietic stem cells [26].

In conclusion, these results confirm our hypothesis that PACAP plays an important role in the pathogenesis of this disease and support the use of this peptide in clinical practice as a valuable biomarker.

4. Materials and Methods

4.1. Selection of patients for the study

The study included patients with confirmed diagnosis of MM in the Division of Hematology, 1st Department of Internal Medicine, Clinical Centre, University of Pécs. Sample collection and patient data follow-up were conducted between July 2019 and January 2023. A total of 66 patients with MM were included in the examination, most of whom were on treatment. At the time of the study, there were a total of 8 patients who had not received treatment. The mean age of the patients was 63.97 $\pm$ 9.807 years; 35 women and 31 men participated in the study. The youngest patient was 39 and the oldest was 84 years old. The control blood samples were taken from mostly healthy volunteers who did not suffer from hematological diseases, but three of them had hypertension, the others were completely healthy and were not on any medications. The mean age of the control group was 62.10 $\pm$ 9.643 years, the oldest participant was 77 years old and the youngest was 47 years old. The study involved 6 women and 4 men.

4.2. Collection and handling of blood samples and patient data

Several routine laboratory and histological tests were performed in most of the participants: total and ionized calcium, renal function (serum creatinine and urea nitrogen or carbamide levels), inflammatory parameters (serum C-reactive protein level and ESR), complete blood count (Hgb, Htc, white blood cell count (WBC), red blood cell count, platelet count), LDH, serum and urine protein determinations (electrophoresis, immunofixation and light chain assay: STP and UTP, albumin, B2M, BJ protein, M-protein, KLR) and percentage of plasma cells in bone marrow (analyzed with histological and FCM examinations) were determined. These markers can be used to monitor the progression of disease and organ damage. Where these tests were not performed on the day of sampling, the results were analyzed retrospectively up to 1 month for laboratory tests and up to 6 months for histological reports. All laboratory tests were performed in the Department of Laboratory Medicine, and Department of Pathology, Clinical Centre, University of Pécs. In addition, patients with MM were evaluated according to age, gender, OS and PFS, disease status, ECOG score, different staging (ISS, ISS-R) and risk classifications (cytogenetic risk), presence of end-organ damage or myeloma defining event: anemia, hypercalcemia, bone lesion, renal failure. Furthermore, we considered the therapeutic protocol according to which the patients were treated, whether they underwent stem cell transplantation or not, and if the patient was submitted for mobilization and collection of stem cells, this process was also accompanied by sampling.

For PACAP-38 determination, peripheral venous blood was taken in tubes including EDTA (ethylene-diamine tetra-acetic acid). Because of the presence of dipeptidyl-peptidase IV (DPP-IV) in the plasma, a protease inhibitor (20 µL of aprotinin solution at 1.4 mg/mL for 1 mL of blood) was added to the blood samples and an ice water bath was used for storing the tubes to avoid peptide degradation. The EDTA-tubes were centrifuged twice immediately after the collection (first 1000 rpm, 4 °C, 5 min; then 3500 rpm, 4 °C, 15 min), then the supernatant was collected and stored in polypropylene tubes (Sarstedt, Budapest, Hungary) at -80 °C until ELISA analysis.

Patients with MM had their blood drawn once (6 mL blood in an EDTA tube) and their disease stage and treatments were accurately documented. Patients (n=15) who came for stem cell mobilization had a blood sample drawn on the first day of their hospital admission (6 mL blood in an EDTA tube). Thereafter, most patients received chemotherapy according to the mobilization protocol, followed by once-daily sc. granulocyte colony-stimulating factor (G-CSF) injections. The patients' WBC count was continuously monitored, and after the aplasia period, if the absolute neutrophil count exceeded 2 G/L, blood was drawn and the CD34⁺ cell count was determined by FCM. When the CD34⁺ cell count in peripheral blood reached $\geq 20/\mu\text{L}$, the patient underwent cytapheresis. At the same time, an additional 6 mL of blood was collected in an EDTA tube to measure endogenous PACAP-38 levels by ELISA. If one collection did not yield at least one graft sufficient for one autologous stem cell transplantation - two in younger patients - the procedure was repeated the next day. If the collections were repeated, we also repeated our sampling.

If the MM patient was submitted for mobilization and collection of stem cells, only those samples were included in the baseline MM samples, which were collected before the start of the mobilization protocol, because a preliminary study by our research group showed that PACAP-38 was increased in plasma samples during the process. Therefore, samples from patients who had already received mobilization chemotherapy and G-CSF injections at that time were excluded from this part of the study. These samples were evaluated in a follow-up study as a series of contiguous to evaluate changes in PACAP-38 levels during mobilization and collection in individual patients.

All human sample collections were carried out according to a protocol approved by the Institutional Ethics Committee (PTE KK 6383) and followed the Declaration of Helsinki and International Conference on Harmonization (ICH) Good Clinical Practice (GCP) Guidelines to protect the rights of human subjects. In all cases, we obtained informed consent of the volunteers.

4.3. Measurement of PACAP-38-like immunoreactivity (LI) by ELISA

A conventional, sandwich type enzyme-linked immunosorbent assay (ELISA) (human PACAP-38 ELISA kit, MyBiosource, San Diego, CA, USA) was performed to quantify the PACAP-38-like immunoreactivity (LI) in the collected clinical samples. According to our earlier studies [18] the determined concentrations are referred as PACAP-38 levels or values in the manuscript. The assay was performed according to the manufacturer's instructions. Briefly, before the assay, frozen samples and test reagents were thawed and allowed to reach room temperature. Then 50 µL pre-diluted PACAP-38 standards and plasma samples were added to the appropriate wells of the pre-coated 96-well microplate in duplicate. For the blank wells only sample diluent was added to the plate. Then 100 µL of horseradish peroxidase conjugate was added to each well, and the kit was incubated at 37 °C in the dark for 60 minutes. After incubation, the plate was manually washed with 200 µL of wash buffer, and the procedure was repeated four times. Then 50 µL of chromogen solution A and 50 µL of chromogen solution B were added to each well of the plate and incubated for 15 minutes at 37 °C in the dark. Once the appropriate colour reaction occurred, the enzyme reaction was stopped by adding 50 µL of stop solution and the optical density (OD) was read within 15 minutes at a wavelength of 450 nm. The SPECTRO-Star Nano spectrophotometer (BMG Labtech, Ortenberg, Germany) was used to measure the OD of the test wells. Since the obtained OD values were proportional to the content of

PACAP-38 in the test samples, their concentrations were calculated by comparing the OD values of the sample wells with the ODs of the standard curve. All measured PACAP-38 concentrations in plasma are expressed in pg/mL.

4.4. Statistical analysis

GraphPad Prism version 9.5.0 Windows program (GraphPad Software, San Diego, California, USA, www.graphpad.com) was used for statistical analysis. Statistical operations were performed first by performing a descriptive analysis and then determining the distribution of samples. Outliers were identified using the robust regression and outlier removal (ROUT) method and excluded from the finer-grained statistical analysis. For comparisons between groups, the Unpaired t test, Mann-Whitney U test, One-way ANOVA test, Kruskal-Wallis test and Mixed-effect analysis with Tukey's, Dunn's, or Holm-Sídák multiple comparison analysis were used, depending on the number of the compared groups, the distribution of the data sets (normal or not normal) and the types of the variables (dependent or independent). For comparing two or more, normally distributed groups with different homogeneity of variance, Welch or Geisser-Greenhouse correction were applied. Pearson (normal distribution) and Spearman (non-normal distribution) correlation analyses were performed to examine correlations between parameters. The correlation coefficient (r) was used to determine the strength and direction of the linear relationship between variables. In all cases, $p < 0.05$ was considered statistically significant.

5. Conclusions

In conclusion, lower PACAP levels detected in MM and the further significant decrease in peptide levels in active disease support our hypothesis that PACAP may play an important role in MM. We suggest that the differences seen in the patients described in our study are due to a complex microenvironmental effect induced by plasma cells that results in reduced PACAP levels. This mechanism could lead to an additional survival advantage for plasma cells that abrogates the antitumor effect of PACAP and further worsens the condition of patients with progression. In addition, the significant prognostic role of the peptide is supported by the fact that with increasing depth of therapeutic response, PACAP levels of MM patients rising until it reaches plasma values of healthy subjects. Our hypothesis is further supported by the finding that PACAP is also associated with patients' age, survival, plasma cell percentage in the bone marrow, B2M, BJ protein, LDH, and ISS. We conclude that PACAP (along with other markers) may be a promising biomarker in the future to aid in the diagnosis of MM, assess prognosis and potentially monitor the efficacy of clinical therapy.

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Institutional Review Board Statement: All human sample collections were carried out according to a protocol approved by the Institutional Ethics Committee (PTE KK 6383) and followed the Declaration of Helsinki and ICH GCP Guidelines to protect the rights of human subjects. In all cases, we obtained informed consent of the volunteers

Informed Consent Statement: Informed consent was obtained from all subjects involved in the study.

Data Availability Statement: The data presented in this study are available in the article, there is no supplementary data.

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