CAPA

The role of metalloproteinases in Multiple Myeloma – Implications in the pathogenesis and therapeutics

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Ana Sofia Pais[1], Ana Cristina Gonçalves [2,4,5], Catarina Geraldes[3,5,6], Emília Cortesão[3,5,6], Isabel de Sousa[5], Vera Alves[7], Manuel Santos Rosa[5,7], Adriana Teixeira[5,6], José Nascimento Costa [5,8,9] and Ana Bela Sarmento-Ribeiro[2,3,4,5]

[1]Medicine Student/Faculty of Medicine, University of Coimbra (FMUC), Coimbra, Portugal; [2]Applied Molecular Biology Unit, FMUC, Portugal; [3]University Clinic of Hematology, FMUC, Portugal; [4]Center for Neuroscience and Cell Biology (CNC), University of Coimbra, Portugal; [5]CIMAGO - Center of Investigation on Environment Genetics and Oncobiology, FMUC; [6]Clinical Hematology Department, Centro Hospitalar e Universitário de Coimbra (CHUC), Coimbra, Portugal; [7]Immunology Institute, FMUC, Portugal; [8]Oncology and Paliative Care, FMUC, Coimbra, Portugal; [9]Medicine Service, CHUC, Coimbra, Portugal.

TABLE OF CONTENTS

Abstract

Metalloproteinases (MMPs) are known to play a role in cell growth, invasion, angiogenesis, metastasis and bone degradation, all important events in the pathogenesis of cancer. However, the role of MMPs in the development of monoclonal gammopathy of undetermined significance (MGUS) and progression to multiple myeloma (MM) is poorly understood.

We studied the role of MMP-2, -8 and -9 in monoclonal gammopathies (MG), namely in the pathogenesis of MGUS and progression to MM, correlating these results with clinical/laboratory data and prognostic factors. A total of 31 MG patients newly diagnosed, 15 MGUS, 5 SMM (smoldering MM) and 11 symptomatic MM patients, and 2 non neoplasic controls were included in this study. Expression of MMP-2, -8 and -9 was assessed on bone marrow plasma cells (PC) by flow cytometry using monoclonal antibodies labeled with fluorescent probes. We also evaluated the therapeutic potential of a MMP inhibitor, batimastat, in a MM cell line in culture (NCI-H929 [H929] cells).

Our results show that MG patients have higher MMPs intracellular expression levels and in the progression from MGUS to MM these levels seem to decrease, probably due to a higher release. However, the percentage of malignant PC ($CD19^-/CD138^+$) expressing MMPs is higher in SMM, probably due to simultaneously increase in neoplasic cells and/or MMPs production, when compared with MGUS, and higher release, when compared with symptomatic MM. Analyzing MMPs expression according to PC phenotype, malignant cells have lower levels than non-malignant ($CD19^+/CD138^+$). Besides that, the results suggest that the positivity of MMP-9 appears to be a risk factor for symptomatic MM development; however it may be associated with a better outcome in these patients. On the other hand, MMP-2 positivity tends to be a protective factor, but they seem to have a worse survival.

According to clinical/laboratory data, MMPs expression seem to be independent from immunoglobulin subtype. Moreover, the expression of MMP-2 and -8 may be more associated with CRAB symptoms. However, for bone lesion all studied MMPs seem to be important. In addition, an increase of symptoms is probably associated with a higher release of MMPs and this confers poor survival outcomes. According to ISS, MMP-9 seems to be relevant in the progression to a worse prognosis stage.

The results obtained with the MMP inhibitor, batimastat (BB-94), show that it has an anti-proliferative and cytotoxic effect in MM cell line in a time dependent manner, but seems to be concentration independent in a low range drug concentration and dependent from higher variations.

Our preliminary results suggest that PC MMP expression may be correlated with transition of MGUS to MM, promoting extramedullary spreading and disease evolution. Since MM remains incurable, a better understanding of MM biology can lead to new therapeutic approaches.

Keyword: Multiple myeloma, MGUS, metalloproteinases, batimastat, CRAB.

Resumo

As metaloproteinases (MMPs) têm um papel importante na proliferação, invasão, angiogénese, metastização e destruição óssea, todos eventos importantes na patogénese do cancro. No entanto, o papel das MMPs no desenvolvimento da gamapatia monoclonal de significado indeterminado (MGUS) e na progressão para mieloma múltiplo (MM) ainda não está totalmente compreendido.

Nós estudámos o papel da MMP-2, -8 e -9 na gamapatia monoclonal (MG), nomeadamente na patogénese do MGUS e na progressão para MM, correlacionando estes resultados com dados clínicos/laboratoriais e factores de prognóstico.

Neste estudo foram incluídos 31 doentes com MG recém-diagnosticada, 15 MGUS, 5 SMM (MM assintomático) e 11 doentes com MM sintomático, e 2 controlos sem patologia neoplásica. A expressão das MMP-2, -8 e -9 foi avaliada em plasmócitos (PC) de medula óssea, recorrendo à citometria de fluxo utilizando anticorpos monoclonais marcados com sondas fluorescentes. Também foi avaliado o potencial terapêutico de um inibidor das MMPs, o batimastat (BB-94), utilizando uma linha celular de MM em cultura (NCI-H929 [H929]).

Os nossos resultados demonstram que os doentes com MG tem maior expressão intracelular de MMPs e na progressão de MGUS para MM estes tendem a diminuir, provavelmente devido ao aumento da libertação. Contudo, a percentagem de PC malignos ($CD19^{-}/CD138^{+}$) com expressão de MMP é maior nos SMM, provavelmente devido ao simultâneo aumento das células malignas e produção de MMPs, quando comparados com os MGUS, e ao aumento da libertação, quando comparados com os MM sintomáticos. Analisando a expressão das MMP de acordo com o fenótipo dos PC, as células malignas têm menores níveis que as não malignas ($CD19^{+}/CD138^{+}$). Além disso, os resultados sugerem que a positividade para MMP-

9 tende a ser um factor de risco para o desenvolvimento de MM sintomático, contudo, parece conferir maior sobrevivência nestes pacientes. Por outro lado, a positividade para MMP-2 tende a ser um factor protector, mas estes doentes parecem ter pior sobrevivência.

De acordo com os dados clínicos/laboratoriais, a expressão de MMPs parece ser independente do subtipo de imunoglobulina produzida. Por outro lado, a expressão de MMP-2 e -8 parece estar mais associada à presença de sintomas CRAB, enquanto todas as MMPs estudadas tendem a estar correlacionadas com a lesão óssea. Adicionalmente, o aumento do número de sintomas está provavelmente associado à maior libertação de MMPs, o que confere pior sobrevivência. De acordo com o ISS, a MMP-9 parece desempenhar um papel importante na progressão para um estádio de pior prognóstico.

Os resultados obtidos com o inibidor das MMPs, batimastat (BB-94), demonstraram um efeito anti-proliferativo e citotóxico na linha celular de MM, de modo dependente do tempo, mas parece ser independente de pequenas variações da concentração e dependente de grandes variações.

Os resultados preliminares sugerem que a expressão de MMPs nos PC pode estar relacionada com a transição de MGUS para MM, promovendo a invasão extramedular e a evolução da doença. Tendo em conta que o MM permanece uma doença incurável, o melhor conhecimento da biologia do MM pode levar ao desenvolvimento de novas abordagens terapêuticas.

Palavras-chave: Mieloma Múltiplo, MGUS, metaloproteinases, batimastat, CRAB.

Abbreviations

APC – Allophycocyanin

ATCC – American Type Culture Collection

BB-94 – Batimastat

BM – Bone Marrow

BMSC – Bone Marrow Stromal Cells

CLL – Chronic Lymphocytic Leukaemia

CRAB – increased Calcium levels, Renal insufficiency, Anaemia and Bone lesions

EC – Endothelial Cells

ECM – Extracellular Matrix

FC – Flow Cytometry

Ig – Immunoglobulin

IGF-1 – Insulin Growth Factor 1

IgH – Immunoglobulin Heavy chain

IL – Interleukin

ISS – International Staging System

MFI – Mean Fluorescence Intensity

MG – Monoclonal Gammopathy

MGUS – Monoclonal Gammopathy of Undeterminated Significance

MM – Multiple Myeloma

MMPs – Metalloproteinases

MMPI – Matrix metalloproteinases inhibitors

OC – Osteoclast

OPG – Osteoprotegerin

PBS – Phosphate Buffer Saline

PC – Plasma Cells

PE – Phycoerythrin

PerCP – Peridin Chlorophyll Protein Complex

RPMI 1640 – Roswell Park Memorial Institute 1640 medium

SD – Standard Deviation

SMM – Smoldering Multiple Myeloma

STAT3 – Signal Transducer and Activator of Transcription

TGF – Transforming Growth Factor

TIMPs – Tissue Inhibitors Metalloproteinases

TNF- α – Tumor Necrosis Factor-A

VEGF – Vascular Endothelial Growth Factor

β2M – β2-microglobulin

1. Introduction

Monoclonal gammopathies (MG) were first described in 1960 by Jan Waldenström (Waldenström, 1960) and result from the overproduction of a clonal immunoglobulin (Ig) secreted by terminally differentiated B lymphocyte or plasma cell (PC). These cells typically secrete a monoclonal Ig called paraprotein or monoclonal protein, which is recognized as a band of restricted migration on serum and urine electrophoresis (Swerdlow *et al*, 2008). Plasma cell dyscrasias include pre-malignant and malignant conditions, such as monoclonal gammopathy of undetermined significance (MGUS) and multiple myeloma (MM), respectively (Katzel *et al*, 2007).

Although, the first well documented MM case was reported, in 1844, by Samuel Solly, the term MM emerged, in 1873, by J. Von Rustizky, and it was described as a plasma cell neoplasm characterized by the proliferation of malignant plasma cells in the bone marrow (BM), by James H. Wright, in 1900 (Wright, 1900; R. a Kyle & Rajkumar, 2008; Swerdlow et al., 2008). The disease is defined as monoclonal protein in serum higher than or equal to 30g/L and/or BM PC greater than or equal to 10%. When there are no related organ or tissue impairment it is called asymptomatic or smoldering MM (SMM) *versus* symptomatic MM when they are present (Bird *et al*, 2011).

MM first pathogenic step is a premalignant MGUS (Swerdlow *et al*, 2008; Bird *et al*, 2011). The term MGUS emerged in 1978 by Robert Kyle (Kyle, 1978) and it is a pre-malignant plasma cell disorder defined by three criteria: 1) M-protein in serum lower than 30g/L, 2) bone marrow PC less than 10% and low level of PC infiltration in trephine biopsy (if done), 3) no related organ or tissue damage (Bird *et al*, 2011). Approximately 3% of individuals over age 50 and greater than or equal to 5% past 70 years have MGUS (Swerdlow *et al*, 2008).

In MM, median age at presentation is approximately 70 years. It accounts for 1% of all malignancies, representing 10% of hematologic malignancies, and it causes 20% of deaths from hematologic malignancies. Both plasma cell dyscrasias are more common in men than in women (1,4:1) and two times more frequent in African Americans than in Caucasian (Swerdlow *et al*, 2008; Dimopoulos & Terpos, 2010).

Current models assume that MM pathogenesis involve a multistep transformation process (Hallek *et al*, 1998). The earliest changes, that thought to occur in germinal center B cells, include complex genetic and/or epigenetic events, such as primary translocations involving the Ig heavy chain (IgH) gene and multiple trissomies (Hideshima *et al*, 2004). Chromosome translocations lead, directly or indirectly, to cyclin-D dysregulation, which contributes to higher cells susceptibility to proliferative stimuli, leading to a selective expansion (Bergsagel *et al*, 2005; Mahindra *et al*, 2010). Activating mutations of N- or K-RAS appear to mark the progression from MGUS to MM, among others (Bergsagel & Kuehl, 2005).

The pathogenesis of MM involves, not just MM cells, but also BM microenvironment, including several survival signals (IL-6R/STAT3, Ras/MAPK, WNT, NF-κB pathways) and cytokines secretion (vascular endothelial growth factor (VEGF), insulin growth factor (IGF) -1, tumor necrosis factors (TNFs), transforming growth factor (TGF) -β and interleukin (IL) -6), that are crucial to tumor cell proliferation, tumor growth, angiogenesis, invasion, protection from spontaneous and drug-induces apoptosis, metastasis and bone destruction (Podar, 2001; Zhou *et al*, 2005; Bommert *et al*, 2006; Palumbo & Anderson, 2011). IL-6 is the best characterized myeloma growth and survival factor and it is produced by bone marrow stromal cells (BMSC), osteoblasts and, in some cases, by myeloma cells. (Bommert *et al*, 2006; Löffler *et al*, 2007). Furthermore, although MM progression is observed mainly within the BM during the early stages of the disease, these mechanisms promote extramedullary

spreading during the terminal stage of the disease and malignant cells can be detected in peripheral blood of many patients (Hideshima *et al*, 2004).

Matrix metalloproteinases (MMPs) are a family of zinc-dependent endopeptidases structurally and functionally related, that are characterized by the ability to degrade the extracellular matrix (ECM). These proteinases are synthesized as pro-enzymes and then, as inactive pro-MMPs, they are secreted from the cell or anchored to the plasma membrane. Their activation is made by proteolytic cleavage of the propeptide domain. The expression is regulated at transcriptional level by growth factors, hormones, cytokines, and also at the level of activation of the precursors zymogens, interaction with specific ECM compounds and inhibition by endogenous inhibitors, as tissue inhibitors metalloproteinases (TIMPs) (Nagase & Woessner, 1999; Visse & Nagase, 2003; Valckenborgh *et al*, 2004a).

Based on their substrate specificity and domain structure, MMPs were divided into six subgroups: collagenases, gelatinases, stromelysins, matrilysis, membrane-type MMPs and other MMPs (Visse & Nagase, 2003; Roy *et al*, 2009; Sekhon, 2010). One of these subgroups is represented by gelatinases, which include gelatinase A (MMP-2) and gelatinase B (MMP-9), that cleave gelatins and promotes collagen denaturation, the major constituent of the basement membrane (Sekhon, 2010). MMP-2 also digests type I, II, III collagens and is important for osteogenesis. Collagenases are a MMPs subgroup, which include MMP-8, and degrade interstitial collagens I, II and III and also other ECM and non-ECM molecules (Visse & Nagase, 2003).

MMPs play a major role in several biological process, such embryogenesis, morphogenesis, wound healing and normal tissue resorption and remoldering, and also in pathogenesis of several diseases, such as atherosclerosis, aneurysms, nephritis, multiple sclerosis, rheumatoid arthritis and cancer (Visse & Nagase, 2003; Sekhon, 2010). In cancer, tumor cells overexpress

proteases and/or induce their expression in stromal cells in order to degradate ECM and invade surrounding tissue. Besides invasion, MMPs are known to play a role in cell growth, differentiation, angiogenesis, metastasis and bone degradation, all important events in the pathogenesis of cancer (Valckenborgh *et al*, 2004a; Roy *et al*, 2009).

In MM, MMPs play a role in tumor growth, angiogenesis, homing of MM cells and osteolytic bone disease (Valckenborgh *et al*, 2004a, b). MMPs are expressed by neoplasic cells (MMP-2, -7, -8, -9 and -13), BMSC (MMP-1 and -2) and endothelial cells (EC) (MMP-2 and -9) (Barillé *et al*, 1997; Valckenborgh *et al*, 2004a; Zdzisińska *et al*, 2008). The expression of these proteases can be regulated by cytokines (IL-6, IL-1β, TNF- α, etc.), hormones, growth factors, cell-matrix and cell-cell interactions. MMPs might also be able to generate growth promoting signals (IL-6 and IGF-1) (Valckenborgh *et al*, 2004a).

Over the past 20 years a lot of effort was been put off to develop gene therapy and to design MMPs inhibitors (MMPI), as a therapeutic tool for cancer (Rothenberg *et al*, 1998; Syed *et al*, 2004; Valckenborgh *et al*, 2004a; Roy *et al*, 2009). Batimastat, the first MMPI, inhibits MMP-1, -2, -3, -7 and -9 (Stefanidakis & Koivunen, 2006) and prevents osteolytic bone disease, by reducing the number of OC on trabecular surfaces, in prostate cancer metastasis, (Valckenborgh *et al*, 2004a).

Many reports suggest the use of MMPs as biomarkers in solid tumors and associate them with higher risk or worse prognosis. Some studies correlate their expression with MM, exploring its role in tumor growth, homing, angiogenesis and osteolytic bone lesions. However, the role of MMPs in the development of MGUS and progression to MM is poorly understood. As MM remains incurable, with a survival of 3-4 years (6 weeks to 10 years) (Swerdlow *et al*, 2008; Landgren, 2010), the better understanding of MM cell proliferation, survival and migration in the BM microenvironment may enhance knowledge of pathogenesis and provide a framework

for identification and validation of new biomarkers in transition from MGUS to MM and novel molecular therapeutic targets.

In the present study, we aimed to explore the role of MMPs, namely MMP-2, MMP-8 and MMP-9, in the pathogenesis of MGUS and progression to MM and correlate these results with clinical and laboratory data and prognostic factors. We also pretend to evaluate in vitro the therapeutic potential of the MMPI, batimastat (BB-94), in MM cell line in culture.

2. Materials and methods

2.1. Evaluation of MMPs expression

2.1.1. Patients characteristics

To evaluate the role of MMPs in the pathogenesis and the progression of MGUS to SMM and symptomatic MM, 33 subjects were included: 2 controls (subjects without neoplasia) and 31 MG patients, a total of newly diagnosed 15 MGUS patients, 5 SMM and 11 symptomatic MM patients. PCs were isolated from BM aspirations, performed at diagnosis.

Data collected include: age, gender, bone marrow PC, type and amount of abnormal protein in blood, skeletal survey results and serum levels of β2M, albumin, calcium, creatinine, haemoglobin and time for disease progression or death in 1,5 years of follow-up.

The research studies were approved by local ethic committee and all subjects provided written informed consent in accordance with the Declaration of Helsinki.

2.1.2. Flow cytometry

Expression of MMP-2, MMP-8 and MMP-9 was assessed on bone marrow PC by flow cytometry (FC) using monoclonal antibodies labelled with fluorescent probes. Briefly, about 1×10^6 cells were incubated for 10 min at room temperature with 1 μg monoclonal anti-CD138 antibody labeled with allophycocyanin (APC) and anti-CD19 labeled with peridinin chlorophyll protein complex (PerCP) (BD Biosciences). Then cells were washed by centrifugation at 300 ×g for 5 min with phosphate buffer (PBS) and fixated by incubation

with 100 µL of fixation solution (Intracell, Immunostep), for 15 min. Cells were washed, by centrifugation at 300 ×g for 5 min, permeated by incubation with 100 µL of permeabilization solution (Intracell, Immunostep) and with 1 µg of monoclonal antibody anti-MMP-2 labeled with the fluorescent probe FITC (R&D Biosystems) and/or anti-MMP-8 or MMP-9 labeled with phycoerythrin (PE) (R&D Biosystems). After washing with PBS, the cells were resuspended and analyzed on a FACScalibur (BD, Becton, Dickinson and Company) flow cytometer. Results were expressed in percentage and mean fluorescence intensity (MFI). Negative controls were established with isotype immunoglobulin G and submitted to the same procedures. Phenotypically normal PC are CD19 and CD138 positive and phenotypically malignant PC are CD19 negative and CD138 positive.

2.2. Evaluation of therapeutic potential of a metalloproteinase inhibitor in a MM cell line in culture

2.2.1. Cell culture conditions

The therapeutic potential of a metalloproteinase inhibitor, batimastat (BB-94), was evaluated using a MM cell line, the NCI-H929 [H929] cells, provided by American Type Culture Collection (ATCC). The cell line was routinely grown in Roswell Park Memorial Institute 1640 medium (RPMI 1640), containing 2 mM L-glutamine, 25 mM HEPESNa, 100 U/ml penicillin and 100 µg/ml streptomycin (Gibco, Invitrogen) supplemented with 2-mercaptoethanol to a final concentration of 0.05mM and fetal bovine serum (Gibco, Invitrogen) to a final concentration of 10%. Cells were seeded at a density of $0.5x10^6$ cells/ml and kept in culture at 37ºC in a humidified atmosphere with 5% CO2.

In order to evaluate the effect of a metalloproteinase inhibitor, batimastat (BB-94), cells were cultured for 72 hours in the absence and presence of BB-94 in concentrations ranging from 1 nM to 100 μM.

2.2.2. Cell viability evaluation

Cell viability was evaluated by the resazurin assay, based on desidrogenases (mainly in mitochondria) activity. Resazurin was prepared as a stock solution of 100 μg/ml in phosphate-buffered saline (PBS). Stock solution was filtered with a sterile 0.20 μm pore filter and stored in the dark at -20°C. After treatment, a final concentration of 10 μg/ml of resazurin solution was added to the cells, which were then incubated at 37°C for 4 hours. Following this, we collected 200 μl from each well and transferred to 96 well plates. We measured the absorbance at 570 nm and 600 nm colorimetrically, using a Synergy™ HT Multi-Mode Microplate Reader (BioTek Instruments), and calculated cell viability as a percentage of the control cells according to the formula:

$$\frac{[(A_{570} - A_{600})\text{sample}] - [(A_{570} - A_{600})\text{blank}]}{[(A_{570} - A_{600})\text{control}] - [(A_{570} - A_{600})\text{blank}]} \times 100$$

2.2.3. Cell death analysis

Cell death was examined by flow cytometry using annexin and propidium iodide double staining. After an incubation period of 48 hours in the conditions described above, cells were washed with PBS and centrifuged at 1 000 xg for 5 minutes, in order to obtain a density of 1 x 10^6 cells/ml. Untreated and treated cells were resuspended in 100 μl of binding buffer and then in 5 μl of annexin V-FITC (AV) and 2 μl of propidium iodide (PI) staining solution

(ImmunoStep, Salamanca, Spain) were added. Cells were gently stirred in a vortex and incubated for 15 minutes at room temperature (25ºC) in the dark. Finally, 300 µl of binding buffer were added to each tube and cells were then analyzed in a FACS Calibur (Becton Dickinson) flow cytometer equipped with an argon laser. Green fluorescence of AV was collected with a 525 nm band pass filter and red fluorescence of PI with a 610 nm band pass filter. CellQuest software (Becton Dickinson) was used for the acquisition of data and these were analyzed with the Paint-a-Gate software. Results were expressed in percentages of viable cells (AV^-/PI^-), early apoptotic (AV^+/PI^-), late apoptotic/necrotic (AV^+/PI^+) and necrotic cells (AV^-/PI^+).

2.3. Statistical analysis

In this study we used SPSS, 19th version, and for all analysis it was evaluated the normality and homogeneity of variances. According to the results it was selected a parametric or non parametric test. All data was statistically analyzed using one-way ANOVA and unpaired Student's t-test and is reported as mean ± standard deviation (SD). Survival was analyzed using Kapplan-Mayer. Differences were considered statistically significant when $p<0.05$.

3. Results

3.1. Evaluation of MMPs expression

3.1.1. Patients characteristics

To evaluate the role of MMPs in the pathogenesis and in the progression from MGUS to SMM and symptomatic MM, were included 2 controls (subjects without neoplasia) and a total of newly diagnosed 31 MG patients, 15 MGUS, 5 SMM and 11 symptomatic MM patients. PCs were isolated from BM aspirations, done at diagnosis.

As we can se in table I, the mean age is 68 years (±2,8) for control subjects, 70,1 years (±2,9) for MGUS patients, 73,2 years (±2,9) for SMM and 76,5 years (±1,7) for symptomatic MM. According with gender, the populations are manly female, all controls and SMM (100%), 60% of MGUS and 72,7% of MM patients.

BM plasmocytosis and Ig concentration increase with disease progression, being the mean of BM PC 5,21% (±0,6) for MGUS patients, 13,2% (±1,77) for SMM and 32,64% (±8,91) for symptomatic MM. The average of Ig concentration is 13,41 g/L (±1,61), 21,74 g/L (±4,64) and 34,57 g/L (±10,05), for MGUS, SMM and symptomatic MM patients, respectively. Monoclonal protein subtype in MGUS patients was 60% IgG, 33,3% IgA and 6,7% IgM, while in SMM patients is 60% IgA and 40% IgG, and in MM patients 45,5% IgG, 36,4% IgA and 18,2% light chains (Table I).

When collected the data about the presence of CRAB symptoms, most MM patients have bone lesion (81,8%) and 18,2% have hypercalcaemia, 18,2% renal lesion and 45,5% anaemia. According to the International Staging System (ISS) 66,7% of MGUS patients are in stage I

and 33,3% in stage II. In SMM patients 40% are in stage I, 40% in stage II and 20% in stage II. On the other hand, 81,8% of symptomatic MM patients are in stage III (Table I).

In a 1,5 year of follow-up, just one patient progressed, from MGUS to symptomatic MM, and 10 patients died, all with MM, but just half of them by progressive disease.

Table I. Patient characteristics.

		Total n = 33	Control n = 2	MGUS n = 15	SMM n = 5	Symptomatic MM n = 11
Variable		n (%) or median (±SD)				
Age (years)		73,5 ±1,7	68 ±2,8	70,1 ±2,9	73,2 ±2,9	78,5 ±1,7
Gender M/F		9/22 (29%/71%)	0/2 (0/100%)	6/9 (40%/60%)	0/5 (0/100%)	3/8 (27,3%/72,7%)
PC (%)		16,6 ±3,95	-	5,21 ±0,6	13,2 ±1,77	32,64 ±8,91
Ig subtype	G	17 (54,8%)	-	9 (60%)	3 (60%)	5 (45,5%)
	A	11 (35,5%)	-	5 (33,3%)	2 (40%)	4 (36,4%)
	M	1 (3,2%)	-	1 (6,7%)	0	0
	Light chain	2 (6,5%)	-	0	0	2 (18,2%)
Ig concentration (g/L)		21,41 ±3,64	-	13,41 ±1,61	21,74 ±4,64	34,57 ±10,05
CRAB symptoms	Hypercalcaemia	3 (9,7%)	-	1 (6,7%)	0	2 (18,2%)
	Renal lesion	2 (6,5%)	-	0	0	2 (18,2%)
	Anaemia	5 (16,1%)	-	0	0	5 (45,5%)
	Bone lesion	11 (35,5%)	-	2 (13,3%)	0	9 (81,8%)
ISS	I	13 (41,9%)	-	10 (66,7%)	2 (40%)	1 (9,1%)
	II	8 (25,8%)	-	5 (33,3%)	2 (40%)	1 (9,1%)
	III	10 (32,3%)	-	0	1 (20%)	9 (81,8%)

3.1.2. Evaluation of PC MMPs expression in patients with Monoclonal Gammopathies

In this preliminary study, our results show that patients with monoclonal gammopathies (MG) have higher MMPs expression levels when compared with controls, especially for MMP-8

and -9 (Fig 1.B), however, these differences are not statistically significant. In the percentage of cells expressing MMPs, there are no differences between groups (Fig 1.A).

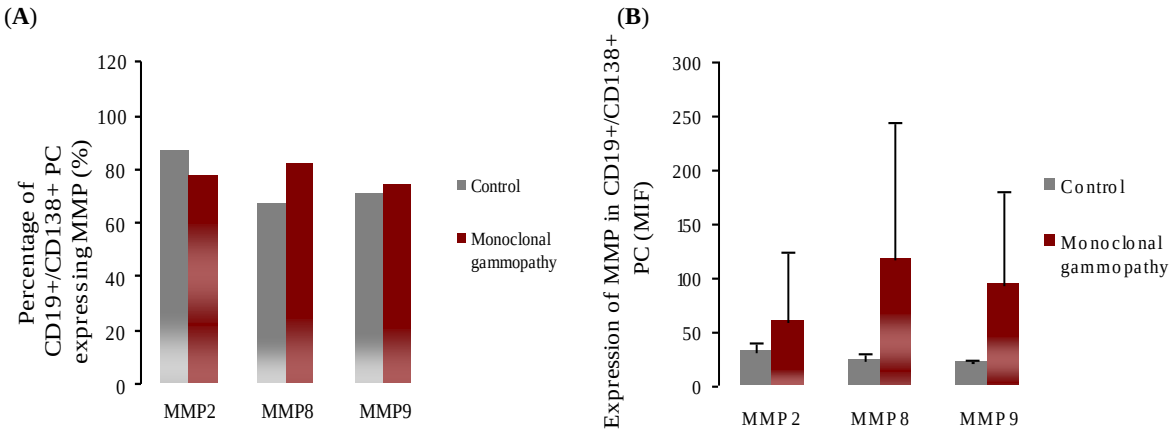

Fig 1. Evaluation of MMPs in controls and patients with Monoclonal Gammopathies. In (**A**) is represented the percentage of $CD19^{+}/CD138^{+}$ PC expressing the MMP-2, -8 and -9 and in (**B**) the intracellular expression levels of MMPs. The results are expressed in percentage (%) and mean intensity fluorescence (MIF) (**A** and **B**, respectively) and represent the mean ± SD of the values obtained in controls *versus* MG patients.

When analysed the results, in MG patients, according to PC phenotype we observe that malignant PC have about half of the percentage of PC expressing MMP-2 (37%) and MMP-9 (40%), when compared with non-malignant PC (78% and 74% respectively), being the results statistically significant (p=0,000 for MMP-2 and p=0,012 for MMP-9) (Fig 2.A). Intracellular expression levels of MMPs are also lower in phenotypically malignant PC than normal PC, being statistically significant just for MMP-2 (26 ±16 MIF *versus* 61±62 MIF, p=0,037) (Fig 2.B).

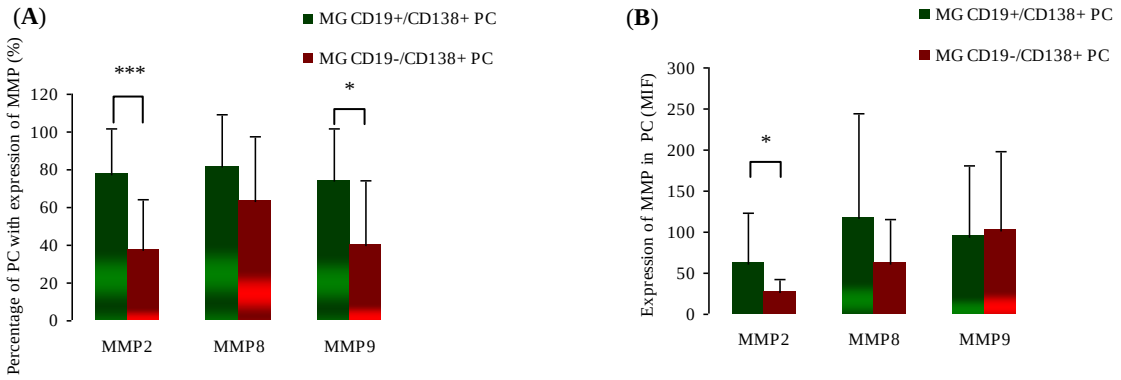

Fig 2. Analysis of MMPs in patients with MG according to PC phenotype. The percentage of PC expressing the MMP-2, -8 and -9 is represented in (**A**) and the intracellular expression levels of MMPs in (**B**), for non-malignant ($CD19^+/CD138^+$) *versus* malignant ($CD19^-/CD138^+$) PC. The results are expressed in percentage (%) and mean intensity fluorescence (MIF) (**A** and **B**, respectively) and represent the mean ± SD of the values obtained in MG patients. (* p<0,05, ** p<0,01, *** p<0,001)

After, we evaluated intracellular expression levels of MMPs in patients based on MG subtype and we observed that they tend to be higher in MGUS compared with SMM and symptomatic MM. As we can observe in figure 3.B, for phenotypically normal PC, MMP-2 in MGUS patients is approximately four times higher than SMM (90 ±83 MIF *vs* 21 ±3 MIF, respectively) and about twice of MM patients (42 ±14 MIF) (p=0,026 and p=0,004, respectively). For MMP-9, the expression is also higher is MGUS, being statistically significant when compared with symptomatic MM (148 ±97 MIF *versus* 37 ±14 MIF, p=0,033) (Fig 3.B). However, in malignant PC of symptomatic MM patients, we observe the lowest levels of MMP-9 (36 ±33 MIF) compared with MGUS (147 ±112 MIF) and SMM patients (97 ±48 MIF), with statistically significant differences, p=0,005 and p=0,047, respectively (Fig 3.D). Besides that, SMM patients show about two times more percentage of malignant PC expressing MMP-2 (67%) and -8 (95%) when compared with MGUS (35% and

54%, respectively) and symptomatic MM (28% and 61%, respectively), with p-value lower than 0,05 (Fig 3.C).

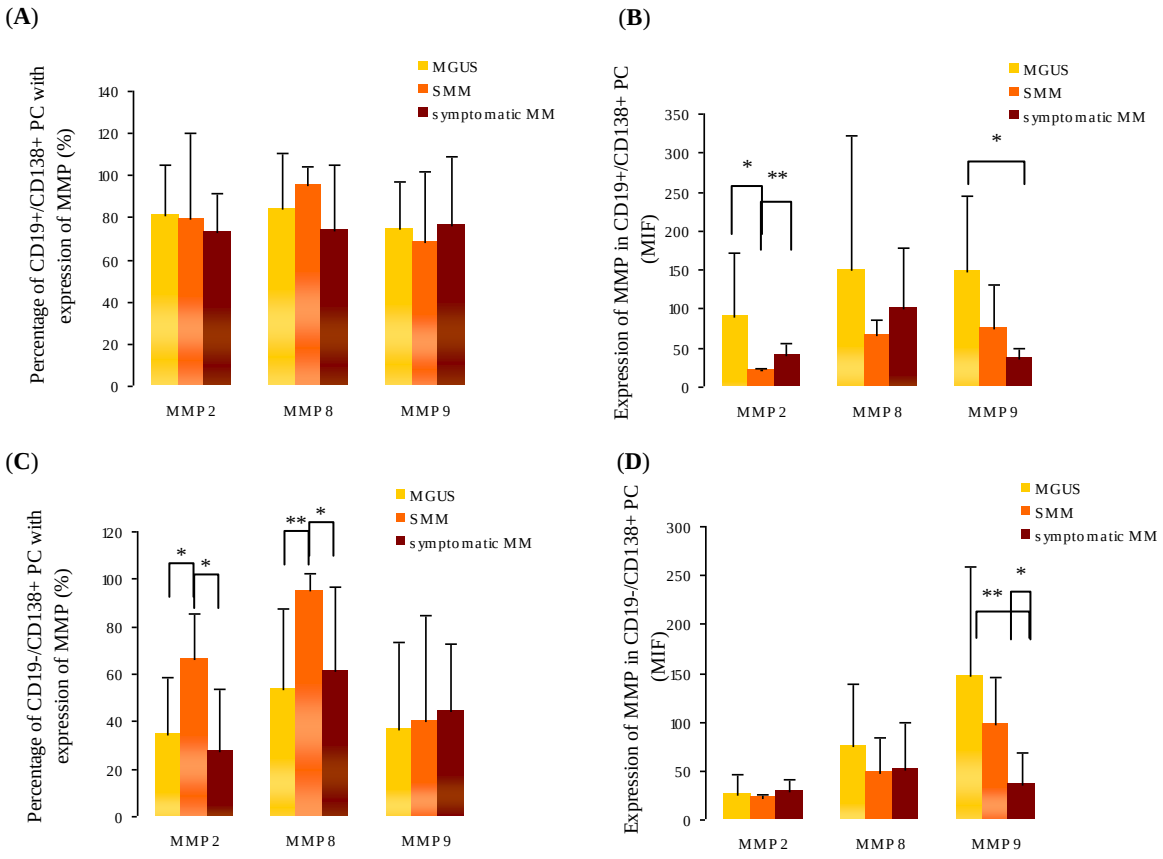

Fig 3. Evaluation of MMP-2, -8 and -9 in MGUS, SMM and symptomatic MM patients. The percentage of $CD19^+/CD138^+$ (**A**, **B**) and $CD19^-/CD138^+$ PC (**C**, **D**) expressing the MMPs and their intracellular expression levels are represented. The results are expressed in percentage (%) (**A**, **C**) and mean intensity fluorescence (MIF) (**B**, **D**) and represent the mean ± SD of the values obtained in MG patients (* $p<0,05$, ** $p<0,01$).

Similarly to the analysis above mentioned in MG patients, we have compared the expression levels of MMP-2, -8 and -9 in MGUS and MM patients according to PC phenotype, non-malignant ($CD19^+/CD138^+$) *versus* malignant ($CD19^-/CD138^+$). As represented in figure 4, we observed that in MGUS and symptomatic MM patients, the percentage of $CD19^-/CD138^+$

PC expressing MMPs is approximately half when compared with non-malignant PC, being statistically significant for MMP-2 (p=0,004 and p=0,000, respectively) and for MMP-9 (p=0,036 and p=0,045, respectively) (Fig 4.A and C). MMP-2 intracellular expression levels in malignant PC are also lower when compared with $CD19^{+}/CD138^{+}$ PC, in MGUS (26 ±20 MIF *versus* 90 ±83 MIF) and in symptomatic MM (28 ±12 MIF *versus* 42 ±14 MIF); while MMP-8 are significant decreased in symptomatic MM (51 ±48 MIF *versus* 100 ±79 MIF) (Fig 4.B and D). In SMM group no differences have been detected (data not show).

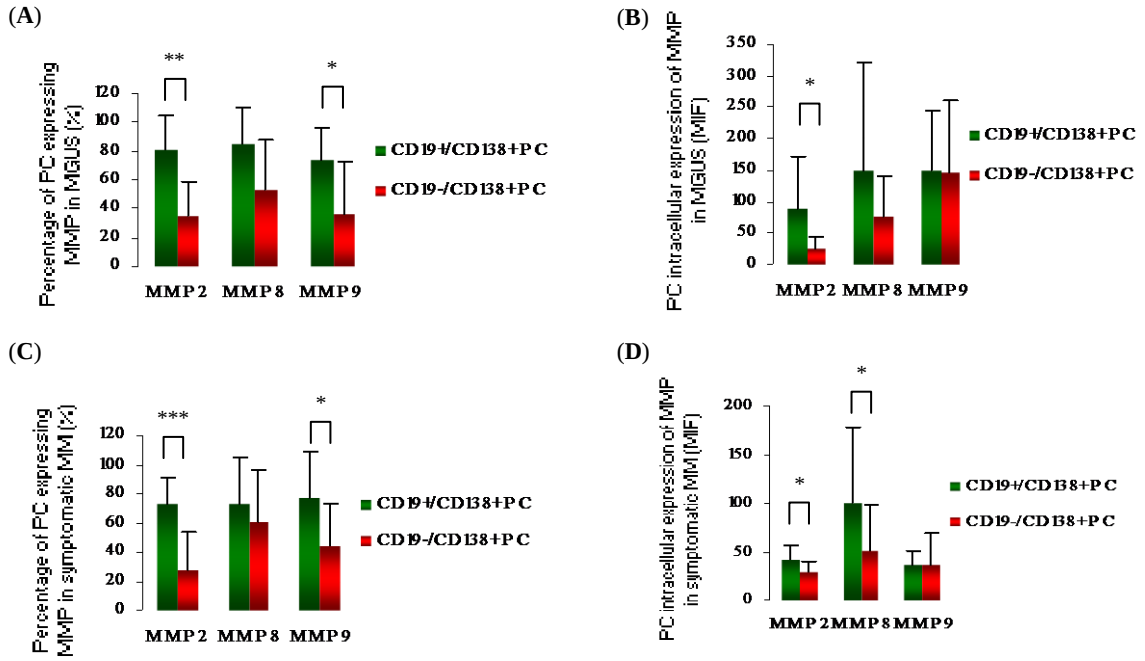

Fig 4. Comparative analysis of MMPs in MGUS and symptomatic MM patients according to PC phenotype. The MMP-2, -8 and -9 expression levels in MGUS (**A**, **B**) and symptomatic MM patients (**C**, **D**) are represented, for phenotypically normal ($CD19^{+}/CD138^{+}$) *versus* neoplasic ($CD19^{-}/CD138^{+}$) PC. The results are expressed in percentage (%) (**A**, **C**) and mean intensity fluorescence (MIF) (**B**, **D**) and represent the mean ± SD of the values obtained in patients PC. (* p<0,05, ** p<0,01, *** p<0,001)

To calculate the frequency of MMPs expression in MGUS, SMM and symptomatic MM, we had considered that a patient is positive for one MMP, if there is more than 20% of phenotypically malignant PC that express the MMP. As we can see in table II, all SMM patients are positive for MMP-2 and -8. Positivity for MMP-9 is observed in 72,7% of symptomatic MM patients *versus* just 53,3% and 40% for MGUS and SMM, respectively. When analysed different combinations of these MMPs, 46,7% of MGUS patients are positive for MMP-2 and -9, all SMM (100%) are positive for MMP-2 and -8, and 63,6% of symptomatic MM are positive for MMP-8 and -9.

Table II. Analysis of the frequency of patients showing MMP-2, -8 and -9 positivy.

	MGUS n = 15 (%)	**SMM** n = 5 (%)	**Symptomatic MM** n = 11 (%)
MMP-2	9 (60%)	5 (100%)	6 (54,5%)
MMP-8	11 (73,3%)	5 (100%)	8 (72,7%)
MMP-9	8 (53,3%)	2 (40%)	8 (72,7%)
MMP-2 + MMP-8	9 (60%)	5 (100%)	5 (45,5%)
MMP-8 + MMP-9	7 (46,7%)	2 (40%)	7 (63,6%)
MMP-2 + MMP-9	7 (46,7%)	2 (40%)	4 (36,4%)
MMP-2 + MMP-8 + MMP- 9	7 (46,7%)	2 (40%)	4 (36,4%)

We have consider a Cut-Off of 20%

3.1.3. Influence of Ig subtype in MMPs expression

MMPs expression was also analysed in MG patients according to Ig subtype. As we can observe in figure 5.A, in patients with monoclonal gammopathy Ig G we did not observe any differences in the percentage of phenotypically normal PC expressing MMPs. However, there are already statistically significant differences in malignant PC, being the percentage of these cells that express MMP-8 higher in SMM patients (97%) than in MGUS (59%) and

symptomatic MM (51%, p=0,035) (Fig 5.C). Furthermore, in these cells, MMP-9 intracellular expression levels are about five times higher in MGUS patients (126 ± 92 MIF), when compared with symptomatic MM (23 ±2 MIF), with the differences statistically significant (p=0,026) (Fig 5.B and D).

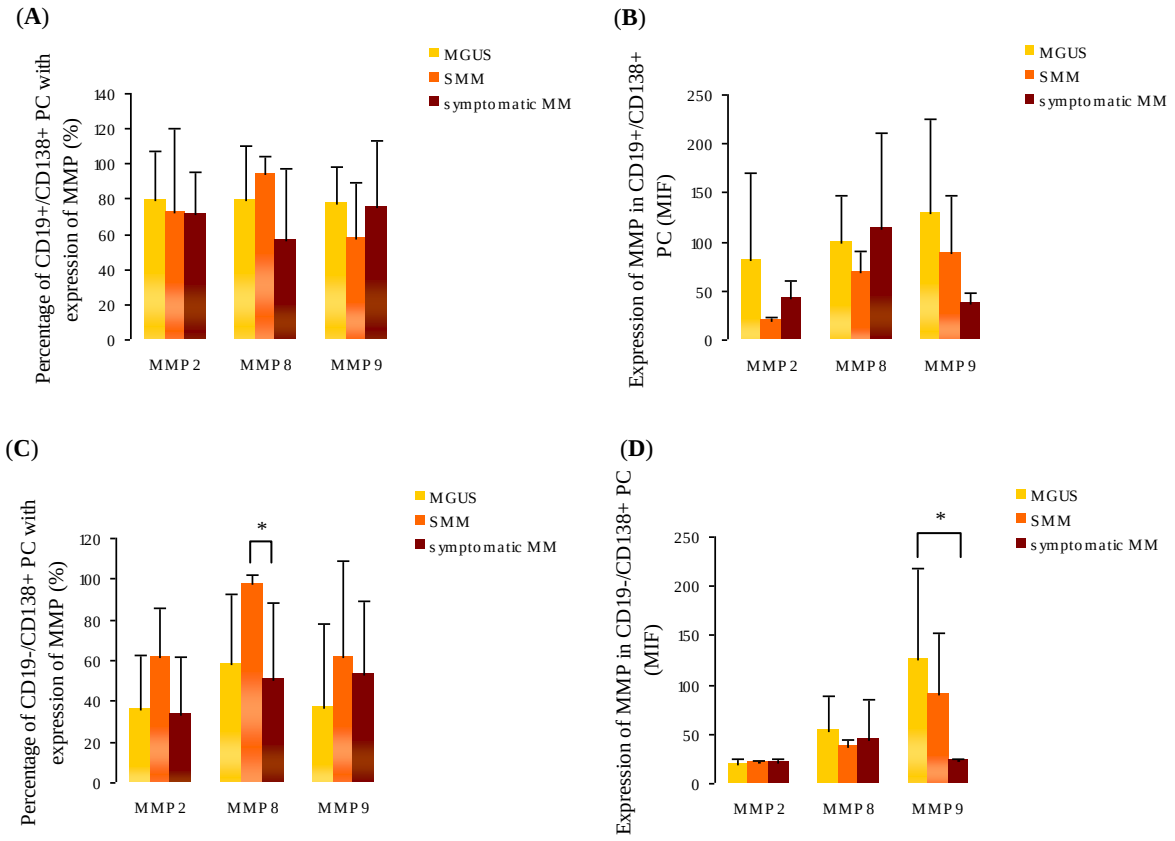

Fig 5. Evaluation of the differences in percentage of cells expressing MMPs and in intracellular expression levels for Ig G MGUS, SMM and symptomatic MM patients. The results are expressed in percentage (%) (A and C) and mean intensity fluorescence (MIF) (B and D) and represent the mean ± SD of the values obtained in $CD19^{+}/CD138^{+}$ (**A**, **B**) and $CD19^{-}/CD138^{+}$ PC (**C**, **D**). (* $p<0.05$)

In Ig A patients, the expression of MMPs shows the same tendency, but the results are not statically significant (data not show).

Besides that, we compare MMPs expression levels for each diagnosis subgroup according with Ig subtype and no differences were observed in MGUS and SMM patients (data not show). For symptomatic MM patients, the results show a significant increase in the percentage of malignant PC ($CD19^-/CD138^+$) expressing MMP-8 only in Ig A patients, when compared with Ig G (p=0,05) and light chain MM patients (p=0,013) (Fig 6.C). For normal PC ($CD19^+/CD138^+$), no statistically significant differences are observed (Fig 6.A), as well as in both PC population's intracellular expression of MMPs (Fig 6.B and D).

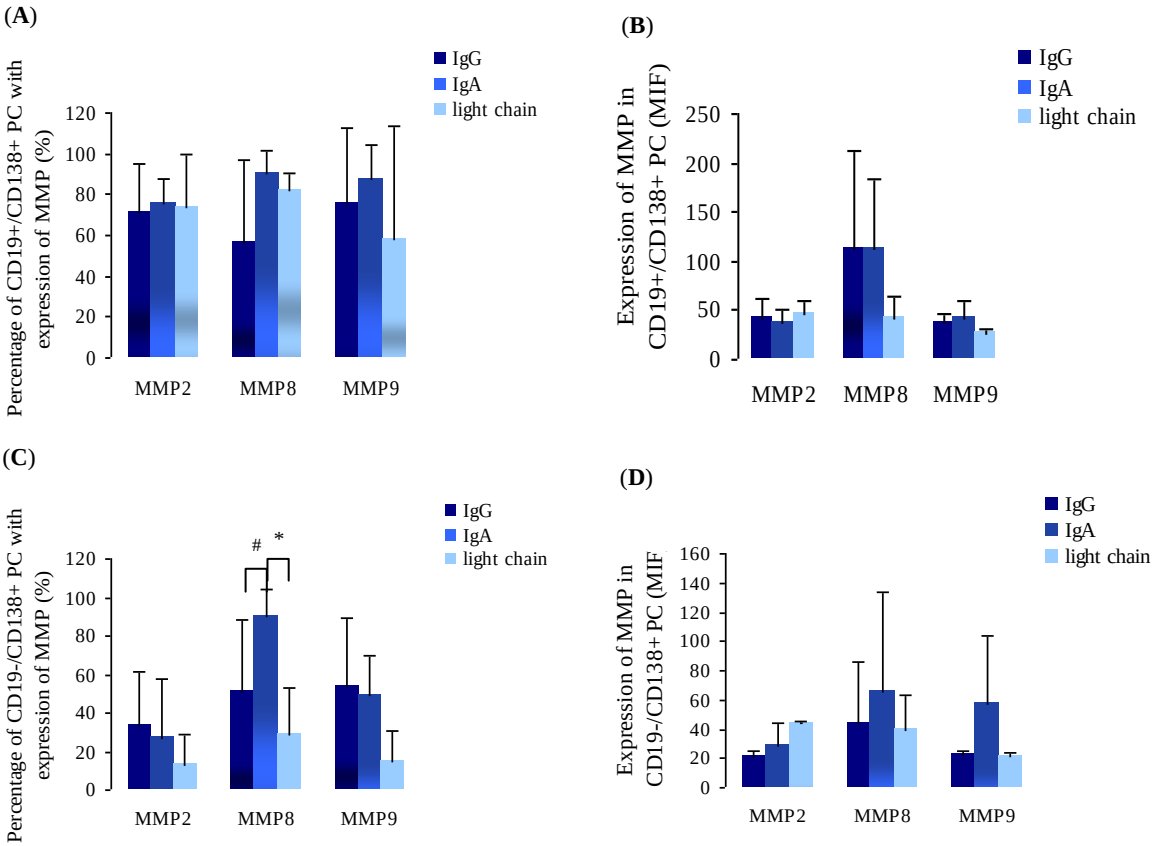

Fig 6. Influence of Ig subtype in MMPs expression for symptomatic MM patients. The expression of MMP-2, -8 and -9 is represented in (**A**) and (**B**) for $CD19^+/CD138^+$ PC and in (**C**) and (**D**) for $CD19^-/CD138^+$ PC. The results are expressed in percentage (%) (**A**, **C**) and mean intensity

fluorescence (MIF) (**B**, **D**) and represent the mean ± SD of the values obtained in PC of MG patients. (* p<0,05, # p=0,05)

When analysed PC MMPs expression levels in each diagnosis subgroup (MGUS, SMM, symptomatic MM), there were no differences between subgroups according to Ig G and non Ig G expression (data not show).

3.1.4. Correlation of MMPs expression with CRAB symptoms

The MMPs expression levels were also evaluated in symptomatic MM patients according to the presence of CRAB symptoms, namely increased calcium levels, renal insufficiency, anaemia and bone lesions, individually (Fig 7-10) and jointly (Fig 11).

Our results, represented in figure 7.A, show that patients with hypercalcaemia have a significant increase in the percentage of normal PC ($CD19^+/CD138^+$) expressing MMPs, especially MMP-2 (p=0,045), when compared with patients with normal serum calcium (93% *versus* 69%). Besides that, intracellular expression levels of MMP-8 and -9, both in non-neoplasic and in neoplasic PC ($CD19^+/CD138^+$ and $CD19^-/CD138^+$ PC), tend to be lower in patients with increase calcium serum levels (Fig 7.B e D, respectively).

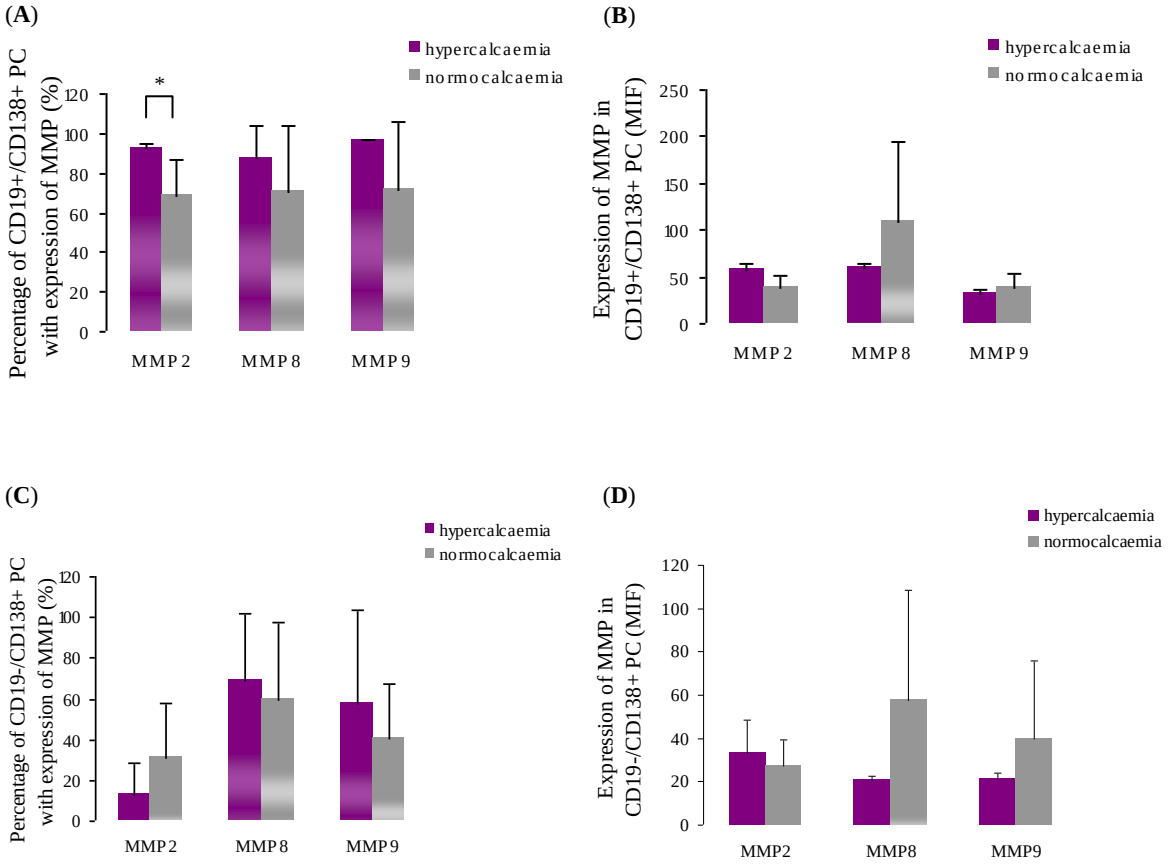

Fig 7. Evaluation of the percentage of cells expressing MMPs and intracellular expression levels, in symptomatic MM patients with hypercalcaemia and normocalcaemia. The results are expressed in percentage (%) (**A**, **C**) and mean intensity fluorescence (MIF) (**B**, **D**) and represent the mean ± SD of the values obtained in $CD19^+/CD138^+$ (**A**, **B**) and $CD19^-/CD138^+$ PC (**C**, **D**). (* $p<0,05$)

In figure 8, we observe the levels of MMPs in symptomatic MM patients with or without renal lesion. Patients with renal lesion have about a half of MMP-2 intracellular expression levels (24 ±4 MIF *versus* 46 ±12 MIF, p=0,033) and also approximately half of percentage of normal PC that express MMP-2 (47% *versus* 79%, p=0,012) and MMP-8 (33% *versus* 83%, p=0,032), when compared with patients without renal lesion (Fig 8.B and A, respectively). In neoplasic PC ($CD19^-/CD138^+$) no statistically differences were found (Fig 8.C and D).

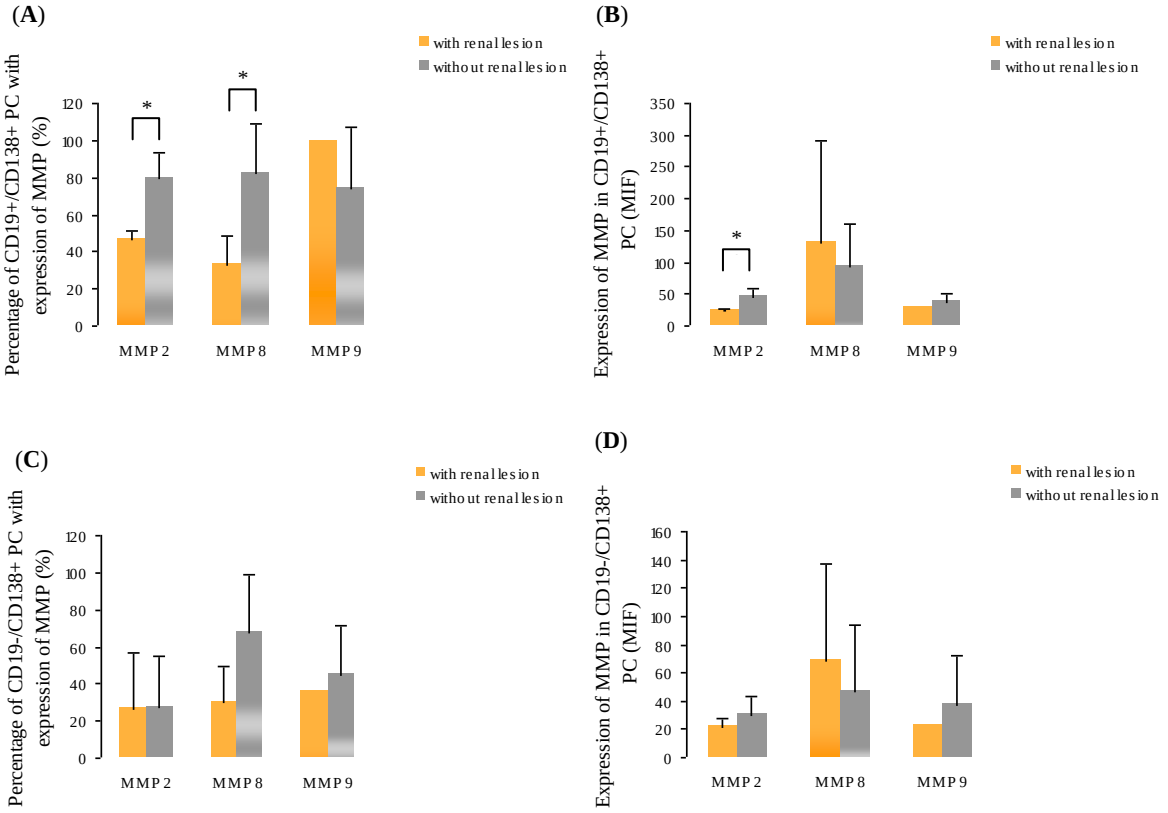

Fig 8. Analysis of the percentage of cells expressing MMPs and intracellular expression levels in symptomatic MM patients according with the presence (with) or absence (without) of renal lesion. The results are expressed in percentage (%) (**A**, **C**) and mean intensity fluorescence (MIF) (**B**, **D**) and represent the mean ± SD of the values obtained in normal ($CD19^+/CD138^+$) (**A**, **B**) and neoplasic PC ($CD19^-/CD138^+$) (**C**, **D**). (* p<0,05)

When we analysed the relationship between the presence or absence of anaemia with the MMPs expression levels, we observe that MM patients with anaemia have a tendency to expressed higher intracellular MMP-8 levels compared with patients without this symptom, both in $CD19^-/CD138^+$ and $CD19^+/CD138^+$ PC (Fig 9.A and B), but it is not statistically significant. In the same way, no differences were observed in the percentage of PC that expresses MMPs (data not show).

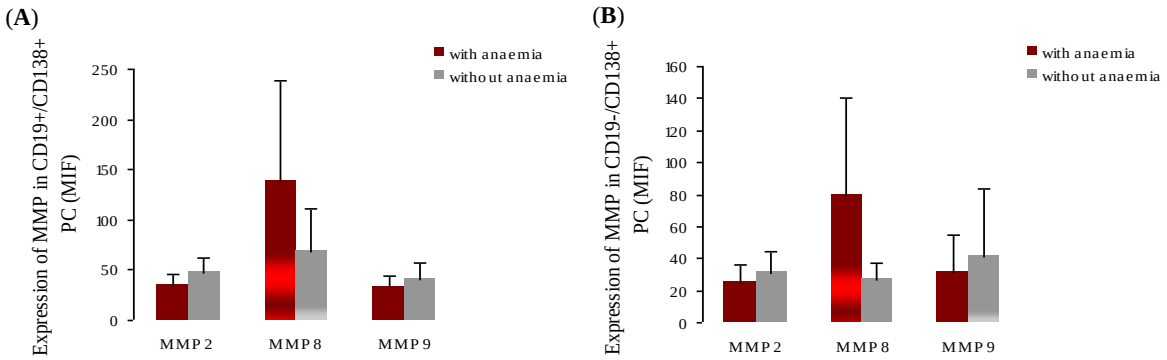

Fig 9. Comparative analysis of MMPs intracellular expression levels in symptomatic MM patients according to the presence (with) or absence (without) of anaemia. The results are expressed in mean intensity fluorescence (MIF) (**A, B**) and represent the mean ± SD of the values obtained in $CD19^{+}/CD138^{+}$ (**A**) and $CD19^{-}/CD138^{+}$ PC (**B**).

Just one symptomatic MM patient do not have bone lesion, so the evaluation of MMPs according to the presence of this CRAB criteria could not be done in this population. However, as MMPs are associated with the osteolytic bone lesions, we compared the MMPs expression levels in all MM patients, including those that are asymptomatic (SMM) and symptomatic (Fig 10). Patients with bone lesion have a significant decrease in the percentage of phenotypically malignant PC expressing MMPs, namely MMP-2 (26%) and -8 (60%) compared with those without bone lesion (64% and 95%, respectively), with p-value lower than 0,05 (p=0,009 for MMP-2 and p=0,032 for MMP-8) (Fig 10.C). In phenotypically normal PC, there are no statistically significant differences, but the tendency is the same (Fig 10.A). However in these cells, MMP-2 intracellular expression levels are significantly higher in patients with bone lesion compared with those without this lesion (44 ±14 MIF *versus* 24 ±9 MIF, p=0,006) (Fig 10.B and D). In opposition, MMP-8 and -9 intracellular expression levels tend to be lower in patients with bone lesion (Fig 10.B and D).

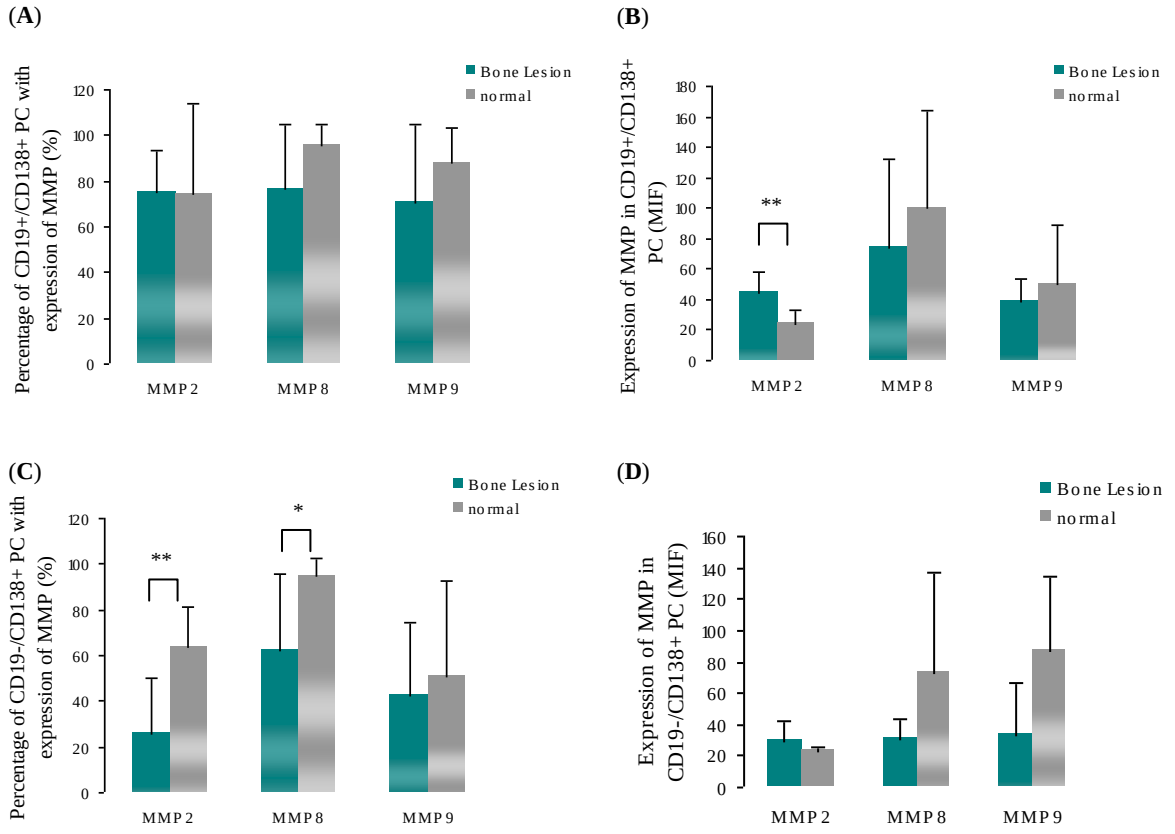

Fig 10. Evaluation of the percentage of cells expressing MMPs and intracellular expression levels in MM patients according to the absence (normal) or presence of bone lesion. The results are expressed in percentage (%) (**A**, **C**) and mean intensity fluorescence (MIF) (**B, D**) and represent the mean ± SD of the values obtained in $CD19^{+}/CD138^{+}$ (**A**, **B**) and $CD19^{-}/CD138^{+}$ PC (**C**, **D**). (* p<0,05, ** p<0,01)

When analysed all MM patients (SMM and symptomatic MM) according to the number of CRAB symptoms present, we observe that MMPs expression levels tends to be inversely related with these symptoms (Fig 11). In fact, the percentage of $CD19^{-}/CD138^{+}$ PC expressing MMP- 2 and -8 is higher in the absence of CRAB symptoms, compared with the presence of two of these symptoms (p=0,049 and p=0,021) (Fig 11.C). Moreover, in neoplasic PC of patients without any CRAB symptoms, the MMP-9 intracellular expression levels are about

two and four times higher than those who have one and two of these symptoms (p=0,044), respectively (Fig 11.D). However, in non-neoplasic PC, the MMP-2 intracellular expression levels in patients without CRAB symptoms are approximately half than in patients with one (p=0,023) or two (p=0,028) of these symptoms (Fig 11.B). No differences in the percentage of phenotypically normal PC expressing MMPs is detected (Fig 11.A).

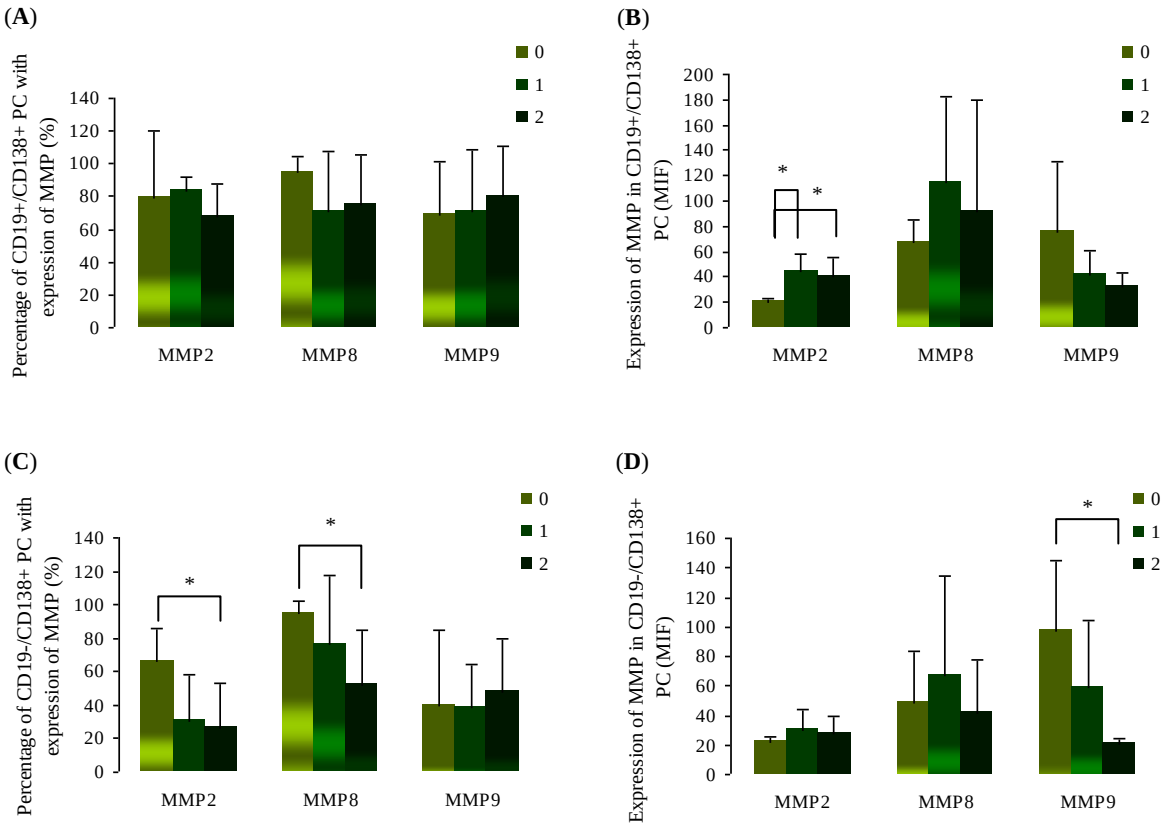

Fig 11. Influence of the number of CRAB symptoms (0, 1 and 2) in the percentage of cells expressing MMPs and intracellular expression levels in MM patients. The results are expressed in percentage (%) (**A, C**) and mean intensity fluorescence (MIF) (**B, D**) and represent the mean ± SD of the values obtained in non malignant ($CD19^+/CD138^+$) (**A**, **B**) and malignant ($CD19^-/CD138^+$) PC (**C**, **D**). (* p<0,05)

3.1.5. Expression of MMPs in MM patients according to ISS stage

When we analysed the MMPs, in MM patients, according to ISS stage system (Fig 12), we observe that patients in ISS II or III show lower expression levels of intracellular MMP-9 compared with those in stage I (Fig 12.A and B). However, only in neoplasic PC these differences tend to be statistically significant (p=0,05) (Fig 12.B). No differences in the percentage of PC expressing MMPs were detected (data not show).

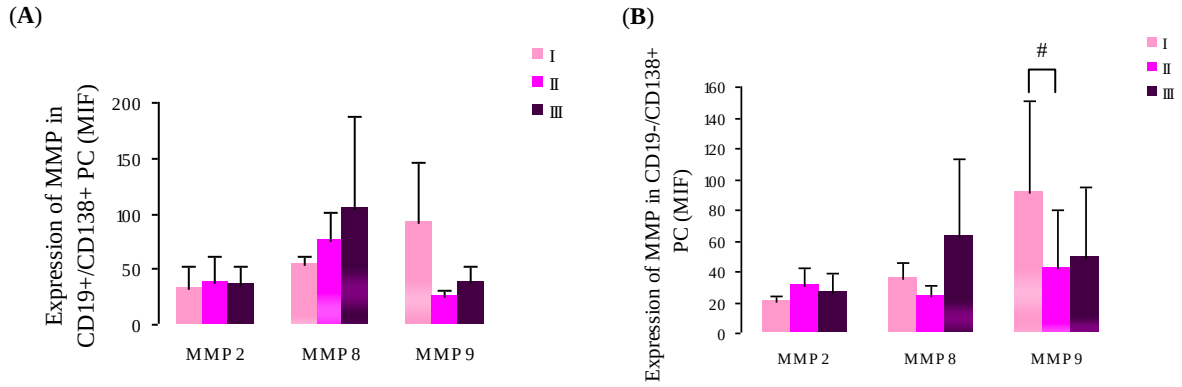

Fig 12. Influence of ISS stage in intracellular expression levels of MMPs in MM patients. The results are expressed in mean intensity fluorescence (MIF) (**A, B**) and represent the mean ± SD of the values obtained in $CD19^{+}/CD138^{+}$ (**A**) and $CD19^{-}/CD138^{+}$ PC (**B**). (# p=0.05)

3.1.6. Survival analysis according to MMPs expression

Finally, we evaluated the role of MMPs, namely the percentage of patient's positives for each MMP expression alone or in association with other, with the risk for symptomatic MM development and/or progression. As we can see in table III, according to the confidence interval, we only can say that certain MMP expression tend to be a protective or risk factor, to confirm our results we need to enlarge the study population. However, the positivity for

MMP-2 and -8 isolated or in association seem to be a protective factor for symptomatic MM development (OR=0,514, 0,667 and 0,357, respectively). In opposition, the positivity for MMP-9 and for the association of MMP-8 and -9 tend to be a risk factor (OR=2, 667 and 2,139, respectively).

Table III. Frequency of symptomatic MM patients showing MMP-2, -8 and -9 positivity and Odds Ratio analysis.

	Symptomatic MM n = 11	**Odds Ratio** (OR)	**Confidence interval** (CI)
MMP2	6 (54,5%)	0,514	0,112-2,361
MMP8	8 (72,7%)	0,667	0,119-3,726
MMP9	8 (72,7%)	2,667	0,544-13,08
MMP2 + MMP8	5 (45,5%)	0,357	0,078-1,64
MMP8 + MMP9	7 (63,6%)	2,139	0,472-9,699
MMP2 + MMP9	4 (36,4%)	0,698	0,154-3,167
MMP 2 + 8 + 9	4 (36,4%)	0,698	0,154-3,167

We have consider a Cut-Off of 20%

With the purpose of investigate, in symptomatic MM patients, if MMPs positivity affects the overall survival, we used Kaplan-Meier method. As expected, in figure 13.A, we can see that the global survival is lower in symptomatic MM patients than in other MG patients. In this group of patients, positive patients for MMP-2 have about 55% survival in 1,5 years of follow-up, while negative patients have approximately 30%, but this difference is not statistically significant (Fig 13.B). When patients are positives for both MMPs, -2 and -8, survival outcomes seem to be lower at an early stage and approximately the same at the end of our follow-up (Fig 13.C). On the other hand, patients that show positivity for MMP-8 and -9 have higher survival after 1,5 years of follow-up (60%), compared with patients that are

negative fot those MMPs, however the results are not statistically significant (Fig 13.C, D and F).

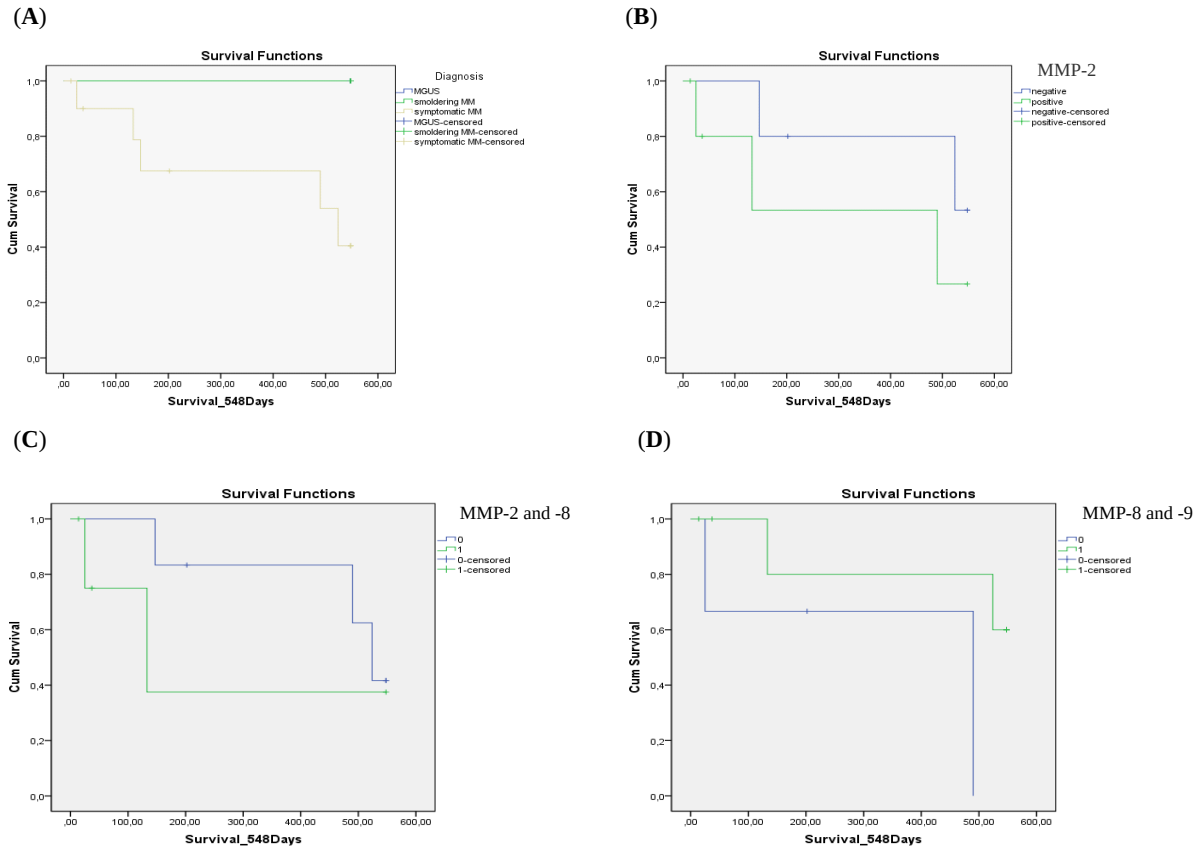

Fig 13. Multiple Myeloma patient´s Kaplan Meier analysis. MM patients overall survival in 1,5 years follow-up (**A**), and survival according to MMPs positivity (negative/0 and positive/1) for MMP-2 (**B**), MMP-2 and MMP-8 (**C**), and MMP-8 and-9 (**D**). All panels' survival was calculated using Kaplan-Meier method.

In MM patients, the survival and the number of CRAB symptoms are inversely related. As we can see in figure 14, patients with one CRAB symptom have 50% survival after 1,5 years of follow-up and patients with two symptoms have approximately 25%, but it is not statistically significant.

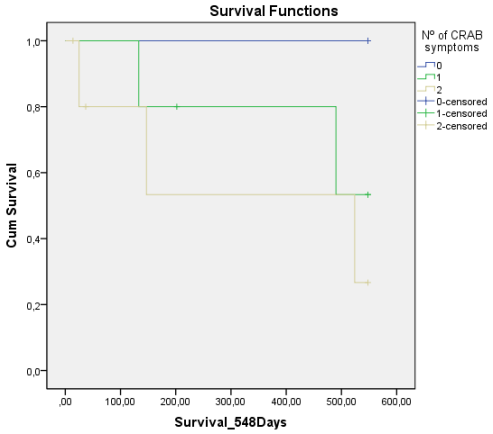

Fig 14. Multiple Myeloma patient´s Kaplan Meier analysis according to the number of CRAB symptoms. Panel' survival was calculated using Kaplan-Meier method.

3.2. Evaluation of therapeutic potential of a metalloproteinase inhibitor in a MM cell line in culture

After analysed the expression of MMPs in MG patients we studied the therapeutic potential of the metalloproteinase inhibitor (MMPI), batimastat, in MM cell viability and death using a cell line in culture (the NCI-H929 cells).

3.2.1. Cell viability evaluation

Batimastat, BB-94, induces a decreased in MM cell proliferation in a time and dose dependent manner and the IC50 at 48 hours is between 5 and 10 μM. However, the BB-94 effect may be independent of low range variations in concentration, but dependent on higher range variations (Fig 15).

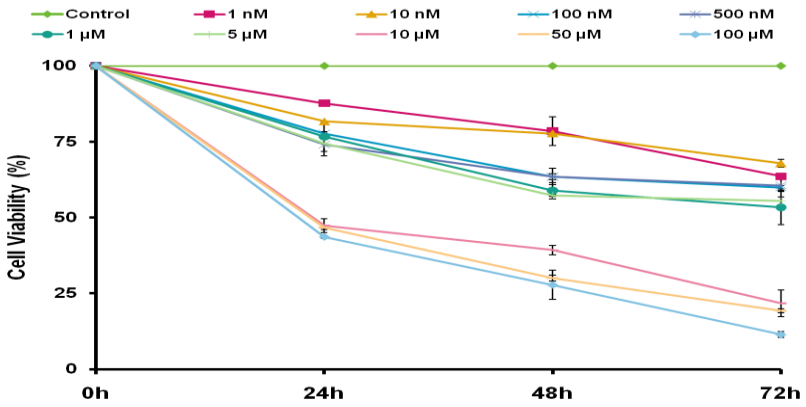

Fig 15. Dose-response curve of Batimastat in a Multiple Myeloma cell line. Multiple Myeloma cells (NCI-H929) were maintained in culture in absence and presence of increasing concentrations of BB-94 during 72h and cell viability was determined by resazurin assay, according with described in Material and Methods. The results are expressed in percentage (%) and represents the mean ± SD of more than 3 independent experiences

3.2.2. Cell death analysis

In order to confirm the previous results and evaluate the proportion of apoptosis and necrosis, we used flow cytometry assay based on cell double staining with AV-FITC and IP. As it can be observed in figure 16, in all conditions, there was a decrease in the percentage of viable cells and an increase in the percentage of apoptotic cells that are dose dependent. Cell death analysis confirms that IC50 of batimastat (BB-94) is about 5 µM.

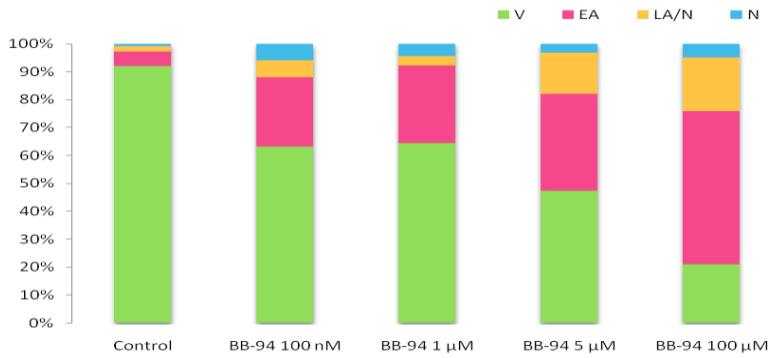

Fig 16. Cell death analysis by flow cytometry. NCI-H929 cells were incubated in the absence (control) and in the presence of BB-94 during 48h in concentrations referred in figure. Cell death was detected by annexin V (AV) and propidium iodide (IP), according with described in material and methods. Viable cells (V) are AV and PI negatives; cells in early stage of apoptosis (EA) are AV positive and PI negative; cells in late stage of apoptosis or in necrosis are AV/IP (LA/N) positive and necrotic cells (N) are AV negative and PI positive. Results are expressed in percentage of cells (%).

4. Discussion and Conclusion

4.1. Evaluation of MMPs expression

Monoclonal gammophaties result from the overproduction of a Ig clone secreted by terminally differentiated B lymphocyte (plasmocyte, PC) and include pre-malignant and malignant conditions, such as MGUS and MM, respectively (Attaelmannan & Levinson, 2000; Katzel *et al*, 2007). As MM remains incurable, with a median survival of 3-4 years (6 weeks to 10 years), the better understanding of MM cell proliferation, survival and migration in the BM microenvironment may enhance knowledge of pathogenesis and provide a framework for identification and validation of novel molecular prognostic markers and therapeutic targets.

MMPs, characterized by the ability to degrade ECM, are known to play a role in cell growth, invasion, angiogenesis, metastasis, and bone degradation, all important events in the pathogenesis of cancer (Valckenborgh *et al*, 2004a; Roy *et al*, 2009). However, the role of MMPs in the development of MGUS and progression to MM is poorly understood.

Our preliminary results show that patients with monoclonal gammopathy (MG) have higher MMPs expression levels when compared with controls, especially MMP-8 and -9. Besides that, when we analyzed MMPs expression in each different MG patient's subgroup (MGUS, SMM and MM), we observed higher MMP-2 and -9 intracellular expression levels in MGUS patients, especially when compared with symptomatic MM. In opposition, symptomatic MM patients have the lowest MMP-9 intracellular levels, while in SMM is MMP-2.

As matrix metalloproteinases are synthesized as pro-enzymes and then secreted as inactive pro-MMPs from the cell or anchored to the plasma membrane (Nagase & Woessner, 1999; Visse & Nagase, 2003; Valckenborgh *et al*, 2004a), these results suggest that in the progression of the plasma cell dyscrasia, from a pre-malignant to a malignant phase, MMPs intracellular expression decreases probably associated with a higher release, in an advanced stage, and a higher production, in an earlier stage of the disease.

Besides that, the percentage of phenotypically malignant PC expressing MMP-2 and -8 is increased mainly in SMM. These results may be related with the increase of malignant PC in the transition from MGUS to SMM. However, an increased in MMPs production may also occur. As described, MM is defined by plasmocytosis in BM greater or equal than 10%, due to the proliferation of malignant plasma cells in the BM, while in MGUS the values are lower (Swerdlow *et al*, 2008; Dimopoulos & Terpos, 2010; Bird *et al*, 2011). Moreover, symptomatic MM, represents an advanced phase of the disease and is related with organ or tissue impairment (CRAB symptoms) (Bird *et al*, 2011). Thereby, the observed decrease in the percentage of neoplasic PC (CD19-/CD138+) expressing MMPs, from asymptomatic to symptomatic MM patients may be associated with a higher release, suggesting a role of MMPs in the progression of the disease and in the onset of symptoms.

Our results are consistent with several studies that show that MMPs, namely MMP-2 and -9, are overexpressed in plasma or serum in several solid tumors and also hematologic malignances, like leukemia and lymphoma. Due to that fact MMPs may represent new potential cancer biomarkers, for diagnostic, prognostic, and risk assessment and also as a therapeutic target (Kader *et al*, 2006; Stefanidakis & Koivunen, 2006; Roy *et al*, 2009; Sekhon, 2010) .

Differences between neoplasic and non-neoplasic plasmocytes in MG patients were also observed. In this context, we verified that phenotypically malignant PC tend to have lower percentage of cells expressing MMPs and lower intracellular expression of MMPs than non-malignant PC. These results may be associated with the release of these proteases mainly from phenotypically malignant PC, which is in agreement with other studies (Valckenborgh *et al*, 2004a).

For some prognostic biomarkers in hematologic neoplasias, as ZAP-70 in chronic lymphocytic leukaemia (CLL), a cut-off had been made to define the positivity value (Schroers *et al*, 2005). According with this, we considerer that a MG patient show positivity for each MMP, if at least 20% of phenotypically malignant PC express this MMP. In our preliminary study, all SMM patients were positive for MMP-2, MMP-8 and for these two MMPs simultaneously. However, the positivity for these MMPs seem to be a protective factor for symptomatic MM, as Odds ratio is lower than one (Table III). On the other hand, MMP-9 is more frequent in symptomatic MM patients, where it seems to represent a risk factor (Table III). These findings suggest that MMP-9 tend to be an important factor in symptomatic MM development, but enlarged population studies should be done to have solid conclusions.

Besides the important role of MMPs as risk factors in MM development, it could influence the course of the disease and, consequently, the overall survival. Our results showed that positivity for MMP-2 seems to confer lower overall survival. In opposition with positivity for MMP-8 and -9 in combination that appears to confer better survival, further confirming the potential prognostic role of MMPs expression in MM.

In other words, in symptomatic MM patients, MMP-9 are more frequent and appears to be a disease risk factor, but it seems to be associated with better outcomes. Besides that, MMP-2,

although more unusual, is probably a protective factor for disease development, and seems to be associated with reduced survival.

Immunoglobulin subtype may also be related with prognosis, for example, MM Ig D, a rare form of MM, is characterized by its clinical severity and poor prognosis (Benchekroun *et al*, 2011). In our preliminary study, we did not have any Ig D MG patients and MMPs expression seems to be independent on the expressed immunoglobulin subtype.

Besides we did not observe any differences in MMPs expression levels between MGUS and SMM, symptomatic IgA MM patients show higher percentage of phenotypically malignant PC expressing MMP-8 compared with Ig G and light chains MM patients (Figure 6).

Some studies referee that MGUS patients with non-IgG isotype are at great risk of progression, specially when associated with M-protein over 15g/L and abnormal free light chain (Rajkumar *et al*, 2005; Landgren, 2010; Landgren *et al*, 2011). However, in our studied population we find no differences between these groups.

The presence of related organ or tissue impairment, what includes increased calcium levels, renal insufficiency, anaemia and bone lesions, known as CRAB symptoms, is relevant to symptomatic MM diagnosis and contributes to differential diagnosis with SMM (Swerdlow *et al*, 2008; Landgren, 2010; Bird *et al*, 2011). In order to evaluate the association of MMPs with CRAB symptoms, we studied the correlation of these proteases with each organ impairment in symptomatic MM patients. Increase calcium levels are defined by "corrected serum calcium higher than 0,25 mmol/L above the upper limit of normal or greater than 2,75 mmol/L"; renal impairment by "creatinine above 173 μmol/L"; anaemia by "haemoglobin 2g/dL below the lower limit of normal or haemoglobin (< 10 g/dL)" and bone lesion by "lytic lesions or osteoporosis, with compression fractures" (Bird *et al*, 2011).

In our study population, two MM patients have hypercalcaemia and they show a higher percentage of phenotypically normal PC expressing MMPs, especially MMP-2. However, intracellular expression levels of MMP-8 tend to be lower in these patients. Furthermore, the percentage of cells that express MMP-2 and -8 and the intracellular expression levels of MMP-2, is lower in patients with renal insufficiency, namely in phenotypically normal PC. In the five patients with anaemia, our results show a tendency to higher intracellular expression levels of MMP-8, when compared with the rest of symptomatic MM patients. Besides that, just one symptomatic MM patient, does not show bone osteolytic lesion, so the evaluation of MMPs according to the presence of this symptom could not be done in this population.

There are no studies correlating MMPs expression with the presence of myeloma organ or tissue impairment, except for osteolytic bone disease. So, these results are extremely interesting, because they suggest that the expression of MMP-2 and -8 may be associated with the presence of CRAB symptoms, but the study population should be enlarged to have solid conclusions.

As previously refereed, MMPs play a role in osteolytic bone disease and some studies mention that they are involved in osteoclast (OC) recruitment and can degrade mineralized bone matrix (Valckenborgh *et al*, 2004a). Furthermore, in MM, bone destruction is associated with an enhancement of bone resorption, due to development and activation of OCs by the increase in RANK ligand and decrease in osteoprotegerin (OPG). The suppression of bone formation, due to inhibition of WNT-mediated osteoblast differentiation may also be implicated (Giuliani, 2001; Dimopoulos & Terpos, 2010; Abe, 2011).

In this context, we compared the MMPs expression levels in all MM patients, asymptomatic (SMM) and symptomatic, and we observe that the presence of bone lesion is associated with less percentage of PC expressing MMPs, namely MMP-2 and -8 in phenotypically malignant

PC. Intracellular expression levels are higher for MMP-8 and -9 and lower for MMP-2. These results suggest that all studied MMPs seem to be important in MM bone lesion, which is in agreement with Valckenborgh *et al.* (2004) study. These authors show that MMP-8 have the ability to degrade collagen I, the major constituent of the bone and the denatured collagens I becomes a substrate for MMP-2 and -9.

As the decision to start treatment is based on the diagnosis of symptomatic MM, which includes CRAB criteria (Moreau, 2011), we divided MM patients according to the number of CRAB symptoms and correlated with MMPs expression. Our results show that they tend to be inversely related, as an increase of symptoms is associated with a decrease of MMPs expression. The only exception is MMP-2, which has a lower intracellular expression levels in SMM, especially in phenotypically normal PC.

CRAB criteria is also associated with poor outcome, being a parameter of stage III in Durie and Salmon prognostic staging system previously used (Swerdlow *et al*, 2008; Bird *et al*, 2011). Our data confirm that in MM patients, the survival and the number of CRAB symptoms are inversely related. These findings suggest that an increase of symptoms is probably associated with a higher release of MMPs and this confers poor survival outcomes.

As previously refereed, MM remains incurable, so the analysis of prognostic factors is important to compare outcomes (Katzel *et al*, 2007; Swerdlow *et al*, 2008). International Staging System (ISS) for MM has replaced the Durie-Salmon staging system and it is based on serum albumin and β2-microglobulin (β2M), grouping patients in prognostic stages according to median survival, which is 62, 44 and 29 months for stage I, II and III, respectively (Greipp *et al*, 2005; Bird *et al*, 2011). In order to evaluate if MMPs are associated with the prognosis outcome, we analysed the MM patient's population according

to ISS stage and we observe that stage II and III MM patients show lower MMP-9 intracellular expression levels compared with stage I.

MMP-9 expression levels is largely studied in many solid tumors as a biomarker in diagnosis and/or for prognosis, but these studies mainly analyzed plasma or serum MMPs levels (McGowan & Duffy, 2008; Roy *et al*, 2009; Sekhon, 2010), while in our study we have determined intracellular levels, which could explain the differences.

Summarizing, monoclonal gammophaties patients have higher MMPs intracellular expression levels and in the progression from MGUS to symptomatic MM these levels seem to decrease, possibly due to a higher release, which could contribute to ECM degradation. SMM have higher percentage of PC expressing MMPs probably because of simultaneously increase in malignant cells and MMPs production when compared with MGUS, and higher release when compared with symptomatic MM patients. When analysed the MMPs expressing according to PC phenotype, malignant PC have lower intracellular expression levels of MMPs and percentage of cells expressing MMPs.

The frequency of symptomatic MM patient's positives for MMP-9 is higher and it appears to be a risk factor to the development of the disease; however, in these patients, MMP-9 positivity seems to be associated with better outcomes. On the other hand, MMP-2 is more frequent when MM patients are asymptomatic, tending to be a protective factor for symptomatic MM patients and, in them, MMP-9 positivity leads to a worse survival.

According to laboratory/clinical data, MMPs expression seem to be independent from Ig subtype expressed. Furthermore, MMP-2 and -8 expressions may be associated with the presence CRAB symptoms, but for bone lesion all studied MMPs seem to be important. In addition, an increase of symptoms is probably associated with a higher release of MMPs and

confers poor survival outcomes. According to ISS, MMP-9 seems to be relevant in the progression to a worse prognosis stage.

In conclusion, our preliminary study suggests that PC MMPs expression may be correlated with transition of MGUS to MM, promoting extramedullary spreading and disease evolution. However, study population should be enlarged, in order to confirm our results.

4.2. Evaluation of therapeutic potential of a metalloproteinase inhibitor in a MM cell line in culture

MM treatment is actually focused in targeting molecular pathways, including immunomodulatory agents, thalidomide and lenalidomide and the proteasome inhibitor, bortezomib (Gahrton, 2004; Merchionne *et al*, 2007). The need to develop effective new treatments remains urgent, because MM remains incurable, despite of recent advances in basic biology and treatment (Anderson *et al*, 2005).

Given the important role of MMPs in tumor growth and metastasis, the results observe in the evaluation of MMPs expression in patients and due to the lack of therapeutic success in MM treatment, the inhibition of this enzyme family may be a promise therapeutic approach. Batimastat, the first synthetic MMP inhibitor to enter in clinical trials in 1994 (Rothenberg *et al*, 1998), have shown, in our preliminary study, an anti-proliferative effect in MM cell line in a time dependent manner, but it seems to be independent at lower range drug concentration and dependent from higher variations. According to cell death analysis, batimastat show a cytotoxic effect in MM cell line.

There are some data relating MMPI with increase survival. Marimastat prolong survival in patients with glioblastoma or gastric and gastro-oesophageal adenocarcinoma, and showed

comparable effect with conventional chemotherapy in pancreatic cancer (Bramhall *et al*, 2002; Stefanidakis & Koivunen, 2006). Neovastat also showed modest survival benefit in patients with refractory renal cell carcinoma. In MM, Neovastat (AE-941), a shark cartilage extract, that inhibits MMP-2, -9 and -12, is in phase II of clinical trial as an antiangiogenic agent (Ryoo *et al*, 2002; Béliveau *et al*, 2002; Roy *et al*, 2009;).

This study suggests that the MMPI, batimastat, could be a new therapeutic approach in multiple myeloma in monotherapy.

5. Acknowledgments

I wish to express my gratitude to my supervisor, Professora Doutora Ana Bela Sarmento Ribeiro, my co-supervisor, Doutora Catarina Geraldes, and also Mestre Ana Cristina Gonçalves, who were profusely helpful and offered invaluable support and guidance. I also would like to tank Raquel Alves for her helpful assistance and the Faculty of Medicine of the University of Coimbra, C.H.U.C. – Centro Hospitalar e Universitário de Coimbra and CIMAGO - Center of Investigation on Environment Genetics and Oncobiology for supporting our research.

6. Authors contributions

Concept and design and obtaining funding: ASP, CG and ABSR. Analysis and interpretation of data and Drafting the article: ASP, ACG and ABSR. Collection and assembly of data: CG, EC, AT and ASP. Critical revision of the article for important intellectual content and final approval of the article: JMNC and ABSR. Technical support: ACG and RA.

7. Conflict of interest

All authors have no conflicts of interest to declare.

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