Bone Marrow Mast Cell Density Correlates with Circulating Biomarkers of Bone Disease in Multiple Myeloma

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Authors' contributions

This work was carried out in collaboration between all authors. Authors RV and MGA designed the study, wrote the protocol and wrote the first draft of the manuscript. Authors GT and MD managed the literature searches, and performed the ELISA analysis. Authors CAP and ANA managed the experimental process. Authors SM and AP analyzed the data. All authors read and approved the final manuscript.

Article Information

Original Research Article

ABSTRACT

Aims: There is increased mast cell density (MCD) in multiple myeloma (MM) bone marrow (BM) that is correlated with advanced disease stage. Mast cells (MCs) produce various mediators promoting MM growth and bone metabolism. The aim of this study was to evaluate whether BM MCD correlates with markers of bone metabolism, such as circulating levels of osteoprotegerin (OPG), soluble receptor activator of nuclear factor-κB ligand (sRANKL), osteopontin (OPN) and macrophage inflammatory protein-1 alpha (MIP-1 alpha).

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Study Design: This is a cross-sectional study.

Place and Duration of Study: Department of Medicine, University of Crete, Department and Laboratory of Haematology, University Hospital of Heraklion, Department of Haematology and Internal Medicine, Venizelion Hospital of Heraklion, Department of Haematology, General Hospital of Chania, between January 2010 and December 2014.

Methodology: All parameters were analyzed by ELISA. We studied 56 active MM patients (32 males, 24 females) and 20 healthy controls. According to ISS 14 patients were in stage I, 22 in stage II and 20 in stage III. MCs were highlighted in BM immunohistochemically, using monoclonal antibody to MC tryptase.

Results: All values were higher in active MM patients compared to healthy subjects ($p<0.001$ for all cases). OPG was decreasing in advanced stages ($p= 0.018$), whereas all other values were in parallel with ISS disease stages ($p<0.001$ for all cases). Moreover, BM MCD correlated positively with sRANKL, OPN and MIP-1 alpha ($p<0.0001$ for all cases), and negatively with OPG ($r= -0.279$ $p<0.03$).

Conclusion: MCs are increased in MM BM and participate in many aspects of the disease. They may release various cytokines increasing myeloma growth and also may participate in the skeletal disease of MM rendering them as potential targets for therapy.

Keywords: Bone disease; mast cells; multiple myeloma; osteolysis.

ABBREVIATIONS

Mast Cell Density (MCD); Multiple Myeloma (MM); Bone Marrow (BM); Monoclonal Gammopathy of Undetermined Significance (MGUS); Macrophage Inflammatory protein-1 alpha (MIP-1 alpha); Receptor Activator of Nuclear factor-κB (RANK); RANK Ligand (RANKL); osteoprotegerin (OPG); osteopontin (OPN); Mast Cells (MCs); International Staging System (ISS); Stem-cell Factor (SCF); Interleukin-3 (IL-3); Microvascular density (MVD); N-terminal telopeptide (Ntx).

1. INTRODUCTION

Multiple myeloma (MM) is a debilitating B cell neoplasia, part of a spectrum of diseases, ranging from monoclonal gammopathy of undetermined significance (MGUS) to plasma cell leukemia. This progression is based on a quite complex molecular basis, with both genetic aberrations in MM cells and evolving interactions between different cell types. Malignant plasma cells rely heavily on their interactions with elements of the surrounding microenvironment, in order to survive and further to expand. By these means, several cell types and cytokines are involved, playing major roles in various aspects of MM progression, including angiogenesis and skeletal disease [1].

Myeloma skeletal disease is unique among other malignant bone diseases. It is considered to be the result of the impaired balance between osteoclasts and osteoblasts: Increased osteoclastic activity enhances bone resorption, whereas decreased osteoblastic efficiency reduces bone formation. This disturbed bone remodeling may cause skeletal-related events with hypercalcemia, following significant morbidity. The study of MM bone disease is rather complex, with the participation of various mediators [2,3]. Among them, macrophage inflammatory protein-1 alpha (MIP-1 alpha) acts as chemoattractant for cells of the monocyte-macrophage lineage, including osteoclast precursors, stimulating their differentiation and bone resorption [4-8]. The system of receptor activator of nuclear factor-κB (RANK)/RANK ligand (RANKL), along with osteoprotegerin (OPG) [9] also participates in these procedures. RANK is a transmembrane receptor on the surface of osteoclast precursors and binding to RANKL induces their maturation and enhances their survival [10,11]. OPG is a soluble decoy receptor for soluble RANKL (sRANKL), produced, among others, by osteoblasts, blocking sRANKL/RANK interactions, thereby limiting osteoclastogenesis. Normally, RANKL/OPG ratio is very low, but in MM there is enhanced sRANKL and reduced OPG production, resulting a higher RANKL/OPG ratio and increased osteoclast activity [12]. Another participant in MM bone disease is osteopontin (OPN), a non-collagenous matrix protein, responsible for the migration and attachment of osteoclasts to mineral matrix of bone surfaces [13,14].
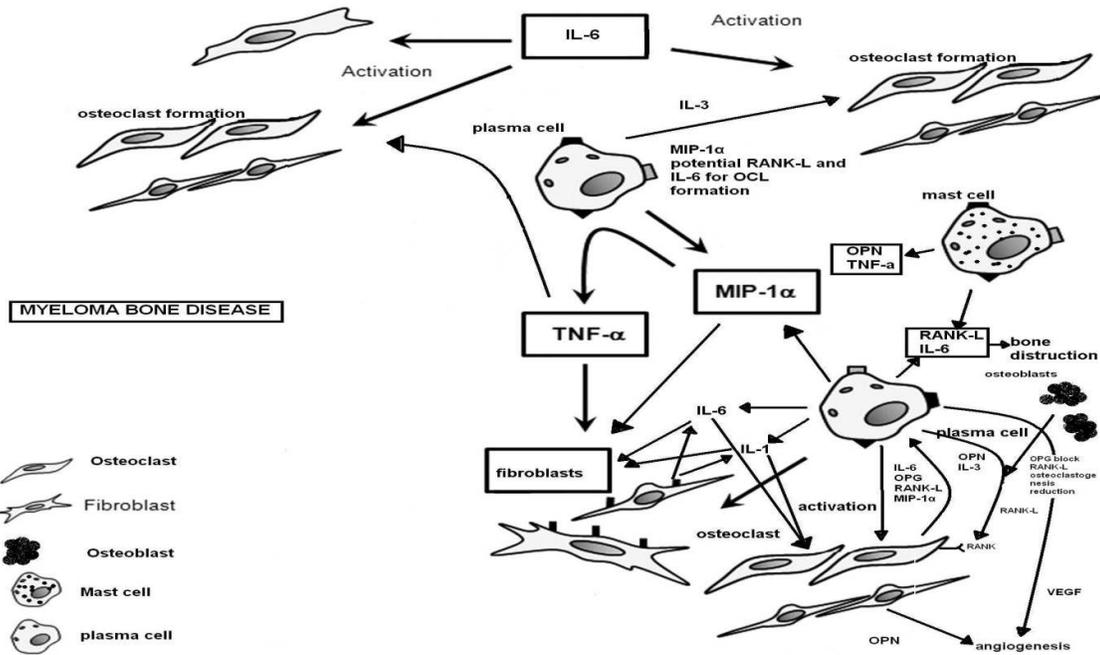
Fig. 1. Mechanisms responsible for myeloma bone disease. Myeloma cells produce factors that activate osteoclast formation. In addition they induce RANK-Ligand and IL-6 production and enhance bone destructive process. Myeloma cells also produce IL-3, MIP-1 a and VEGF and in combination with OPN produced by osteoclasts mediate the process of angiogenesis

There is also a plethora of cells implicated in the various aspects of MM progression. Among them, inflammatory cells are active participants and mast cells (MCs) possess multiple roles: they are accumulated in BM microenvironment as an initial host response, with potential tumorcidial activity. By these means, myeloma cells secrete attractants for MCs, but in turn, they secrete several factors favoring MM growth: MCs accumulation, although with an initial anti-tumor mood, aims disease progression [15,16]. There is an extensively studied participation of MCs in the angiogenic process: MCs secrete various direct and indirect angiogenic factors and also may participate in vasculogenesis mimicry [15,17,18]. We have already correlated BM MCs density (MCD) with international staging system (ISS) stages [19] and proliferation rate of myeloma plasma cells [20]. These correlations could be justified through their participation in the angiogenic process. On the other hand, since MCs may produce various versatile factors, it seems that their participation in MM growth is more complex.

The aim of the present study is to ascertain whether the degree of MCs accumulation in MM BM is associated with bone disease. Therefore, we estimated MCD in active MM BM and correlated it with circulating levels of known factors of bone disease, such as MIP-1 alpha, OPN, IL-6, OPG, and sRANKL.

2. MATERIALS AND METHODS

2.1 Materials

Fifty-six active MM patients (32 males, 24 females) were included in this study. The median age was 64 years (range 38–81 years). Patients with history of previous or current other malignancies, other BM diseases, renal or liver impairment, HIV, uncontrolled infectious diseases, use of immunomodulatory drugs or incapability to consent, were excluded from the study. According to the ISS classification system [21], 14 patients were classified as stage I, 22 as stage II, and 20 as stage III. The types of monoclonal protein were 31 IgG, 18 IgA, and light chain disease for 7 patients. Patients had not received any myeloma-related treatment, including corticosteroids and biphosphonates, before entering the study. Twenty age- and sex-

matched healthy subjects (12 males and 8 females, median age 64, range 41-75 years) were recruited as controls among blood donors and people following a physical training program (over 60 years of age). All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional research committee and with the 1964 Helsinki declaration and its later amendments. Informed consent was obtained from all individual participants included in the study.

2.2 Methods

Serum samples from patients were collected before starting any myeloma-related treatment. Sera, collected from patients and controls, were aliquoted into separate vials and stored at −70°C. All assays were performed at the end of the study, in order to avoid interassay variability. Before starting the analysis, the samples were thawed to room temperature and mixed by vortexing in order to eliminate substances potentially affecting reproducible results. Hemolyzed or lipemic samples were excluded from the analysis. The detection of MIP-1alpha, OPN, IL-6, OPG, and sRANKL, in the serum was performed by a solid-phase sandwich ELISA, using monoclonal antibodies against MIP-1alpha, OPN, IL-6 (Quantikine, R&D systems Inc. Mineapolis, MN, USA), OPG and sRANKL antibodies (Bio Vendor-Laboratorni Medicina as Brno, Czech Republic).

MM patients and controls underwent trans iliac trephine biopsies. The histological diagnosis was made on hematoxilin-eosin-stained slides. Initially, biopsies were fixed in 10% formalin decalcified in 10% EDTA for 48h and embedded in paramat extra. Hematoxylin and eosin stained, 3 µm thick sections were examined by light microscopy. MCD was estimated immunohistochemically, as has been described previously [19]. Briefly, MCs were highlighted by immunostaining with the monoclonal antibody to MC tryptase (Ab-2 of Thermo-Scientific, Clone AA1) using the alkaline phosphatase – antialkaline phosphatase method (ultravision LP Detection System- Thermo-Scientific-TL12S-AL). After deparaffization and gradual rehydration, sections were incubated with the primary antibody (dilatation 1/3000, 30 min, room temperature without requirement of antigen retrieval, with the use of negative and positive controls (non-malignant tonsillar tissue from the files of the laboratory). In each specimen BM

MCs were quantified in three areas of neoplastic infiltration containing the highest number of MCs (hot spot) in ten high-power fields (HPF) (x400) for each hot spot separately. MCD was the mean percentage of positive staining MCs calculated in the three hot spots for each specimen and expressed as mast cells/HPF (MCs/0.0625 mm^2).

2.3 Statistical Analysis

Data are presented as means ± SD. The Mann-Whitney *U*-test was applied to evaluate any difference in serum cytokine levels in patients and healthy individuals. Non- parametric tests were also used (Mann-Whitney and Wilcoxon for independent and paired two samples statistics). The investigation of potential correlations among variables was carried out with the Spearman's rho correlation co-efficient. *P* values <0.05 were considered to be statistically significant.

3. RESULTS

Mean± SD levels of the measured parameters in active MM patients and in controls are shown in Table 1.

Table 1. Values of bone marrow mast cell (MC) density (MCD) and serum levels of osteoprotegerin (OPG), soluble receptor activator of nuclear factor κB ligand (sRANKL), osteopontin (OPN) and macrophage inflammatory protein-1 alpha (MIP- 1alpha) in patients with active multiple myeloma (MM) compared to healthy controls. (p<0.001 for all cases)

	MM patients	Controls
	(n= 56)	(n= 20)
MCD (MCs/0.0625 mm^2)	10.0±5.2	1.6±0.6
OPG (pmol/l)	13.1±11.6	6.4±3.5
sRANKL (pmol/l)	368.5±391.5	20.7±12.6
OPN (pg/ml)	78.7±58.4	40.0±32.8
MIP-1alpha (pg/ml)	46.9±29.7	21.0±2.8

Serum levels of OPG, sRANKL, OPN, MIP-1alpha and BM MCD were significantly higher in MM patients compared to controls (p<0.001, in all cases). Table 2 shows their values in ISS stages, where it can be noted that OPG was decreasing in advanced stages (p= 0.018), whereas all other parameters were increasing in parallel with ISS stages (p<0.001 for all cases).

Table 2. Values of bone marrow mast cell density (MCD) and serum levels of osteoprotegerin (OPG), soluble receptor activator of nuclear factor κB ligand (sRANKL), osteopontin (OPN) and macrophage inflammatory protein-1 alpha (MIP-1alpha) in various stages of multiple myeloma, according to International Staging System (p<0.001 for MCD, sRANKL, OPN and MIP-1 alpha and p= 0.018 for OPG)

MCD (MCs/0.0625 mm2)	
Stage I (n= 14)	5.1±1.2
Stage II (n= 22)	8.4±3.4
Stage III (n= 20)	15.2±4.0
OPG (pmol/l)	
Stage I (n= 14)	20.9±17.8
Stage II (n= 22)	12.1±9.0
Stage III (n= 20)	8.8±4.5
sRANKL (pmol/l)	
Stage I (n= 14)	68.2±68.7
Stage II (n= 22)	248.9±154.2
Stage III (n= 20)	710.2±456.3
OPN (pg/ml)	
Stage I (n= 14)	25.5±9.4
Stage II (n= 22)	63.3±37.9
Stage III (n= 20)	132.9±52.6
MIP-1 alpha (pg/ml)	
Stage I (n= 14)	23.8±6.2
Stage II (n= 22)	36.6±13.9
Stage III (n= 20)	74.5±31.4

A significant negative correlation was found between MCD and OPG (r= -0.279, p<0.03), whereas positive correlations between MCD with sRANKL (r= 0.572), OPN (r= 0.605) (Fig. 1).

4. DISCUSSION

MCs are found in most tissues, serving a fundamental role within the immune system [22]. They arise from pluripotent CD34+ stem cells and differentiate along the myeloid pathway. After leaving BM, MCs progenitors are influenced by a variety of signals directing their migration into tissues, where they subsequently mature [23]. A major step is the interaction with endothelial cells and various tissue factors via adhesion molecules. MCs chemotaxis occurs in response to a variety of growth and differentiation factors, such as stem-cell factor (SCF) and interleukin-3 (IL-3), and to numerous chemokines. Among others, MIP-1-alpha, elicit migration of mouse BM MCs after Fc epsilon RI activation on laminin, vitronectin, and fibronectin

[24]. MCs can be activated after exposure to allergens or by other triggers, such as cytokines and neuropeptides. Their activation can be enhanced by SCF and IL-33 [25]. Upon activation, MCs release various mediators upon activation. These mediators can be divided into three overlapping categories: preformed and newly-synthesized lipid mediators, cytokines and chemokines. There is a large body of evidence supporting their involvement in tumor angiogenesis. Therefore, among others, MCs secrete several classical (vascular endothelial growth factor, fibroblast growth factor-2, platelet-derived growth factor beta, IL6) and nonclassical (IL8, chymase, tryptase) proangiogenic factors. Tryptase is a very powerful factor, inducing proliferation of endothelial cells and degradation of extracellular matrix components, through activation of latent metaloproteinases, releasing latent pro-angiogenic factors [16,23].

MCs accumulation has been associated with enhanced growth and invasion in various human cancers, including breast, stomach, esophagus and oral cavity [26]. There is an increasing study of MCs participation in MM progression [27,28]. The major aspect of MCs participation in MM progression is enhancing angiogenesis: BM MCD has been correlated with BM microvascular density (MVD) in MGUS, non-active and active MM [29]. It seems that MCs produce various angiogenic factors and may participate in vasculogenesis mimicry. We already have demonstrated that MM BM MCD is in parallel with ISS stages and also correlates with proliferation rate of myeloma plasma cell [19,20]. We suggest that these correlations are justified due to participation of MCs in the angiogenic process. Angiogenesis is a complex procedure, favouring direct MM growth and the implicated participants possess multiple and versatile properties.

Previously was reported that there is a clear association of MCD with the angiogenic potential in several inflamations and malignant diseases suggesting that angiogenesis is the most significant role of MCs [30]. There are several angiogenic procedures indicating the significant role of MCs in osteolytic disease [28]. This observation is enhanced by the positive correlation of MCD with levels of MMP-9 and mainly with RANKL and N-terminal telopeptide (Ntx). The correlation of MCD with the urine Ntx levels are among the most sensitive biochemical markers of osteoclastic activity [31].
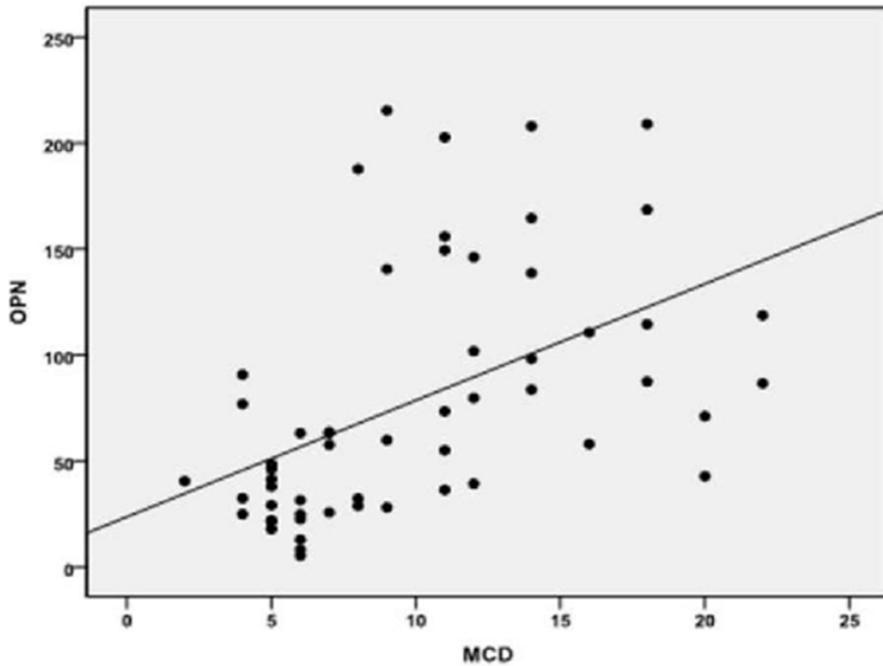
Fig. 2. Positive correlation of mast cell (MC) density (MCD) (MCs/0.0625 mm^2) with osteopontin (OPN) (pg/ml) in patients with active multiple myeloma (r= 0.605, p<0.0001)

During the last decade, immunomodulatory drugs, icluding thalidomide and lenalidomide and proteasome inhibitors, such as bortezomibe, have been increasingly used for the treatment of MM. The role of these drugs in bone metabolism has been evaluated in several studies [31]. Proteasome inhibitor bortezomibe is a potent, highly selective, and reversible proteasome inhibitor that targets 26S proteasome complex and inhibits its function [32]. The proteasome regulates key cellular processes, including modulation of transcription factors, such as NF-kB, cell cycle progression, inflammation, immune surveillance, growth arrest, and apoptosis [33]. Bortezomib has inhibitory effects on the NF-κB activity in MM cells. NF-κB is a major transcriptional factor which mediates the expression of many proteins including cytokines, chemokines, cell adhesion molecules, as well as those involved in anti-apoptosis and cellular growth control [33]. Thalidomide has a direct tumoricidal activity, an antiangiogenic effect and modulates TNF-α signalling through direct and/or indirect effects on the tumour microenvironment [34], reduces FGF-2, VEGF and IL-6 secretion in bone marrow stromal cells and by MM cells [35]. Lenalidomide, a derivative of thalidomide, is less toxic and more potent than the parent drug [36]. In patients with relapsed or

refractory MM, lenalidomide can overcome resistance not only to conventional chemotherapy but also to thalidomide [37,38]. The bisphosphonates are other compounds that, although originally used to reduce bone loss in MM due to an anti-osteoclast activity, have also been shown to have a direct effect on MM cells [39]. In fact, zoledronic acid has a direct cytotoxic activity on tumor cells and suppresses angiogenesis [40,41], inhibits FGF-2 - and VEGF-dependent proliferation of endothelial cells and inhibits VEGFR-2 in an autocrine loop [39] .Neridronate exerts its antiangiogenic activity through both a direct effect on endothelial cell proliferative activity and inhibitory effect on the responsivity of the endothelial cells to the proliferative stimuli mediated by angiogenic cytokines [15]. No drugs that target biomarkers have been used in this study.

In the present study, we found that BM MCD in active MM patients is increased, compared to healthy population and also in parallel with ISS stages. This is in accordance with our previous reports [19,20]. The important findings are the significant correlations with the markers of abnormal bone metabolism. This can be justified through the angiogenic process: since the major role of MCs in MM growth is angiogenic
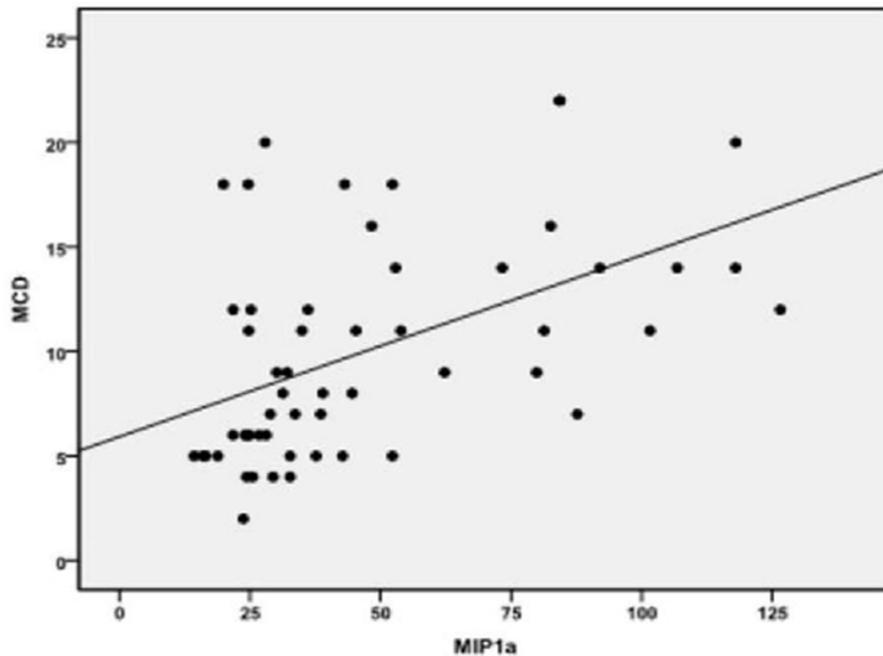
Fig. 3. Positive correlation of mast cell (MC) density (MCD) (MCs/0.0625 mm^2) with macrophage inflammatory protein-1 alpha (MIP-1a) (pg/ml) in patients with active multiple myeloma (r= 0.528, p<0.0001)

progression, which in turn is correlated with both disease activity and bone disease [28], it can be suggested that the participation of MCs in osteolytic disease is indirect. On the other hand, there are several angiogenic procedures participating in osteolytic disease, such as degradation of extracellular matrix. Our observation is enhanced by the positive correlations of MCD with levels of RANKL, MIP-1alpha and OPN. The correlation with RANKL may be partially direct, since MCs may secrete RANKL, along with IL-6, prostaglandin D2, tumor necrosis factor, and tryptase, all of which may cause bone disease in MCs disorders [23,29]. Similarly, the correlation with OPN may also be partially direct since MCs may produce OPN [42,43]. It is obvious that for both cases, MCs are not their unique source. On the other hand, the positive correlation of MCD with MIP-1alpha and the negative correlation with OPG are rather indirect and can be justified due to the modified BM microenvironment, favouring all aspects of MM growth. To the best of our knowledge, no study until now has displayed a correlation between MCD and parameters of bone disease, such as MIP-1alpha, OPG, sRANKL and OPN, in MM.

5. CONCLUSION

Our study showed that MCs play a significant role in MM growth and progression. They mainly promote angiogenesis, but it seems that they also may participate, indirectly and directly, in skeletal disease, as well. Therefore, the participation of MCs in MM biology is getting more complex and more studies are needed in order to explore the precise implicated mechanisms.

CONSENT

All authors declare that 'written informed consent was obtained from the patient (or other approved parties) for publication of this article and accompanying images.

ETHICAL APPROVAL

All authors hereby declare that all experiments have been examined and approved by the appropriate ethics committee and have therefore been performed in accordance with the ethical standards laid down in the 1964 Declaration of Helsinki.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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