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THE ROLE OF ADIPOKINES IN MONOCLONAL GAMMOPATHIES

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Abstract

In the pathogenesis of monoclonal gammopathies (MG), namely in multiple myeloma (MM), the bone marrow microenvironment displays a critical role. As there is accumulating evidence supporting a link between obesity and MM, it is possible that this association is made through altered adipokines secretion levels. Whereas these hormones are important in several physiologic functions, there are studies showing that they also participate in the carcinogenesis process of some solid tumors. However, adipokine's role in hematological malignancies is poorly understood.

We studied the role of adiponectin, leptin, resistin, MCP-1, and TNF-α in patients with MG, by correlating the peripheral blood (PB) and bone marrow (BM) levels of these adipokines with patient's clinical and laboratorial characteristics, as well with prognostic factors.

A total of 111 MG patients - 52 with monoclonal gammopathy of undetermined significance (MGUS), 20 with smoldering multiple myeloma (sMM) and 39 with symptomatic MM - and 69 non-neoplastic controls were included in this study. PB and BM adipokines levels were accessed using ELISA commercial kits. Comparison between groups of patients and controls was performed using nonparametric Mann-Whitney test. Survival analysis was made by the Kaplan-Meier method.

Our results show an increase in leptin and resistin PB levels in MG patients compared with controls. In MGUS patients we observe the highest adiponectin PB and BM levels, and in symptomatic MM the lowest, having sMM patients an intermediate value. On the other hand, leptin levels were higher in the PB and BM of sMM individuals when comparing with MGUS and symptomatic MM. MCP-1 tended for higher levels in patients in the later stages of the disease. sMM was also associated with higher PB TNF-α levels. Results from BM of patients were somewhat overlapping with those from the PB.

When considering laboratorial characteristics, adipokine secretion seems to be dependent of the type of heavy chain produced. Light chain symptomatic MM patients were associated with the higher levels of adiponectin and TNF-α, as well with the lower levels of leptin and resistin, when compared with IgG and IgA symptomatic MM patients. In what concerns the type of light chain that is produced, MGUS individuals producing κ light chains, presented higher BM levels of MCP-1.

Additionally, the presence of hypercalcemia and bone lesions in symptomatic MM patients, is associated with higher PB and BM leptin levels. Also MCP-1 levels in the BM of patients who had anemia were higher than those who did not have. According to the ISS, stage III patients, seem to have higher PB levels of resistin than those in stage I. However they show the inverse tendency in the BM. Finally, higher levels of PB adiponectin and BM leptin were associated with decreased overall survival.

These results suggest that, adipokines may be involved in MG, namely in MM, by creating a pro-inflammatory BM microenvironment, that could contribute to the carcinogenesis process, and by participating in the immunological changes underlying the pathogenesis of MM. As MM remains an incurable disease, better understanding of the MM pathogenesis, is of critical importance to create new therapeutic approaches and improve the patient's prognosis.

Keywords

Monoclonal Gammopathy; Multiple Myeloma; Adipokines; Microenvironment; Prognosis

Resumo

Na patogénese das gamapatias monoclonais (GM), nomeadamente no mieloma múltiplo (MM), o microambiente da medula óssea exibe um papel importante. Com a evidência acumulada que suporta uma ligação entre obesidade e o MM, é possível que esta associação seja feita através de uma alteração dos níveis de secreção das adipocitocinas. Enquanto estas hormonas são importantes em diversas funções fisiológicas, existem estudos que mostram que estas também participam no processo de carcinogénese de alguns tumores sólidos. No entanto, o papel das adipocitocinas em neoplasias hematológicas é pouco compreendido.

Nós estudámos o papel da adiponectina, leptina, resistina, MCP-1 e TNF-α em doentes com GM, correlacionando os níveis de adipocitocinas no sangue periférico (SP) e medula óssea (MO), com as características clínicas e laboratoriais dos doentes, bem como com factores prognósticos.

Um total de 111 doentes com GM - 52 com gamapatia monoclonal de significado indeterminado (MGUS), 20 com mieloma múltiplo indolente (sMM) e 39 com MM sintomático - e 69 controlos não neoplásicos foram incluídos neste estudo. Os níveis de adipocitocinas no SP e MO foram quantificados através de kits comerciais de ELISA. A comparação entre grupos de doentes e controlos foi realizada através do teste não paramétrico de Mann-Whitney. A análise de sobrevivência foi conseguida pelo método de Kaplan-Meier.

Os nossos resultados mostram um aumento dos níveis de leptina e resistina no SP dos doentes com GM, comparado com os controlos. Observámos nos doentes com MGUS níveis maiores de adiponectina no SP e na MO, e menores nos doentes com MM sintomáticos, tendo os doentes com sMM um valor intermédio. Por outro lado, os níveis de leptina no SP e MO de doentes com sMM estavam elevados, quando comparados com MGUS e MM sintomático. MCP-1 tendeu para níveis superiores em doentes nas fases mais tardias da doença. sMM

também estava associado a níveis superiores de TNF-α no SP. Os resultados da MO dos doentes eram, de algum modo, sobreponíveis aos encontrados no SP.

Quando consideradas características laboratoriais, a secreção de adipocitocinas parece ser dependente do tipo de cadeia pesada produzida. Doentes com MM sintomático de cadeias leves, estavam associados a maiores níveis de adiponectina e TNF-α, bem como a menores níveis de leptina e resistina, comparativamente ao doentes com MM sintomático IgG e IgA. No que diz respeito ao tipo de cadeia leve produzida, os indivíduos com MGUS produtores de cadeias leves κ, apresentaram níveis superiores de MCP-1 na MO.

Adicionalmente, a presença de hipercalcémia e lesões ósseas em doentes com MM sintomático, está associada a níveis superiores de leptina no SP e MO. Também, os níveis de MCP-1 na MO dos doentes com anemia, estavam mais altos dos que os que não tinham. De acordo com a ISS, doentes em estádio III, parecem ter níveis superiores de resistina no SP em relação aos do estádio I. No entanto, eles mostram a tendência inversa na MO. Finalmente, níveis elevados de adiponectina no SP e de leptina na MO, foram associados com diminuição da sobrevivência geral.

Estes resultados sugerem que, as adipocitocinas podem estar envolvidas nas GM, nomeadamente no MM, ao criarem um estado pró-inflamatório no microambiente da MO, que poderá contribuir para o processo de carcinogénese, e ao participarem nas alterações imunológicas subjacentes à patogénese do MM. Como o MM permanece uma doença incurável, uma melhor compreensão da patogénese do MM, é de grande importância para criar novas abordagens terapêuticas e melhorar o prognóstico dos doentes.

Palavras-chave

Gamapatia monoclonal; Mieloma Múltiplo; Adipocitocinas; Microambiente; Prognóstico

Abbreviations

AKT/PKB - Protein kinase B

BM - Bone marrow

BMI - Body mass index

BMM - Bone marrow microenvironment

BMSC - Bone marrow stem cells

CCR2 - C-C chemokine receptor type 2

CHUC - Centro Hospitalar e Universitário de Coimbra

c-MYC - c-MYC protein

CRAB - Hypercalcemia, Renal lesion, Anemia and Bone lesion

CT - Computed tomography

DNA - Deoxyribonucleic acid

ECM - Extracellular matrix

EDTA - Ethylenediamine tetraacetic acid

ELISA - Enzyme-Linked Immunosorbent Assay

Ig - Immunoglobulin

IGF-1 - Insulin-like growth factor 1

IgH - Immunoglobulin heavy chain

IL-6 - Interleukin 6

IQR - Interquartile range

ISS - International Staging System

JAK - Janus Kinase

K-RAS - Kirsten rat sarcoma viral oncogene homolog

LC - Light chain

MAPK - Mitogen activated protein kinases

MCP-1 - Monocyte chemoattractant protein 1

MCP-2 - Monocyte chemoattractant protein 2

MCP-3 - Monocyte chemoattractant protein 3

MG - Monoclonal gammopathy

MGUS - Monoclonal gammopathy of undetermined significance

MM - Multiple myeloma

mTOR - Mammalian target of rapamycin

n - Number of cases

NF-κB - Nuclear factor κB

N-RAS - Neuroblastoma rat sarcoma viral oncogene homolog

PB - Peripheral blood

PET-CT - Positron emission tomography - computed tomography

PI3K - Phosphatidylinositol-3 kinases

RAF - Rapidly accelerated fibrosarcoma related proteins

RANKL - Receptor activator of nuclear factor κB ligand

RAS - Rat sarcoma related proteins

SD - Standard deviation

sMM - Smoldering multiple myeloma

STAT-3 - Signal transducer and activator of transcription 3

TNF-α - Tumor necrosis factor α

VEGF - Vascular endothelial growth factor

VLA-4 - Very late antigen 4

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1. Introduction

Monoclonal gammopathies (MG) are a condition that arises from an overproduction of one plasma or lymphoplasmacytic cell clone, which is recognized through serum or urine electrophoresis that detects the abnormal amounts of immunoglobulins produced by those cells (M-protein). MG that derive from plasma cells include pre-malignant and malignant conditions such as monoclonal gammopathy of undetermined significance (MGUS) and multiple myeloma (MM), respectively [(1)].

MGUS is a plasma cell derived pre-malignant disorder, affecting at least 3% of the population above the age of 50, that is clinically defined by the presence of a serum M-protein inferior to 3g/dL, bone marrow plasma cells lower than 10% and absence of end-organ damage related symptoms [(2–4)]. MM cases are usually preceded by MGUS, which is considered the first pathogenic step into this plasma cell malignancy[(5,6)]. MGUS has a 1% annual risk of progression to MM or related disorder [(7)].

MM is a malignant plasma cell disorder that represents approximately 10% of all hematological cancers and 1% of neoplastic diseases, with an annual age-adjusted incidence of 5.6 cases per 100,000 persons. The median age of diagnosis is 70 years, and it's two times more frequent in African Americans compared with Caucasians, and slightly more common in males than females [(5,8,9)]. The disease is defined by the following three criteria: presence of a serum M-protein superior to 3g/dL; bone marrow plasma cells higher than 10% and presence of end-organ damage related symptoms, defined as CRAB symptoms (hypercalcemia, renal insufficiency, anemia and lytic bone lesions). When the patient has no end-organ damage related symptoms, the disorder is classified as smoldering or asymptomatic multiple myeloma (sMM) [(7,10)]. sMM has an annual risk of progression to MM of 10% in the first five years, 3% in the following five years, and then 1% [(4)].

Relatively to the pathogenesis of MM, it has been proposed a model that involves two different, but perhaps overlapping, pathways. One, with a high incidence of gene translocations of the immunoglobulin heavy chain (IgH) gene, leading to nonhyperdiploid tumors, and another with multiple trisomies, leading to hyperdiploid tumors [(11)]. Primary translocations occurs in the germinal center B cells, and they lead to a deregulation of a variety of oncogenes such as cyclin *D1* or *D3*, which leaves the cells more susceptible to proliferative stimuli, resulting in the expansion of a certain clone. Secondary translocations are responsible for the progression to MM, and occur later in the disease. They usually involve activating mutations of *c-MYC*, *N-* and *K-RAS* oncogenes [(6,11)].

Besides the genetic and epigenetic changes, also the bone marrow microenvironment (BMM) displays a major role in the pathogenesis of MM. The BMM includes extracellular matrix (ECM) proteins and various cell types such as bone marrow stromal cells (BMSCs), endothelial cells, osteoclasts and osteoblasts [(12)]. Homing of MM cells to the bone marrow mediated through the release of specific chemokines, such as monocyte chemotactic proteins 1, 2 and 3 (MCP-1, -2 and -3), and adhesion molecules, such as syndecan-1 and very late antigen 4 (VLA-4), leads to an interaction of the tumor cells with the BMSCs and ECM proteins [(11,13)]. This interaction promotes activation of proliferative/anti-apoptotic signaling pathways (e.g JAK/STAT3, RAS/RAF/MAPK, and PI3K/AKT/mTOR) and secretion of various types of cytokines such as interleukin 6 (IL-6), insulin-like growth factor 1 (IGF-1), vascular endothelial growth factor (VEGF), tumor necrosis factor α (TNF-α) and receptor activator of NF-κB ligand (RANKL). Such interactions are crucial for tumor cell growth and proliferation, angiogenesis, bone destruction, resistance to pro-apoptotic stimuli (including conventional therapies) and metastasis [(11,12,14)]. Another cell type that also belongs to the bone marrow, and it is often forgotten, is the adipocyte.

The adipose tissue, not only serves as an energy storage depot and mechanical barrier, but it also acts as an active endocrine organ through the secretion of adipokines [15], which play an important role in energy balance, insulin-sensitivity, lipid metabolism, blood pressure, angiogenesis, hemostasis, immunity, inflammation and acute-phase response [16]. These hormones are produced predominantly by adipocytes of white adipose tissue (e.g. leptin and adiponectin) in adipocytes and other tissues (e.g TNF-α, and IL-6) and in adipocytes and others adipose tissue cells (e.g resistin) [17].

The role of adipokines in inflammation has been extensively studied due to its implications in obesity-related pathologies. They have pro-inflammatory (resistin, TNF-α, MCP-1) and anti-inflammatory properties (adiponectin), or play a role in inflammation in accordance to its levels and tissue's resistance (leptin) [18]. Furthermore, there is strong evidence that adipose tissue expansion is on the basis of several obesity related disorders such as metabolic syndrome, hypertension, atherosclerosis, chronic kidney disease diabetes, respiratory disorders and, more recently, cancer [19]. One mechanism behind this relationship is the crosstalk between adipocytes and the immune system through deregulation of the adipokines secretion [20,21]. In fact, obesity is characterized by a state of low-grade inflammation [16], with secretion of high levels of pro-inflammatory adipokines and cytokines, at least in part due to an increased number of resident macrophages in the adipose tissue [22]. Whereas pro-inflammatory adipokines are overproduced in obesity, adiponectin production is reduced [15].

The role of adipokines in cancer is being studied to clarify their participation in the carcinogenesis process, given the relationship between obesity and certain types of cancer such as breast, endometrial and colon cancer [17]. A recent review also linked obesity with the risk for hematological malignancies such as leukemia, lymphoma and multiple myeloma [23]. *In vitro* studies on different cancer cell lines using leptin showed that this adipokine had

various effects such as increased cell proliferation, growth, transformation, invasion, and apoptosis suppression [17], suggesting a role of this hormone in the carcinogenesis process. Moreover, accumulating evidence suggests that adiponectin can have anti-neoplastic effects by acting directly on cancer cells, and perhaps indirectly by modulating insulin-sensitivity at the target tissue site regulating inflammatory responses and influencing tumor angiogenesis [24]. The influence of other adipokines is not so widely studied.

In MM, following the compelling evidence that obesity is positively related with incidence and mortality of this malignancy [9,25,26], some studies aiming to discover the role of adipokines in the pathogenesis of MM have been undertaken. As the mechanisms behind the observed association between obesity and MM are not very clear, only now the focus has started to change to the adipocyte. As stated above, the adipocyte is a bone marrow cell that is often forgotten. The number of adipocytes increases with age, resulting in adipocyte deposits occupying up to 70% of the bone marrow cavity in elderly persons [27]. It has been shown that increased visceral adiposity is associated with more bone marrow fat, despite no correlation between bone marrow fat and body mass index (BMI), subcutaneous or total abdominal adipose tissue [28]. Knowing that MM median age of diagnosis is 70 years and giving the positive association with obesity, it is plausible to think that bone marrow adipocytes may interact with MM cells, namely through deregulation of adipokines production.

However the role of the adipokines in MM, and in all obesity-related cancers is poorly understood. A better understanding of its role in the pathogenesis of MM may provide a new approach to this malignancy, prevent clinical MM, design new strategies of treatment and improve prognosis classification.

1.1 Aims

In the present study, we aim to explore the role of pro- and anti-inflammatory adipokines, namely adiponectin, leptin, resistin, MCP-1 and TNF-α in the pathogenesis of MG, namely in MGUS, sMM and symptomatic MM, and correlate its levels with patient's clinical and laboratorial characteristics, as well with prognostic factors.

2. Materials and Methods

2.1. Ethical statement

The present study was conducted in accordance with the Helsinki declaration. The Ethics Committee of Faculty of Medicine of University of Coimbra (Coimbra, Portugal) approved all research procedures. All participants provided their informed consent for participation in this study prior to enrollment.

2.2. Study population

To fulfill our objectives, we studied a total of 111 MG patients followed in the Hematology Service of Centro Hospitalar e Universitário de Coimbra (CHUC): 52 were patients with MGUS, 20 with sMM and 39 with symptomatic MM. We also studied 69 non-neoplastic controls with mild cognitive impairment followed in the Neurology Service of CHUC for Alzheimer's disease screening (they did not had any kind of dementia). We collected demographic characteristics for patients and controls, and recorded patient's clinical characteristics, namely the type of paraprotein, ISS stage and CRAB symptoms. We also maintained patient's follow-up in order to collect survival data and correlate it with adipokines levels.

2.3 Evaluation of adipokines levels in MG patients and controls

We analyzed samples obtained from MG patients and control's peripheral blood (PB), and from bone marrow (BM) of MG patients, that were collected before any therapeutic approach. PB and BM samples were collected into EDTA tubes, and were centrifuged for 10 minutes at 1000 g. After this, samples were immediately aliquoted and stored at -80ºC.

PB and BM adiponectin, leptin and resistin levels were measured using ELISA sets from R&D Systems® (human Adiponectin Duoset® ELISA; human Leptin Duoset® ELISA; human

Resistin Duoset® ELISA). The levels of MCP-1 and TNF-α were also measured using ELISA sets from BD Biosciences (BD OptEIA™ Human MCP-1 ELISA Set; BD OptEIA™ Human TNF-α ELISA Set). We did not analyze MCP-1 and TNF-α levels in the control's PB samples.

2.4 Statistical analysis

Statistical analysis of the data was performed with IBM® SPSS® Statistics Version 22. We performed descriptive analysis of the characteristics of patients and controls. PB and BM adipokines levels are presented in median and interquartile range (IQR), and presented as whisker and box plots. Comparison between cases and controls, and between the subgroups of cases were conducted using nonparametric Mann-Whitney test, after testing for the normality of our parameters. The same test was performed to compare the levels of adipokines according to the case's clinical characteristics. Survival analysis was performed using the Kaplan-Meier method. To indicate statistical significance we used p value < 0.05.

3. Results

3.1 Subjects characteristics

As we can see in **Table 1**, we studied 69 controls and 111 Monoclonal Gammopathies (MG) patients, being 52 with MGUS, 20 with sMM and 30 with symptomatic MM. The mean age was similar in all four groups of subjects, being approximately 70 years. Controls were mainly females, and MG patients were mainly males. BM plasmocytosis and serum Ig concentration increased with disease progression, as they were lower in MGUS subjects (4.6 ± 2.2 % and 13.6 ± 5.5 g/L, respectively) and higher in the symptomatic MM patients (35.6 ± 20.9% and 38.4 ± 27.0 g/L, respectively).

In MGUS patients the monoclonal immunoglobulin was mainly of the IgG subtype (n = 35), being the rest IgA, IgM and biclonal IgM/IgG. sMM were mainly of the IgG and IgA subtype (n = 10 and n = 6, respectively) with only one individual with the IgM subtype, one with a biclonal component of IgA/IgG and two individuals with the light chain subtype. In what concerns the symptomatic MM subjects, 18 presented with IgG, 15 with IgA, five with light chain and one with IgD subtype. In what concerns the type of light chain, MGUS subjects had similar distribution between λ and κ light chains, as sMM and symptomatic MM patients produced mainly κ light chains.

CRAB symptoms were defined as: serum calcium value higher than 11 mg/dL or more than 1 mg/dL above the normal value (hypercalcemia), serum creatinine value higher than 2 mg/dL (renal lesion), hemoglobin value lower than 10 g/dL or more than 2g/dL bellow the normal value (anemia) and one or more osteolytic lesions in skeletal radiography, CT or PET-CT (bone lesion). Of the 39 MM patients, the main presenting symptoms were bone lesion and anemia (32 and 31 patients, respectively). Renal lesion and hypercalcemia only accounted for nine and eight patients, respectively. Finally, in what concerns the International Staging

System for Multiple Myeloma (ISS), the grand majority of them (*n* = 25) presented in the ISS stage III at the time of diagnosis, and the remaining 14, were evenly distributed between stage I and stage II. All this characteristics are summarized in **Table 1**.

Table 1. Descriptive characteristics of cases and controls

	Controls	**Cases**		
		MGUS	**sMM**	**symptomatic MM**
n	69	52	20	39
Male (*n*)/ Female (*n*)	28/41	32/20	9/11	18/21
Age (mean ± SD)	70.9 ± 11.8	69.8 ± 10.1	72.4 ± 10.8	69.9 ± 10.8
Plasma cells % (mean ± SD)	-	4.6 ± 2.2	13.1 ± 6.9	35.6 ± 20.9
Ig concentration g/L (mean ± SD)	-	13.6 ± 5.5	26.4 ± 13.4	38.4 ± 27.0
Type of heavy chain (*n*)				
IgA	-	12 (23.1%)	6 (30%)	15 (38.5%)
IgG		35 (67.3%)	10 (50%)	18 (46.2%)
IgM		4 (7.7%)	1 (5%)	-
IgD		-	-	1 (2.5%)
IgM/IgG		1 (1.9%)	-	-
IgG/IgA		-	1 (5%)	-
Light chains		-	2 (10%)	5 (12.8%)
Type of light chain (*n*)				
λ	-	25 (48.1%)	7 (35%)	12 (30.8%)
κ		27 (51.9%)	12 (60%)	26 (66.7%)
κ/λ		-	1 (5%)	1 (2.5%)
CRAB symptoms (*n*)				
Hypercalcemia	-	-	-	8 (20.5%)
Renal lesion				9 (23.1%)
Anemia				31 (79.5%)
Bone lesion				32 (82.1%)
ISS (*n*)				
I	-	-	-	7 (17.9%)
II				7 (17.9%)
III				25 (64.1%)

n - Number of cases; **SD** - standard deviation; **%** - percentage

3.2 Evaluation of adipokines levels in patients and controls

We first compared the levels of adipokines in PB of all the cases (MG patients all together in the same group) with those observed in controls (**Table 2**). We detected significant higher

levels of leptin and resistin in PB of MG patients (13.2 ng/mL and 9.49 ng/mL, respectively) compared with controls (7.95 ng/mL and 7,42 ng/mL, respectively). No significant differences were found in what regards to adiponectin in this analysis.

In order to explore if there were any differences if we considered each subtype of MG separately, we compared control individuals with each MG subtype and between the different subtypes of MG patients (**Table 3 and Figure 1A, B**).

Table 2. Levels of adiponectin, leptin and resistin in the PB of controls and MG subjects

	Controls *n* = 69	**MG**
Adiponectin µg/mL	6.36 (5.65)	6.92 (11.85) *n* = 110
Leptin ng/mL	7.95 (13.64)	13.2 (13.52) **a** *n* = 111
Resistin ng/mL	7.42 (5.13)	9.49 (9.73) **a** *n* = 111

Results in median (IQR); ***n*** - Number of cases; **a** - $p < 0.01$

In the PB samples, adiponectin was significantly higher in the MGUS patients when compared with the controls ($p = 0.012$) and with symptomatic MM subjects ($p = 0.04$). MGUS patients also showed higher levels of resistin than the controls ($p = 0.001$). On the other hand, we found that sMM was associated with increased PB leptin when compared with controls ($p = 0.001$), MGUS ($p = 0.012$) and symptomatic MM ($p = 0.043$). TNF-α levels were also increased in sMM compared with MGUS individuals ($p = 0.034$). Symptomatic MM patients had higher levels of resistin than controls ($p = 0.014$). Although not statistically significant, symptomatic MM individuals tended to have higher levels of MCP-1 than MGUS judging by the IQR (**Table 3**).

In the BM samples, the results were somewhat overlapping with those from the PB samples. We found significant increase in leptin levels of sMM subjects compared with MGUS ($p =$

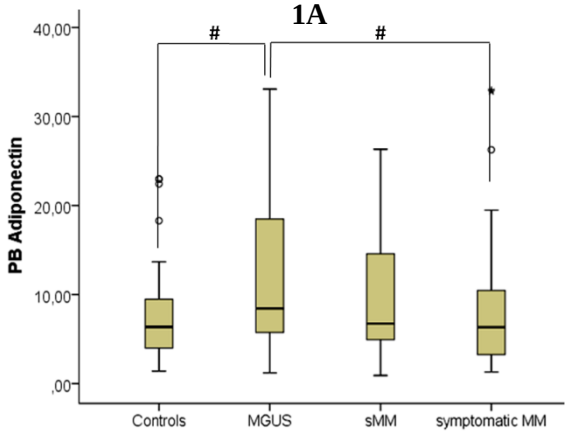
0.003) and symptomatic MM patients (p = 0.021). The BM levels of resistin and MCP-1 in symptomatic MM patients tended for higher levels comparing with MGUS judging by their respective IQR, even though it was not statistically significant. PB and BM results are illustrated in **Figure 1A and B**, respectively.

Table 3. Levels of adipokines in the PB and BM of controls, MGUS, sMM and symptomatic MM subjects

		Controls n = 69	MGUS	sMM	symptomatic MM
Peripheral blood	**Adiponectin µg/mL**	6.36 (5.65)	8.43 (14.14)[a] n = 51	6.73 (10.59) n = 20	6.32 (7.67) n = 39
	Leptin ng/mL	7.95 (13.64)	10.48 (11.14) n = 52	17.9 (16.83)[b] n = 20	13.53 (14.01) n = 39
	Resistin ng/mL	7.42 (5.13)[c]	10.74 (8.51) n = 52	8.45 (5.44) n = 20	8.8 (13.99) n = 39
	MCP-1 pg/mL	-	193.09 (181.0) n = 31	729.8 (770.98) n = 9	148.59 (531.75) n =28
	TNF-α pg/mL	-	7.0 (4.24) n = 21	11.86 (3.9)[d] n = 7	8.73 (8.59) n = 14
Blood marrow	**Adiponectin µg/mL**	-	16.68 (46.4) n = 52	8.34 (19.92) n = 20	9.52 (17.36) n = 37
	Leptin ng/mL	-	13.34 (12.31) n = 52	23.35 (10.73)[c] n = 20	15.38 (14.21) n = 37
	Resistin ng/mL	-	34.02 (47.89) n = 52	21.82 (36.81) n = 20	25.2 (58.52) n = 37
	MCP-1 pg/mL	-	343.52 (271.95) n = 31	738.74 (795.24) n = 10	390.9 (708.57) n = 25
	TNF-α pg/mL	-	8.45 (10.22) n = 21	11.0 (7.24) n = 7	11.3 (10.32) n = 14

Results in median (IQR); ***n*** - Number of cases;
(a) - $p < 0.05$, different from controls and symptomatic MM
(b) - $p < 0.01$, different from controls, and $p < 0.05$, different from MGUS and symptomatic MM
(c) - $p < 0.01$, different from MGUS, and $p < 0.05$, different from symptomatic MM
(d) - $p < 0.05$, different from MGUS

1A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30,00
20,00
10,00
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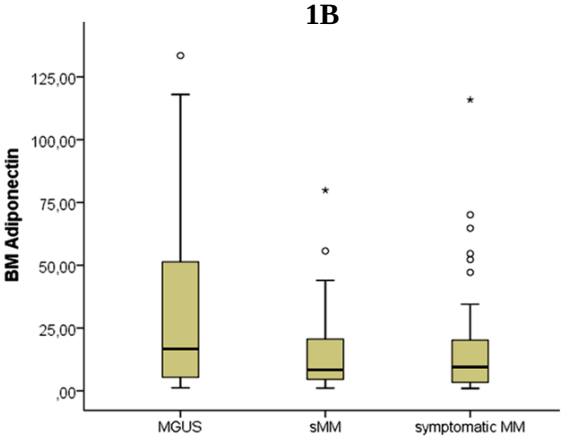
#
#
Controls
MGUS
sMM
symptomatic MM

1B


BM Adiponectin
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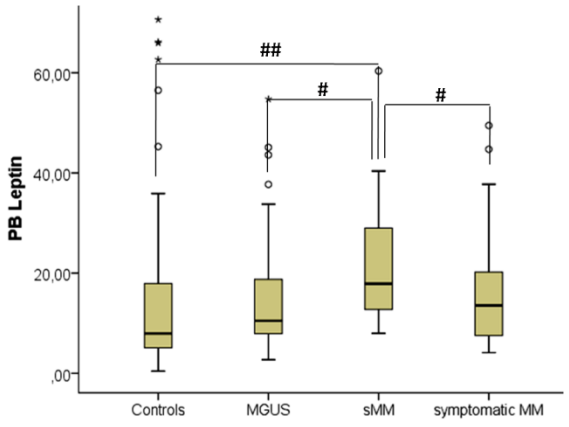
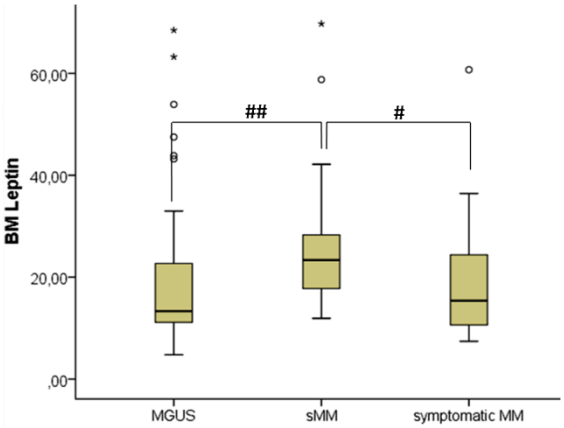
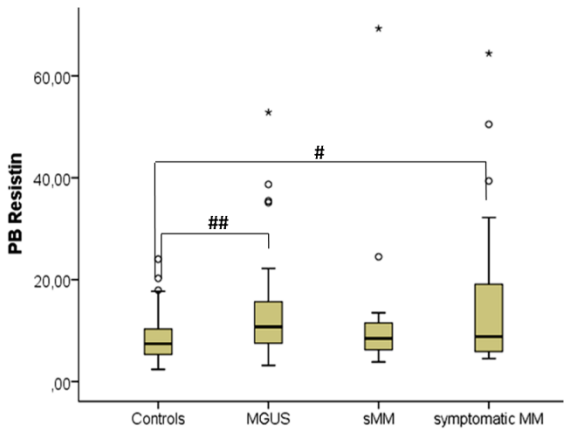
100,00
75,00
50,00
25,00
,00
MGUS
sMM
symptomatic MM


PB Leptin
60,00
40,00
20,00
,00
##
#
#
Controls
MGUS
sMM
symptomatic MM


BM Leptin
60,00
40,00
20,00
,00
##
#
MGUS
sMM
symptomatic MM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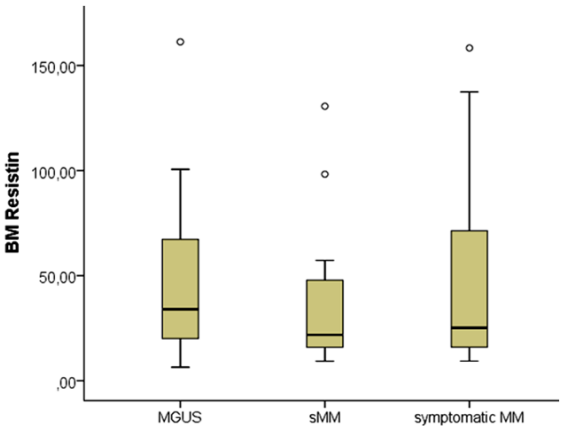
40,00
20,00
,00
#
##
Controls
MGUS
sMM
symptomatic MM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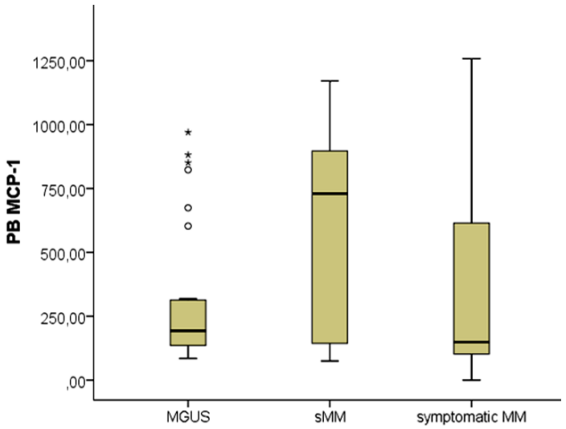
150,00
100,00
50,00
,00
MGUS
sMM
symptomatic MM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750,00
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250,00
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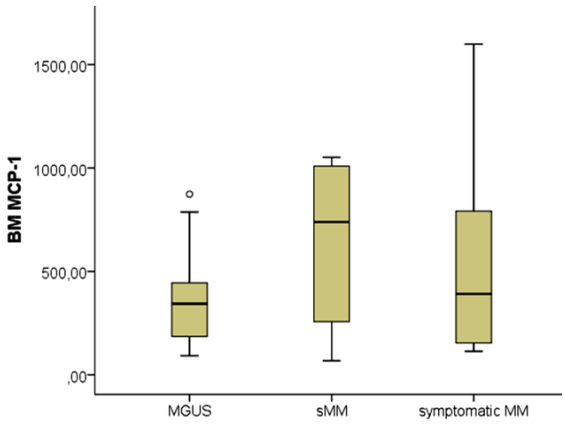
MGUS
sMM
symptomatic MM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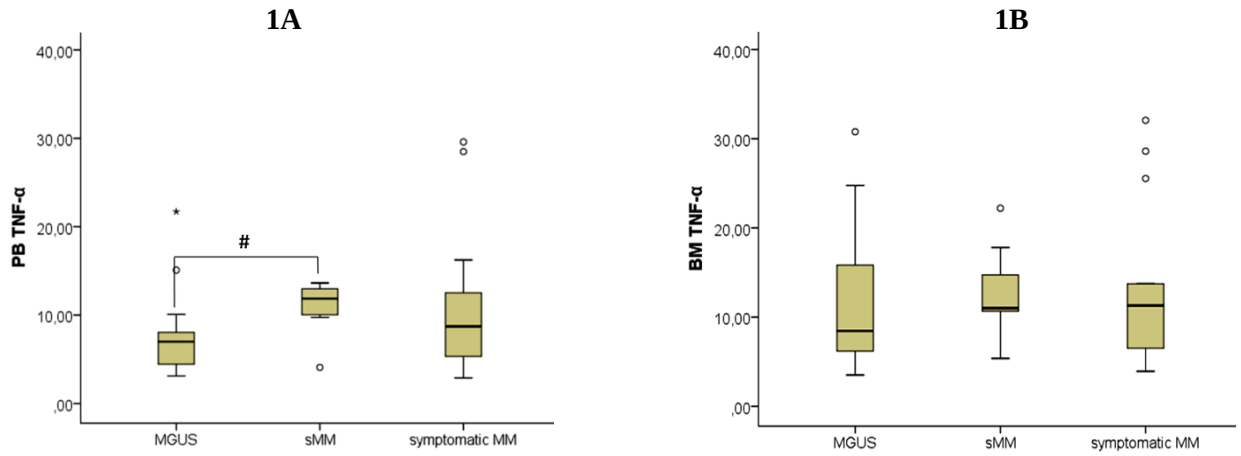


Fig. 1 - Adipokines levels in PB (**A**) and BM (**B**) of controls, MGUS, sMM and symptomatic MM individuals. **#** - $p < 0.05$; **##** - $p < 0.01$; **o and ∗** - outliers

3.3 Correlation of adipokines levels with patient's subtype of paraprotein

Results regarding the type of heavy chain secreted are fully described in **Table 4**, for all subgroups of patients. **Figure 2A and B** illustrates the results for symptomatic MM patients in the PB and BM samples, respectively.

In the PB samples of symptomatic MM patients, when comparing the IgA patients with the IgG subtype, we could not detect any significant difference between them. However, in patients with IgA subtype we observe a tendency for higher levels of adiponectin compared with other Ig subtypes ($p = 0.055$). On the other hand, when we looked for differences in MM patients between these two heavy chain subtypes and light-chain (LC), we found that patients with LC had higher levels of adiponectin than IgA and IgG subtypes ($p = 0.013$ and $p = 0.006$, respectively), and lower levels of leptin when compared only with the IgG subtype ($p = 0.006$). Furthermore, LC MM patients had a tendency for higher TNF-α levels, but the results didn't show statistical significance.

Once again, the results in the BM samples of these individuals were overlapping with those from the PB samples. However, we found more significant differences in the BM. Between the IgA and the IgG subtype, MCP-1 levels were higher in the later ($p = 0.018$). Adiponectin

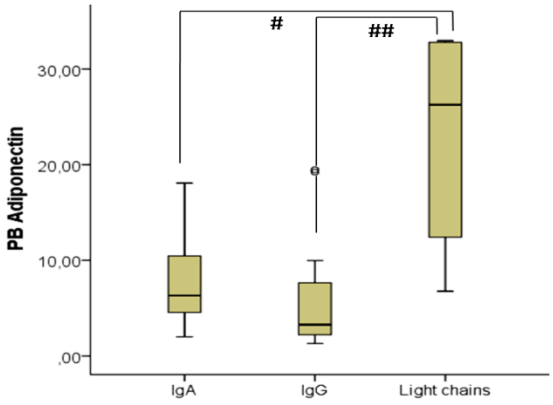
Table 4. Levels of adipokines in the PB and BM of MGUS, sMM and MM subjects according to the type of heavy chain secreted

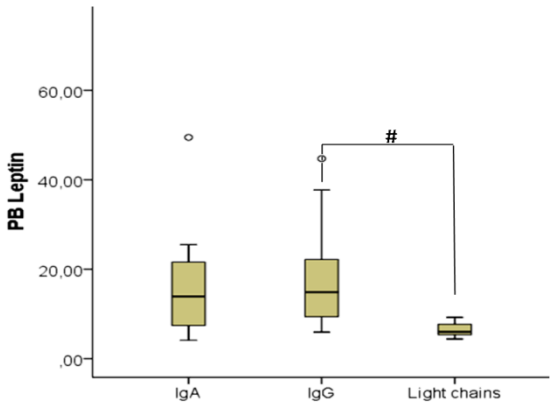
		MGUS			sMM		symptomatic MM		
		IgA	IgG	IgM	IgA	IgG	IgA	IgG	LC
Peripheral blood	Adiponectin µg/mL	6.4 (10.8) (*n* = 12)	8.4 (15.3) (*n* = 34)	12.9 (22.8) (*n* = 4)	4.3 (14.8) (*n* = 6)	7.6 (10.8) (*n* = 10)	6.3 (6.3) (*n* = 15)	3.3 (6.1) (*n* = 18)	26.3 (23.3)[a] (*n* = 5)
	Leptin ng/mL	10.2 (14.4) (*n* = 12)	10.4 (10.1) (*n* = 35)	12.9 (17.5) (*n* = 4)	19.7 (13.2) (*n* = 6)	19.9 (51.1) (*n* = 10)	13.9 (19.2) (*n* =15)	14.9 (15.4) (*n* = 18)	6.0 (3.6)[b] (*n* = 5)
	Resistin ng/mL	10.1 (12.1) (*n* = 12)	11.2 (8.2) (*n* = 35)	8.9 (4.9) (*n* = 4)	11.4 (8.4) (*n* = 6)	8.1 (5.3) (*n* = 10)	7.1 (14.8) (*n* = 15)	14.5 (16.6) (*n* = 18)	5.8 (101.9) (*n* = 5)
	MCP-1 pg/mL	174.8 (595.0) (*n* = 8)	193.1 (164.2) (*n* = 21)	197.7 (*n* = 2)	929.9 (*n* = 2)	729.8 (752.4) (*n* =5)	109.3 (182.0) (*n* = 10)	189.3 (542.6) (*n* = 15)	407.0 (346.1) (*n* = 2)
	TNF-α pg/mL	5.9 (4.1) (*n* = 6)	7.1 (4.5) (*n* = 15)	-	13.0 (*n* = 2)	11.1 (41.7) (*n* = 4)	6.1 (*n* = 3)	8.7 (8.2) (*n* = 8)	17.9 (*n* = 2)
Bone marrow	Adiponectin µg/mL	6.5 (28.2) (*n* = 12)	20.0 (46.1) (*n* = 35)	25.7 (97.4) (*n* = 35)	8.4 (9.0) (*n* = 6)	8.3 (27.6) (*n* = 10)	10.0 (13.8) (*n* =13)	6.7 (12.6) (*n* = 18)	47.2 (65.5)[a] (*n* = 5)
	Leptin ng/mL	13.5 (14.0) (*n* = 12)	13.3 (12.3) (*n* = 35)	16.5 (10.1) (*n* = 4)	20.3 (12.3) (*n* = 6)	24.6 (23.9) (*n* =10)	17.5 (12.6) (*n* = 13)	18.8 (13.8) (*n* = 18)	8.9 (1.3)[a] (*n* = 5)
	Resistin ng/mL	27.8 (23.1) (*n* = 12)	39.7 (53.52) (*n* = 35)	20 (234.4) (*n* = 4)	31.4 (57.5) (*n* = 6)	20.0 (11.3) (*n* = 10)	36.4 (87.0) (*n* = 13)	32.9 (60.6) (*n* = 18)	13.5 (8.8)[c] (*n* = 5)
	MCP-1 pg/mL	322.0 (316.0) (*n* = 8)	345.5 (281.4) (*n* = 21)	292.4 (*n* = 2)	738.5 (*n* = 3)	739.0 (714.6) (*n* = 5)	125.5 (248.3) (*n* = 7)	453.0 (652.6)[d] (*n* = 15)	396.8 (*n* = 2)
	TNF-α pg/mL	13.5 (15.9) (*n* = 6)	7.5 (8.1) (*n* = 15)	-	11.2 (*n* = 2)	10.8 (12.7) (*n* =4)	13.7 (*n* = 3)	9.4 (6.1) (*n* = 8)	28.8[b] (*n* = 2)

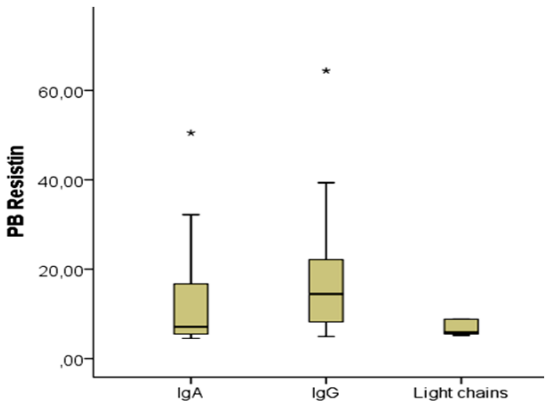
Results in median (IQR) when available; *n* - Number of cases;
(a) - $p < 0.05$, different from IgA, and $p < 0.01$, different from IgG; **(b)** - $p < 0.05$, different from IgG; **(c)** - $p < 0.01$, different from IgG; **(d)** - $p < 0.05$, different from IgA

levels were again higher in the LC subtype when compared with the IgG (p = 0.007) and IgA patients subtypes (p = 0.01).Moreover, we observed higher levels of TNF-α than the IgG MM subjects (p = 0.037). In the same individuals, we also found lower levels of leptin than IgG (p = 0.005) and IgA MM individuals (p = 0.026), as well as lower levels of resistin compared only with the IgG subtype (p = 0.009). Because we only had one case of IgD MM subtype, we did not test if there were any differences with the other Ig subtypes. We performed the same statistical analysis for MGUS and sMM subjects, but we didn't find any significant difference.

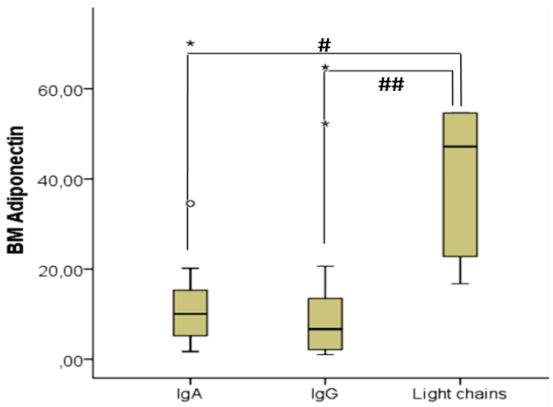
2A

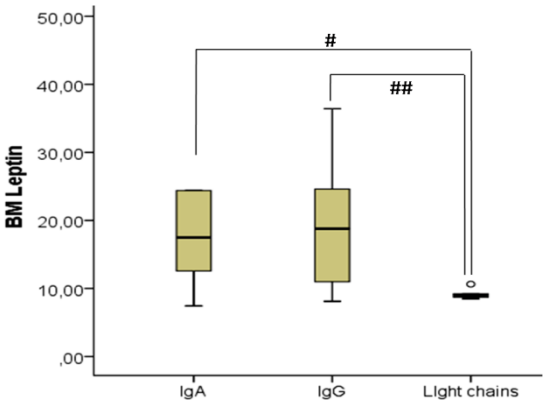


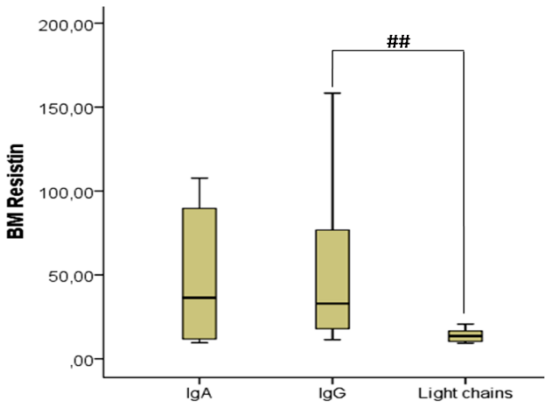




2B







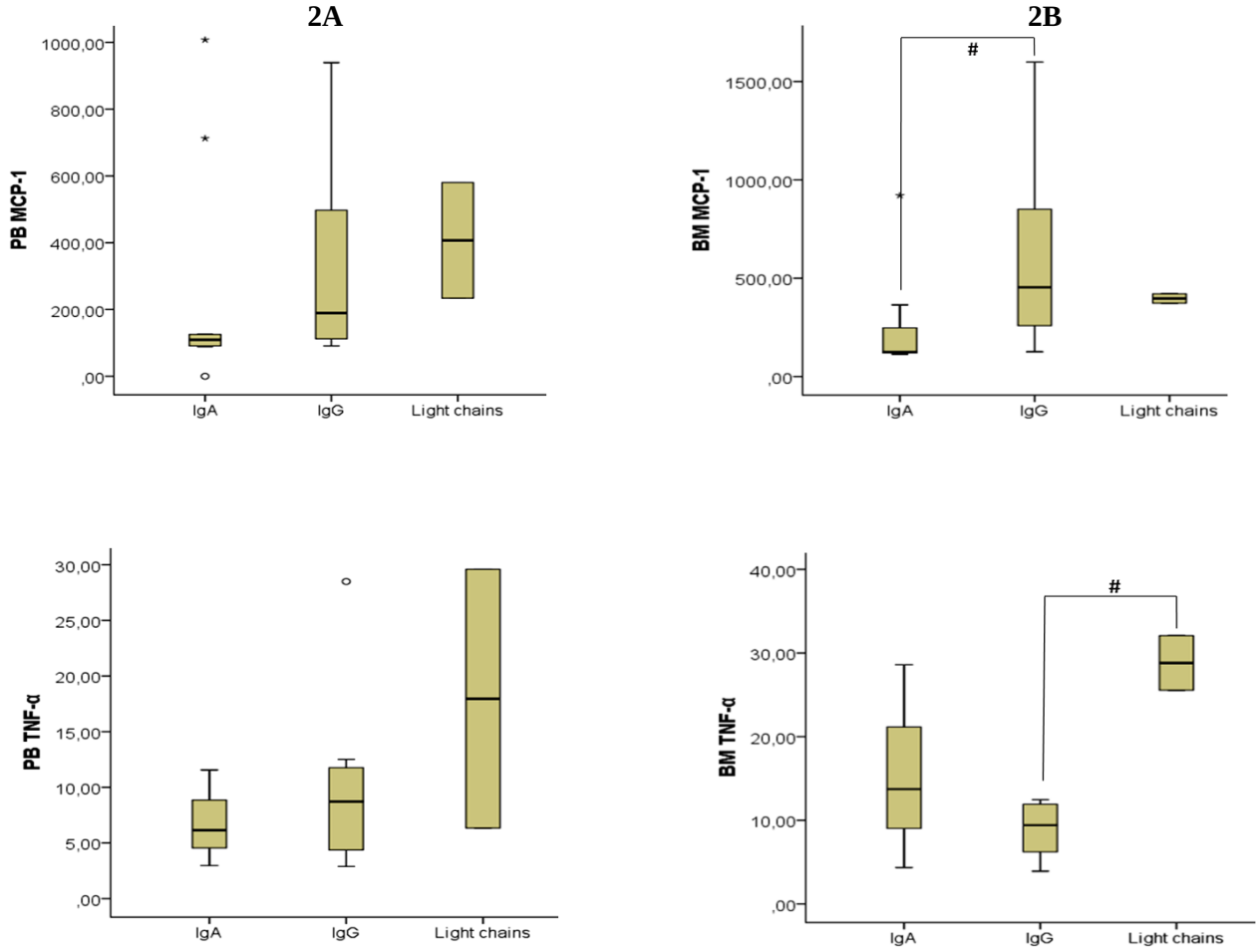


Fig. 2 - Adipokines levels in the PB (**A**) and BM (**B**) of symptomatic MM individuals according to the type of heavy chain secreted.

- $p < 0.05$; **##** - $p < 0.01$; **o and ∗** - outliers

We also analyzed the differences in the pattern of adipokines according to the type of light chain. However, the secretion of adipokines does not seem to be dependent from the type of light chain that is produced in symptomatic MM patients, because no significant differences were found. By performing the same tests on MGUS and sMM subjects, we observed that MGUS subjects who produced κ light chains had higher BM MCP-1 levels than those who produced λ light chains ($p = 0.02$). Results regarding adipokines levels according to the type of light chain produced are fully described in **Table 5**.

Table 5. Levels of adipokines in the PB and BM of MGUS, sMM and MM subjects according to the type of light chain secreted

		MGUS		sMM		symptomatic MM	
		λ	κ	λ	κ	λ	κ
Peripheral blood	Adiponectin µg/mL	9.2 (11.1) (*n* = 24)	8.2 (15.8) (*n* = 27)	11.3 (11.0) (*n* = 7)	6.0 (8.8) (*n* = 12)	5.9 (8.6) (*n* = 12)	6.5 (7.9) (*n* = 26)
	Leptin ng/mL	10,0 (17.8) (*n* = 25)	10.5 (9.7) (*n* = 27)	20.1 (15.1) (*n* = 7)	16.3 (16.9) (*n* = 12)	12.0 (6.9) (*n*= 12)	13.9 (18.3) (*n*= 26)
	Resistin ng/mL	12.2 (9.4) (*n* = 25)	10.0 (7.0) (*n* = 27)	8.7 (5.6) (*n* = 7)	8.0 (6.9) (*n* = 12)	8.7 (10.5) (*n* = 12)	8.5 (20.8) (*n* = 26)
	MCP-1 pg/mL	152.8 (178.3) (*n* = 16)	195.2 (456.9) (*n* = 15)	859.6 (*n* = 3)	708.4 (856.7) (*n* = 6)	119.6 (249.0) (*n* = 10)	217.6 (520.1) (*n* = 17)
	TNF-α pg/mL	6.1 (5.7) (*n* = 11)	7.5 (4.4) (*n* = 10)	10.0 (*n* = 2)	12.3 (28.4) (*n* = 5)	13.9 (*n* = 2)	6.3 (9.1) (*n* = 11)
Bone marrow	Adiponectin µg/mL	15.8 (43.1) (*n* = 25)	20.0 (49.6) (*n* = 27)	9.1 (8.3) (*n*= 7)	8.0 (25.2) (*n* =12)	9.4 (8.0) (*n* =10)	14.3 (25.4) (*n* = 26)
	Leptin ng/mL	13.3 (13.7) (*n* = 25)	13.4 (10.0) (*n* = 27)	22.9 (10.8) (*n* = 7)	22.6 (13.3) (*n* = 12)	13.9 (13.7) (*n* = 10)	16.4 (15.1) (*n* = 26)
	Resistin ng/mL	30.1 (49.9) (*n* = 25)	35.8 (52.0) (*n* = 27)	17.2 (6.1) (*n* = 7)	25.2 (72.2) (*n*= 12)	40.6 (51.1) (*n* = 10)	20.4 (71.4) (*n* = 26)
	MCP-1 pg/mL	221.2 (273.0) (*n* = 16)	383.7 (132.9) [a] (*n* = 15)	1023.1 (744.6) (*n* = 4)	556,6 (534.5) (*n* = 6)	184.0 (676.6) (*n* = 8)	405.9 (622.5) (*n* = 16)
	TNF-α pg/mL	7.7 (5.8) (*n* = 11)	13.0 (11.7) (*n* = 10)	20.0 (*n* = 2)	10.7 (3.4) (*n* = 5)	19.9 (*n* = 2)	11.5 (7.8) (*n* = 11)

Results in median (IQR) when available; *n* - Number of cases; **(a)** - $p < 0.05$

3.4 Analysis of adipokines levels according to CRAB symptoms

Firstly we went to see if there was any difference between those patients who had hypercalcemia, and those who had not (**Table 6**). Despite the tendencies for different levels in all adipokines, the only statistically significant difference detected between those who had hypercalcemia and those who had not, were the levels of leptin in both PB and BM, being lower in the patients with hypercalcemia as represented in **Table 6** (**PB**- $p = 0.037$; **BM** - $p = 0.04$).

Table 6. Adipokines levels according to absence or presence of hypercalcemia

		Absence	Presence
Peripheral blood	**Adiponectin µg/mL**	4.62 (7.11) (*n* = 31)	9.59 (13.11) (*n* = 8)
	Leptin ng/mL	14.1 (13.81) **a** (*n* = 31)	7.23 (9.25) (*n* = 8)
	Resistin ng/mL	9.2 (15.61) (*n* = 31)	6.29 (8.4) (*n* = 8)
	MCP-1 pg/mL	126.16 (469.25) (*n* = 24)	462.57 (922.59) (*n* = 4)
	TNF-α pg/mL	6.34 (9.09) (*n* = 11)	11.0 (*n* = 3)
Blood marrow	**Adiponectin µg/mL**	9.35 (13.33) (*n* = 30)	22.78 (59.59) (*n* = 7)
	Leptin ng/mL	16.97 (13.79) **a** (*n* = 30)	9.23 (13.33) (*n* = 7)
	Resistin ng/mL	32.32 (62.51) (*n* = 30)	17.9 (22.02) (*n* = 7)
	MCP-1 pg/mL	377.8 (584.04) (*n* = 22)	1040.17 (*n* = 3)
	TNF-α pg/mL	10.65 (7.81) (*n* = 11)	12.38 (*n* = 3)

Results in median (IQR) when available; ***n*** - Number of cases; (**a**) - $p < 0.05$;

No significant differences were found in the levels of adipokines in both PB and BM between patients who had renal lesion and those who had not, as described in **Table 7**. However, PB resistin showed a tendency for higher levels in individuals who presented with renal lesion than those who did not ($p = 0.062$).

Table 7. Adipokines levels according to absence or presence of renal lesion

		Absence	Presence
Peripheral blood	**Adiponectin µg/mL**	4.82 (7.24) (*n* = 30)	6,77 (14.06) (*n* = 9)
	Leptin ng/mL	13.42 (12.03) (*n* = 30)	14.1 (62.89) (*n* = 9)
	Resistin ng/mL	8.25 (10.84) (*n* = 30)	18.44 (46.94) (*n* = 9)
	MCP-1 pg/mL	144.05 (500.02) (*n* = 22)	158.05 (880.04) (*n* = 6)
	TNF-α pg/mL	6.67 (8.37) (*n* = 12)	13.63 (*n* = 2)
Blood marrow	**Adiponectin µg/mL**	9.36 (16.8) (*n* = 28)	10.05 (40.78) (*n* = 9)
	Leptin ng/mL	14.81 (14.08) (*n* = 28)	23.4 (61.84) (*n* = 9)
	Resistin ng/mL	27.88 (71.08) (*n* = 28)	25.2 (42.10) (*n* = 9)
	MCP-1 pg/mL	390.9 (665.31) (*n* = 19)	403.7 (904.23) (*n* = 6)
	TNF-α pg/mL	11.05 (16.52) (*n* = 12)	11.77 (*n* = 2)

Results in median (IQR) when available; ***n*** - Number of cases

Results shown in **Table 8** represents the levels of adipokines in symptomatic MM patients related to the presence or absence of anemia. We found that MM patients with anemia had statistically significant higher levels of BM MCP-1, than those without this symptom (p = 0.032). In the PB, we observed the same tendency, but with no statistical significance (p = 0.057). We did not detect any differences in the levels of other adipokines, neither in the PB, nor in the BM.

Table 8. Adipokines levels according to absence or presence of anemia

		Absence	Presence
Peripheral blood	**Adiponectin µg/mL**	4.83 (11.03) (n = 8)	6.7 (7.69) (n = 31)
	Leptin ng/mL	12.94 (7.86) (n = 8)	13.9 (14.8) (n = 31)
	Resistin ng/mL	7.31 (16.8) (n = 8)	8.8 (13.29) (n = 31)
	MCP-1 pg/mL	100.62 (20.10) (n = 4)	203.46 (576.26) (n = 24)
	TNF-α pg/mL	2.89 (n = 1)	10.46 (8.64) (n = 13)
Blood marrow	**Adiponectin µg/mL**	7.19 (16.21) (n = 8)	10.05 (18.49) (n = 29)
	Leptin ng/mL	14.15 (5.46) (n = 8)	18.7 (15.15) (n = 29)
	Resistin ng/mL	57.73 (127.59) (n = 8)	20.02 (47.89) (n = 29)
	MCP-1 pg/mL	140.16 (112.72) (n = 4)	438.1 (676.37) **a** (n = 21)
	TNF-α pg/mL	6.50 (n = 1)	11.45 (12.59) (n = 13)

Results in median (IQR) when available; ***n*** - Number of cases; **(a)** - $p < 0.05$

Finally, we observed a statistical significant differences in leptin PB levels between patients who had bone lesions and those who had not, as the later ones had lower levels (p = 0.05), similar to what we have seen when we looked for associations with hypercalcemia (see **Table 6** above). Patients without bone lesion showed the same tendency for BM leptin levels. The levels of other adipokines were not different whether if the patient had bone lesion or not. Results are described in **Table 9**.

Table 9. Adipokines levels according to absence or presence of bone lesion

		Absence	Presence
Peripheral blood	**Adiponectin µg/mL**	10.46 (6.91) (n = 6)	4.82 (6.06) (n = 32)
	Leptin ng/mL	24.18 (83.51) (n = 6)	12.83 (12.46) **a** (n = 32)
	Resistin ng/mL	7.98 (20.86) (n = 6)	9.0 (15.45) (n = 32)
	MCP-1 pg/mL	311.1 (743.37) (n = 5)	126.76 (481.73) (n = 23)
	TNF-α pg/mL	9.28 (8.3) (n = 4)	8.40 (14.45) (n = 10)
Blood marrow	**Adiponectin µg/mL**	16.15 (30.77) (n = 6)	9.35 (16.2) (n = 30)
	Leptin ng/mL	24.32 (70.84) (n = 6)	14.81 (14.29) (n = 30)
	Resistin ng/mL	31.2 (152.96) (n = 6)	24.19 (49.54) (n = 30)
	MCP-1 pg/mL	438.1 (676.06) (n = 5)	381.79 (711.24) (n = 20)
	TNF-α pg/mL	10.32 (19.27) (n = 4)	11.3 (10.32) (n = 10)

Results in median (IQR); n - Number of cases; **(a)** - p = 0.05;

3.5 Analysis of adipokines according to the ISS stage

The levels of adipokines according the ISS prognostic system are summarized in **Table 10.** As we can observe, in general, we observed in both PB and BM patients with higher ISS stages, a tendency for an increase in pro-inflammatory adipokines, being the differences for resistin PB levels statistically higher in stage III patients comparing with those in stage I (p = 0.006). Interestingly, we found the opposite tendency in the BM of the same group of individuals, but without statistical significance (p = 0.378) (**Table 10 and Fig. 3**).

Table 10. Adipokines levels according to the ISS

		I	II	III
Peripheral blood	**Adiponectin µg/mL**	6.88 (6.74) (n = 7)	7.65 (8.57) (n = 7)	5.19 (8.74) (n = 25)
	Leptin ng/mL	12.35 (12.95) (n = 7)	14.64 (23.18) (n = 7)	13.9 (13.95) (n = 25)
	Resistin ng/mL	5.81 (3.37) (n = 7)	7.36 (11.2) (n = 7)	15.2 (20.71)**a** (n = 25)
	MCP-1 pg/mL	109.59 (420.2) (n = 4)	144.05 (284.77) (n = 6)	203.46 (658.01) (n = 18)
	TNF-α pg/mL	5.33 (n = 1)	3.42 (18.12) (n = 5)	10.73 (8.57) (n = 8)
Blood marrow	**Adiponectin µg/mL**	9.34 (20.59) (n = 6)	9.52 (16.74) (n = 7)	9.78 (18.99) (n = 24)
	Leptin ng/mL	14.03 (9.15) (n = 6)	13.8 (12.16) (n = 7)	20.26 (15.15) (n = 24)
	Resistin ng/mL	49.98 (256.31) (n = 6)	61.09 (126.06) (n = 7)	22.95 (26.23) (n = 24)
	MCP-1 pg/mL	154.18 (n = 3)	381.79 (217.46) (n = 6)	484.53 (768.41) (n = 16)
	TNF-α pg/mL	10.65 (n = 1)	6.5 (18.14) (n = 5)	11.92 (13.68) (n = 8)

Results in median (IQR) when available; ***n*** - Number of cases; **(a)** - $p < 0.01$, different from stage I

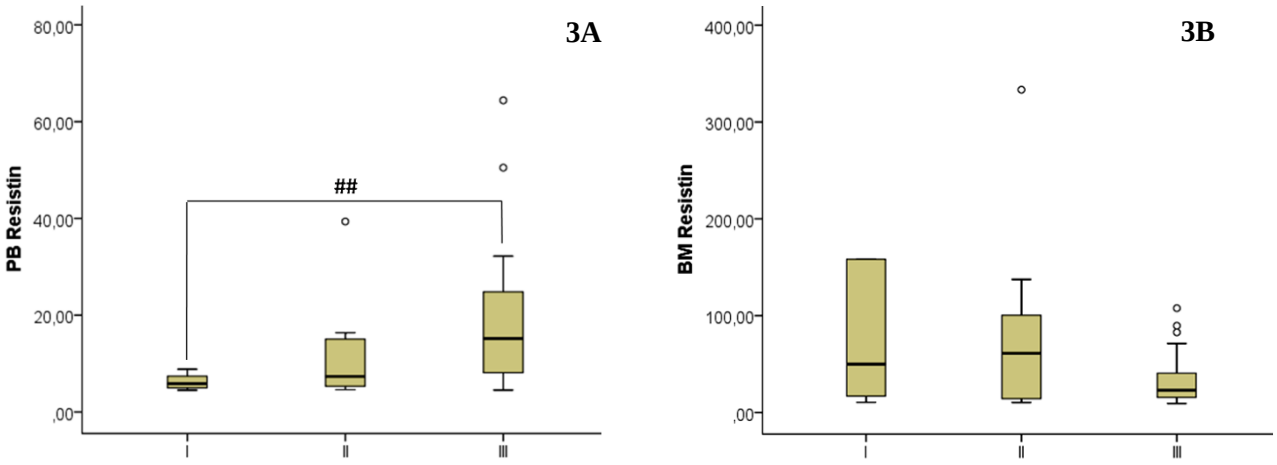


Fig. 3 - Resistin levels in PB (**A**) and BM (**B**) of MM individuals according to the ISS stage.
- $p < 0.01$; **o** - outliers

3.6 Survival curves

We investigated if the overall survival in symptomatic MM patients was affected by any kind of adipokines pattern, using the Kaplan-Meier method. During a 3-year follow-up of these patients, 25 out of 39 died, presenting an overall survival of 12.4 ± 8.9 months. From these 25 patients, seven died of progressive disease, six died of infectious disease and three died of cardiovascular disease. The causes of death of the other patients were not clarified.

We divided the symptomatic MM patients in two groups, using cut-off values based on the mean levels of each adipokine, in both PB and BM (**Group 1** - respective adipokine levels < cut-off; **Group 2** - respective adipokine levels ≥ cut-off). Cut-off values used are shown in **Table 11.**

We found that, patients with PB adiponectin levels lower than 8.6 µg/mL, (group 1) had a better mean survival than those with higher levels (15.1 ± 2.3 months ***vs*** 8.3 ± 2.3 months - $p = 0.018$). When we looked for the same differences in the overall survival, taking into account the BM adiponectin levels (mean = 18.9 µg/mL), although group 1 still had a better overall survival (12.5 months ***vs*** 8.8 months), it was not statistically significant ($p = 0.351$) (**Fig. 4A**).

Additionally, we observed that BM leptin levels (mean = 26.7 ng/mL) affected overall survival. Group 1 had a mean overall survival of 13.1 ± 2.0 months and group 2 had 2.8 ± 1.0 months (p = 0.005). When we took into account the PB leptin levels, overall survival also tended to be superior in group 1 (13.9 ± 1.9 months ***vs*** 6.3 ± 4.1 months), but without statistical significance (p = 0.074) (**Fig. 4B**).

The PB and BM levels of other adipokines did not influence the survival of symptomatic MM patients (results not shown).

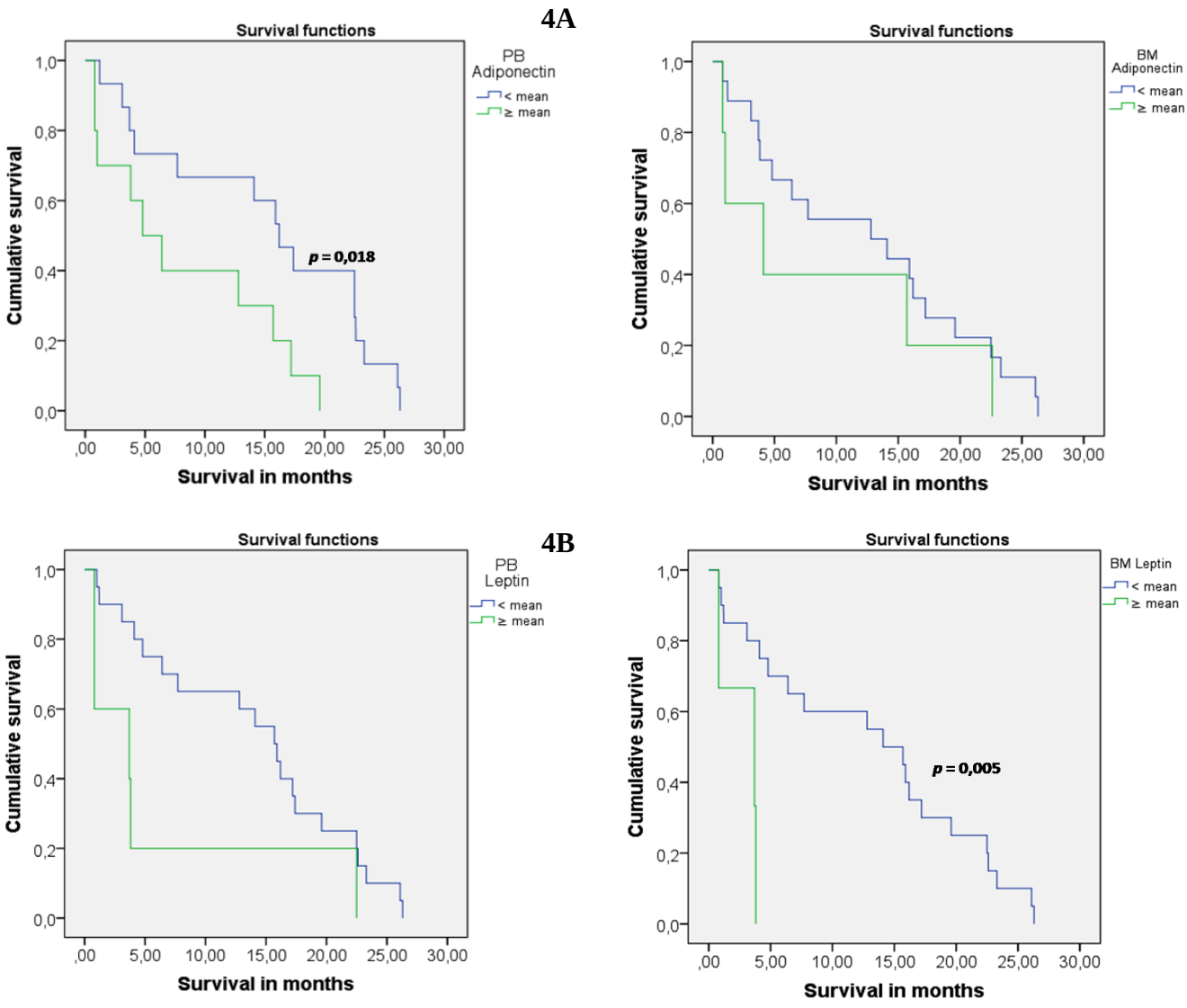


Fig. 4 - Overall survival of symptomatic MM individuals according to adiponectin (**A**) and leptin (**B**) levels in the PB and BM.
Blue line - Group 1; **Green line** - Group 2

Table 11. Cut-off values used for each adipokine in PB and BM of symptomatic MM patients

	Peripheral Blood	Bone marrow
Adiponectin μg/mL	8.6	18.9
Leptin ng/mL	24.2	26.7
Resistin ng/mL	23.1	73.9
MCP-1 pg/mL	342.4	496.1
TNF-α pg/mL	11.0	13.4

Cut-off values are expressed in mean levels

4. Discussion and Conclusion

Adipokines display an important role in several physiological functions in the organism. Obesity, is related with a low-grade inflammation, since there is a decrease in adiponectin and an increase in pro-inflammatory adipokines with an oncogenic potential [18,29]. Although it is well documented that, obesity increase the risk for some types of solid tumors, most of the studies, showing that adipokines participate in this relationship, are *in vitro* studies [17]. However, the role of adipokines in hematological malignancies is poorly understood, and in what regards MM, only a few studies were undertaken.

Our results show that in PB MG patients have higher levels of leptin and resistin than control individuals. The analysis of the adipokines PB levels in each different MG subgroups, showed that MGUS patients had higher levels of adiponectin than symptomatic MM individuals, as well as lower levels of TNF-α. Also, sMM patients had higher levels of leptin compared with controls, MGUS and symptomatic MM patients. PB resistin levels were higher in both MGUS and symptomatic MM patients than control individuals. MCP-1 tended for higher levels in the symptomatic MM group comparing with MGUS. In the BM, the sMM also presented with higher levels of leptin than MGUS and symptomatic MM patients. Adiponectin levels tended as well to be lower in the later stages of the disease. Resistin and MCP-1 were higher in the symptomatic MM subgroup of individuals. As the BM levels appears to have the same pattern of the PB, this indicates that the PB levels reflects the levels in the BM microenvironment.

As adipokines can exhibit anti- and pro-inflammatory properties, these results suggest that in the progression from from MGUS to MM, deregulation of the production of adipokines creates a pro-inflammatory state in the BM microenvironment, which can contribute to the carcinogenic process.

In what concerns adiponectin, Hoffman *et al.* [30] observed an inverse association between MM risk and adiponectin plasma levels. Moreover, Dalamaga *et al.* [31], also reported that MM individuals had lower serum levels of adiponectin. Our results are in agreement with both of these studies. Adiponectin is an anti-inflammatory adipokine that may exert its effects through inhibition of the NF-κB pathway. This in turn, suppresses production of various cytokines such as IL-6 and TNF-α, which are important growth factors for MM cells [12–14,32,33]. Adiponectin may as well increase the production of anti-inflammatory cytokine IL-10 [24]. It is possible that adiponectin may act as a protective factor in MGUS individuals and as a marker of progression from MGUS to MM. A study conducted by Fowler *et al.* [34] discovered that, host-derived adiponectin reduced tumor burden, increased survival of myeloma-bearing mice and prevented bone disease. This gives adiponectin some potential as a novel biomarker and therapeutic target.

Leptin is an adipokine that regulates caloric intake and energy homeostasis. Other studies found that MM individuals presented with higher plasma levels of leptin than controls [31,35–38]. Recently, it was shown that MM cells express leptin receptors, and that blockage of those receptors leads to a decrease in the DNA synthesis [27]. Reseland *et al.*, in 2009, reported that leptin induced the expression of several genes involved in cell growth, survival and signaling in two myeloma cell lines [35]. As stated before, the adipocytes are often forgotten in the studies of MM. Reseland's and our results suggest that adipocytes may have a role in pathogenesis of MM, since they are the major producers of leptin. Interestingly, in our study, leptin levels were higher in the sMM subgroup, rather than in the symptomatic MM subgroup. It could be due to the fact that, during the disease development, BM adipocytes tend to disappear, suggesting that the role of BM adipocytes may be restricted to the initial stages of the disease, before a remodeling of the BMM has occurred [27]. This can mean that the

maximum levels of leptin are produced during sMM phase, before the progression to MM, when symptoms, like bone lesions, change the BMM.

Our results suggest that resistin contributes to the creation of a pro-inflammatory state in the BM. Two other studies [36,37] focusing on leptin and resistin serum levels didn't found any significant differences between MM patients and controls. Interestingly, another study [31] observed significant lower serum levels of resistin in MM patients compared with controls. The authors reported that this finding could be possibly due to a compensatory response of the resistin pathway to the upregulation of other inflammatory and proliferative factors. PB resistin levels in our study, where higher in MGUS and symptomatic MM patients than in controls.

Resistin is an adipokine related with insulin-resistance and activation of inflammatory processes. Whereas in mice, resistin is produced mainly in the adipocytes, in humans it is produced mainly by macrophages and monocytes [18]. This promotes the production of IL-6 and TNF-α, which are important growth factors for MM cells [12–14,18,33]. To our knowledge, there are no studies showing that in MM resistin prodution, is mainly due to BM macrophages.

Additionally, we observed that MCP-1 tended to be higher in PB and BM in patients in the later stages of the disease, when compared with MGUS patients. In what regards to TNF-α, we only found higher levels in the PB of sMM patients compared with MGUS subgroup. MCP-1 is a potent chemoattractant that acts through CCR2 receptor, which is also expressed in MM cells [39], resulting in enhanced migration of the malignant clone. This makes MCP-1 an attractive therapeutic target for MM [39]. In fact, blockage of CCR2 receptor and neutralization of MCP-1, -2 and -3 significantly reduced migration of human MM cells to marrow stromal cell-conditioned medium [39]. TNF-α acts not only as a growth factor, but also

upregulates the production of MCP-1 by the BM stromal cells. We did not find any other studies measuring MCP-1 and TNF-α levels in MM patients.

According with Ig subtype, namely between the IgA and IgG symptomatic MM patients, we only found higher levels of BM MCP-1 in those who produced IgG heavy chains. However, we observed higher PB and BM levels of adiponectin and lower levels of BM leptin of those who only produced light chains relatively to those who produced IgA or IgG heavy chains. Additionally, MM patients with light chains were associated with lower leptin PB levels and lower resistin and TNF-α BM levels than patients with IgG subtype. In what regards the type of light chain secreted, we only observed higher BM MCP-1 levels in κ light chain MGUS patients.

The type of immunoglobulin that is secreted can affect the outcome of patients. For example, the IgA subtype was associated with more severe anemia, and with lower overall survival [(40).] Also the LC subtype is associated with higher incidence of renal lesion. However, in one study, overall survival was comparable to the IgG MM patients [(40)]. Furthermore, one study showed a positive correlation between serum levels of leptin and IgG levels in MM patients [(36)]. Our results suggest that, given the differences especially between the light chain and IgG/IgA subtype, the adipokines may have different roles in the different MM subtypes, as they could participate in the immunological changes underlying the pathogenesis of MM. Some features of MGUS are referred as risk factors for progression to MM. For MGUS, non-IgG subtype is associated with higher progression rates to MM, especially when associated with an M-protein concentration higher than 1,5 g/dL and an abnormal free-light chain ratio[(7)]. However, we did not find any differences in what concerns the type of heavy chain secreted by MGUS patients.

The presence of CRAB symptoms is required to define the diagnosis of symptomatic MM. In our study we observed higher levels of leptin in the PB and BM of patients without hypercalcemia and without bone lesion. Leptin stimulates bone formation through promotion of osteoblast differentiation, bone mineralization and modification of osteoclast differentiation [41]. It is possible that the high levels of leptin in MM act as protective factor for bone disease, and therefore, prevent the occurrence of these symptoms.

We also found higher levels of BM MCP-1 in patients who developed anemia. Anemia in MM is present in about 73% of the patients at diagnosis (5). The origin of the anemia is multifactorial and complex, but it is mainly due to the expansion of malignant cells in the BM. As MCP-1 acts as a chemoattractant to MM cells to the BM, our results suggest that MCP-1 may contribute to anemia related with BM infiltration.

The International Staging System (ISS) for MM is currently used for disease staging. It takes into account albumin and β_2-microglobulin serum levels. Our results show that there is a significant increase in PB resistin levels from patients in stage I to patients in stage III. Interestingly, we found the opposite tendency for BM resistin. We could not find any explanation for this finding. As resistin, in humans, is mainly produced by macrophages, it could be interesting to see if there are any differences in the BM macrophages of MM patients in the different stages of the ISS. However, our results suggest that higher ISS stages tend to be associated with higher levels of pro-inflammatory adipokines. Comparing patients stratified according with the Durie-Salmon staging system, Dalamaga *et al.* [31] found that higher stages tended to present higher levels of leptin, and Esheba *et al.* [36] found that stage II patients had higher leptin levels than those in stage I.

There is some controversy in what regards the ISS, since it was established using data from patients who did not had access to novel therapeutics agents [42], meaning that this staging

system could fail to predict correctly the prognosis of some patients. One study suggested the creation of a new staging system consisting in the presence of anemia and plasmocytoma [42].

With the introduction of novel therapeutics, MM overall 5-year relative survival increased from 28,8% to 34,7%, and 10-year relative survival from 11,1% to 17,4% [43]. By analyzing the impact of the levels of adipokines in overall survival of symptomatic MM patients, we observed that high PB adiponectin and BM leptin levels were associated with significantly reduced overall survival. As sMM and symptomatic MM patients had lower levels of adiponectin compared with MGUS, it is possible that the decrease in the levels of adiponectin act as marker of progression to MM, and, during the disease, as a protective factor.

In summary, as disease progresses from MGUS to symptomatic MM, there is a decrease in the levels of anti-inflammatory adipocytokine adiponectin, and an increase in pro-inflammatory adipokines, which could contribute to the mielomagenesis process through the generation of a pro-inflammatory microenvironment. There seems to exist different roles for adipokines levels according to the type of paraprotein produced, as MM patients producing different subtypes of Igs, have different patterns of adipokines production. Patients with symptomatic MM without bone lesion and hypercalcemia have higher levels of leptin, which could reflect the ability of promoting osteoblastic differentiation and bone mineralization. Moreover, MCP-1 was higher in patients presenting with anemia, which could mean that MCP-1 plays a role in BM infiltration by malignant cells and development of anemia. Resistin levels were higher in the PB of MM patients in stage III, and lower in the BM of the same individuals. Additionally, high levels of adiponectin and leptin affected patient's overall survival, which could translated that these adipokines could have a potential prognostic value.

As MM remains an incurable disease and with poor prognosis, all efforts to better understand the pathogenesis of MM are of major importance. Not only it will allow us to clarify the

mechanisms of the disease, but it will also allow us to discover new therapeutic targets and create groups of risk for the disease, with the objective of improving the prognosis of the patients. However, further studies are needed to investigate the importance of the adipokines in MM and to clarify its therapeutic and prognostic value in the clinical practice.

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6. Conflicts of interest

All authors have no conflicts of interest to declare.

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