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## Angiogenic cytokines profile in smoldering multiple myeloma: No difference compared to MGUS but altered compared to symptomatic myeloma

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# Angiogenic cytokines profile in smoldering multiple myeloma: No difference compared to MGUS but altered compared to symptomatic myeloma

Authors' Contribution:  
Study Design A  
Data Collection B  
Statistical Analysis C  
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Manuscript Preparation E  
Literature Search F  
Funds Collection G

ABCDEF 1,2 **Maria Gkatzamanidou**  
BCDE 1 **Dimitrios Christoulas**  
ABCEF 3 **Vassilis L. Souliotis**  
BCDE 4 **Athanasios Papatheodorou**  
ABCDE 1 **Meletios A. Dimopoulos**  
ABCDEF 1 **Evangelos Terpos**

1 Department of Clinical Therapeutics, National and Kapodistrian University of Athens, School of Medicine, Athens, Greece  
2 Department of Medical Oncology, Dana-Farber Cancer Institute, Harvard Medical School, Boston, MA, U.S.A.  
3 Institute of Biology, Medicinal Chemistry and Biotechnology, National Hellenic Research Foundation, Athens, Greece  
4 Department of Medical Research, 251 General Air Force Hospital, Athens, Greece

**Corresponding Author:** Evangelos Terpos, e-mail: [eterpos@med.uoa.gr](mailto:eterpos@med.uoa.gr) or [eterpos@hotmail.com](mailto:eterpos@hotmail.com)  
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**Background:** Symptomatic multiple myeloma (MM) evolves from an asymptomatic precursor state termed monoclonal gammopathy of undetermined significance (MGUS) and smoldering myeloma (SMM). Angiogenesis plays a key role in the pathogenesis of MM but there are very limited data for angiogenesis in SMM.

**Material/Methods:** We measured the circulating levels of angiopoietin-1 (Ang-1), angiopoietin-2 (Ang-2), vascular endothelial growth factor (VEGF), and angiogenin in 54 patients with SMM. The results were compared with those of 27 MGUS patients, 55 MM patients, and 22 healthy controls. The expression of *VEGF-A* gene was also evaluated in 10 patients with SMM, 10 with symptomatic MM, and 10 with MGUS.

**Results:** The ratio of circulating Ang-1/Ang-2 was reduced in MM patients with symptomatic disease due to a dramatic increase of Ang-2 ( $p < 0.001$ ), but not in patients with SMM or MGUS, in whom it did not differ compared to controls. VEGF and angiogenin were increased in all patients compared to controls. However, circulating VEGF was higher in symptomatic MM compared to SMM and MGUS, while angiogenin was reduced. There were no differences in the expression of *VEGF-A* among the 3 patients categories.

**Conclusions:** SMM has a circulating angiogenic cytokine profile similar to that of MGUS, but has altered profile compared to symptomatic MM. Thus, in the progression of MGUS to SMM, circulating angiogenic cytokines seem to be the same. On the contrary, in symptomatic myeloma, the alterations of angiopoietins along with VEGF contribute to myeloma cell growth, supporting the target of these molecules for the development of novel anti-myeloma agents.

**Key words:** multiple myeloma • angiopoietins • angiogenin • VEGF • smoldering myeloma • angiogenesis

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## Background

Multiple myeloma (MM) is a malignant disorder characterized by multifocal proliferation of clonal, long-lived plasma cells within the bone marrow (BM). MM presents clinically with a broad range of manifestations, including skeletal destruction, immune suppression, and end-organ sequelae [1].

The disease accounts for slightly more than 10% of all hematologic cancers [1]. It is estimated that there will be 22 350 newly diagnosed MM cases and 10 710 MM deaths in the United States in 2013 [2]. Over the past decade, the outcome of MM patients has been dramatically improved after the development of novel agents and the introduction of autologous stem cell transplantation [3,4]. However, MM remains an incurable disease and significant efforts aim to unravel its biology. MM is preceded by a premalignant condition termed monoclonal gammopathy of undetermined significance (MGUS) [5]. MGUS is present in more than 3% of the population above the age of 50 years. Its annual progression rate to MM is of 0.5% to 3% [6]. In some patients, an intermediate but more advanced premalignant stage, termed smoldering multiple myeloma (SMM), is clinically recognized [1]. To date, the biological alterations that lead from MGUS to SMM and finally to symptomatic disease have not been elucidated.

MM evolution is characterized by an avascular stage of slow tumor progression followed by “angiogenic switch” when the balance between pro- and anti-angiogenic factors in the tumor microenvironment is deranged to promote neovascularization and disease progression [7,8]. Angiogenesis is a multistep and tightly regulated process of the formation of new blood vessels, which occurs physiologically during embryonic growth and wound healing, but also in tumor growth and metastasis [9,10]. Our previous study elucidated the role of angiogenic cytokines in MM pathogenesis, and our results were consistent with those of other groups [11–15]. These cytokines mainly include the vascular endothelial growth factor (VEGF), the angiogenin, the angiopoietins, and the basic fibroblast growth factor (bFGF) [11]. Angiopoietin-1 (Ang-1) and its natural antagonist, angiopoietin-2 (Ang-2), are involved in the biology of MM. We have shown that the Ang-1 to Ang-2 ratio is an independent prognostic factor for survival in newly diagnosed MM patients [16,17]. However, to our knowledge, no previous studies have evaluated the above angiogenic cytokines in a large population of each of the MM stages.

The aims of this study were to: i) evaluate the circulating levels of important angiogenesis inducers – VEGF-A, angiogenin, Ang-1, and Ang-2 – as representative angiogenesis inhibitors in patients with MGUS, SMM, and symptomatic MM; and ii) to evaluate the gene expression of *VEGF-A* and determine if any variability of this predominant angiogenic cytokine is present

during the evolutionary process of precursor stages to symptomatic MM, contributing to better understanding the underlying mechanisms of this transition.

## Material and Methods

### Patients and samples processing

We studied 109 consecutive patients, of whom 55 were newly diagnosed and symptomatic with MM (n=55), and 54 asymptomatic MM patients (n=54). We also evaluated 27 patients with MGUS at diagnosis and, as an internal control group, 22 healthy controls of similar age and gender (12M/10F; median age: 67 years, range: 39–83 years). The study was conducted after approval from the institutional Ethical Committee and under the guidelines of the Declaration of Helsinki. All patients provided written consent for participating in this study. The medical history of all subgroups was reviewed to ensure that there was no disease (e.g., cardiovascular disorder, inflammatory disease, renal impairment, or infection) or drug administration that could alter angiogenesis at the time of sampling. Serum samples were collected at the time of diagnosis from all patients, before the initiation of any antimyeloma treatment, including supportive treatment (e.g., bisphosphonate administration) and stored at -80°C until the day of measurement. Patient and control group characteristics are depicted in Table 1.

Peripheral blood mononuclear cells (PBMCs) and plasma cells from bone marrow aspirates used in gene expression study were isolated according to previously described standard methods [18]. Briefly, PBMCs were isolated by using Ficoll-plaque density sedimentation from freshly drawn peripheral blood as previously described [18]. MM plasma cells from bone marrow aspirates were purified by positive selection with anti-CD138 magnetic activated cell-sorting (MACS) separation microbeads, as described by the manufacturer (MACS, Miltenyi Biotec, Auburn, CA). Purity, as confirmed by flow cytometry CD38 and CD45 staining (FACSCalibur cytometer), was above 95% in all MGUS and MM cases, and above 90% in all MM patients.

The gene expression of VEGF-A in PBMCs and in MM plasma cells was estimated in a total of 10 patients with MGUS, 10 patients with SMM, and 10 patients with symptomatic MM. The gene expression of VEGF-A in PBMCs was also studied in 10 healthy volunteers.

### ELISA assay and reagents

Circulating levels of Ang-1, Ang-2, VEGF, and angiogenin were evaluated in all patients, using an ELISA methodology (R&D Systems, Minneapolis, MN), according to the manufacturer's instructions.

**Table 1.** Patient characteristics.

	MM	SMM	MGUS
No	54	55	27
Gender (M/F)	30/24	29/26	15/12
Median age (range)	66(34–80)	65 (38–85)	67 (43–86)
Ig-subtype	IgG 67% IgA 15% Light chain 18%	IgG 72% IgA 28%	IgG 63% IgA 31% Light chain 6%
ISS-stage	I 39% II 38% III 23%	I 100%	
Hb <10 g/dl	32%	0	0
Creatinine >UNL	18%	3.6%	3.7%
Creatinine ≥2 mg/dl	9%	0	0
Albumin <3.5 g/dl	19%	0	0
β2-M >6 mg/L	16%	0	0

### Gene expression – PCR assay

RNA was extracted from peripheral blood lymphocytes as described previously [19]. Total RNA was extracted with Trizol according to the manufacturer's instructions (Invitrogen) and purified from salts and residual DNA using the RNease Mini Kit (Qiagen). Quantity of RNA in each sample was measured by spectroscopy and integrity was determined by gel electrophoresis. Only RNAs with clear 18S and 28S peaks were used. VEGF-A expression was studied using semi-quantitative RT-PCR as described previously [15]. Briefly, RT-PCR was performed as a multiplex using the Titan One Tube RT-PCR System according to the manufacturer's instructions (Roche Diagnostics GmbH, Mannheim Germany). Following RT-PCR, the samples were treated with ExoSAP-IT to remove excess primer and deoxyribonucleotides (dNTPs; USB), as described by the manufacturer. A portion of the RT-PCR product was used in the Fast Start DNA Master SYBR Green I kit. This reaction was run on the Light-Cycler (Roche Diagnostics GmbH) until a sample reached plateau stage. PCR products were run on 1% agarose gel, and relative quantification was performed by spot densitometry comparisons with GAPDH (glyceraldehyde-3-phosphate dehydrogenase) bands. Expression was quantified as a ratio of cytokine to GAPDH mRNA expression. Primer sequences were as follows: for GAPDH, forward 5'-ACCACAGTCCATGCCATCAC-3' and reverse 5'-TCCACCACCCTGTTGCTTGTA-3'; for VEGF-A, forward 5'-GCCCACTGAGGAGTCCAACATC-3' and reverse 5'-TTTTTGAGGAACATTACACG-3'. Melting curve analysis of the PCR products was performed to confirm the presence of a single specific amplicon.

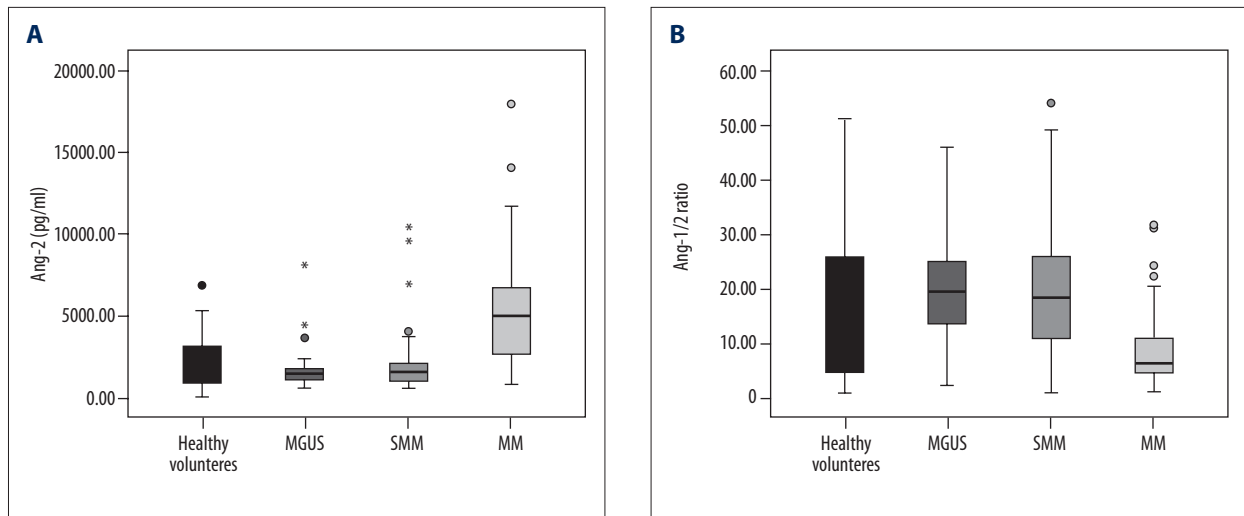
### Statistical analysis

Descriptive statistics were used to characterize baseline variables. Differences between patients and controls, as well as between different patient subsets, were evaluated using the Mann-Whitney U test. Spearman's nonparametric correlation test was used to determine the correlations between evaluated parameters. All p values were 2-sided and the level of significance was <0.05.

## Results

### Measurement of serum levels of angiogenic cytokines

Symptomatic MM patients at diagnosis showed increased levels of serum Ang-2 (mean value ±SD: 5271±3370 pg/ml) compared with SMM patients (2043±1881pg/ml;  $P<0.001$ ), MGUS patients (1851±1516pg/ml;  $P<0.001$ ), and healthy controls (2149±1756 pg/ml,  $P<0.001$ ). There was no statistically significant difference between asymptomatic patients, MGUS patients, and controls (Figure 1A). There was no difference in circulating levels of ang-1 between patients with MM, SMM, MGUS, and healthy controls (Table 2). Consequently, the ang-1/ang-2 ratio was reduced in symptomatic MM patients (mean value ±SD: 8.0±7.3) compared to SMM patients (19.8±12.3;  $P<0.001$ ), MGUS patients (22.2±15.3;  $p<0.001$ ), and healthy controls (18.0±16.6;  $P<0.001$ ), but there was no significant difference between asymptomatic patients, MGUS patients, and controls (Figure 1B). Serum levels of VEGF were increased in



**Figure 1.** Circulating angiopoietin-2 (A) and ratio of angiopoietin-1/angiopoietin-2 (B) in patients and controls.

**Table 2.** Levels of circulating angiogenic cytokines in patients and controls.

	Ang-1 (ng/ml)	Ang-2 (ng/ml)	Ang-1/Ang-2	VEGF (pg/ml)	ANG (ng/ml)
Healthy controls (n=22) (mean value ±SD)	23.3±11.9	2.1±1.8	18.0±16.6	286±267	165.1±34.3
MGUS patients (n=27) (mean value ±SD)	29.6±9.9	1.8±1.5	22.2±15.3	399±188	379.9±155.6
SMM patients (n=54) (mean value ±SD)	27.4±7.9	2.0±1.9	19.8±12.3	403±273	377.1±137.8
MM patients (n=55) (mean value ±SD)	26.4±13.7	5.3±3.4	8.0±7.3	613±408	255.6±137.2

patients with MGUS ( $399 \pm 188$  pg/ml;  $P=0.02$ ), SMM ( $403 \pm 273$  pg/ml;  $P=0.04$ ), and MM ( $613 \pm 408$  pg/ml;  $P=0.001$ ) compared to healthy controls ( $286 \pm 267$  pg/ml). Patients with MM had increased VEGF serum levels compared to MGUS and SMM patients ( $P=0.039$  and  $P=0.009$ , respectively) (Figure 2A). Patients with MGUS, SMM, and symptomatic MM had increased levels of serum angiogenin ( $379.9 \pm 155.6$  ng/ml,  $377.1 \pm 137.8$  ng/ml and  $255.6 \pm 137.2$  pg/ml, respectively) compared with the healthy controls ( $165.1 \pm 34.3$  ng/ml;  $P<0.001$  for all comparisons). Patients with MM had decreased angiogenin serum levels compared to MGUS and SMM patients ( $P<0.001$ ) for both comparisons. There was no statistically significant difference between SMM and MGUS patients (Figure 2B). A trend was observed in the correlation of angiogenin serum levels with ang-2 ( $r=-0.250$ ,  $P=0.008$ ) and Ang-1/Ang-2 ratio ( $r=0.217$ ,  $P=0.022$ ).

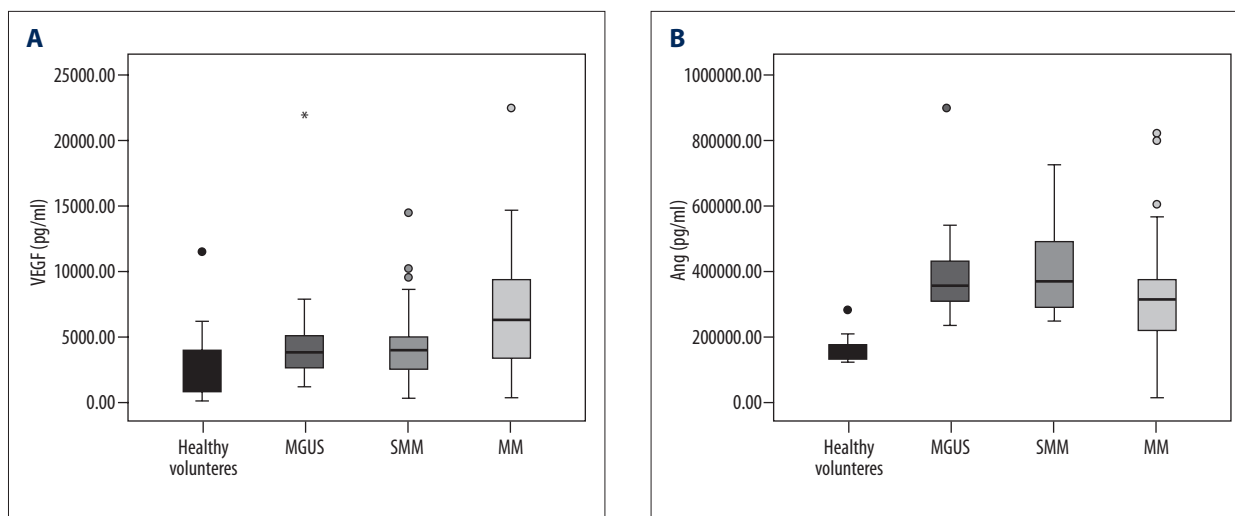
#### VEGF-A expression by semi-quantitative RT-PCR

We found that VEGF-A is expressed in PBMCs and MM plasma cells from all patients in all groups analyzed. In PBMCs, we observed no significant difference in the levels of VEGF-A mRNA expression between healthy volunteers, MGUS, SMM,

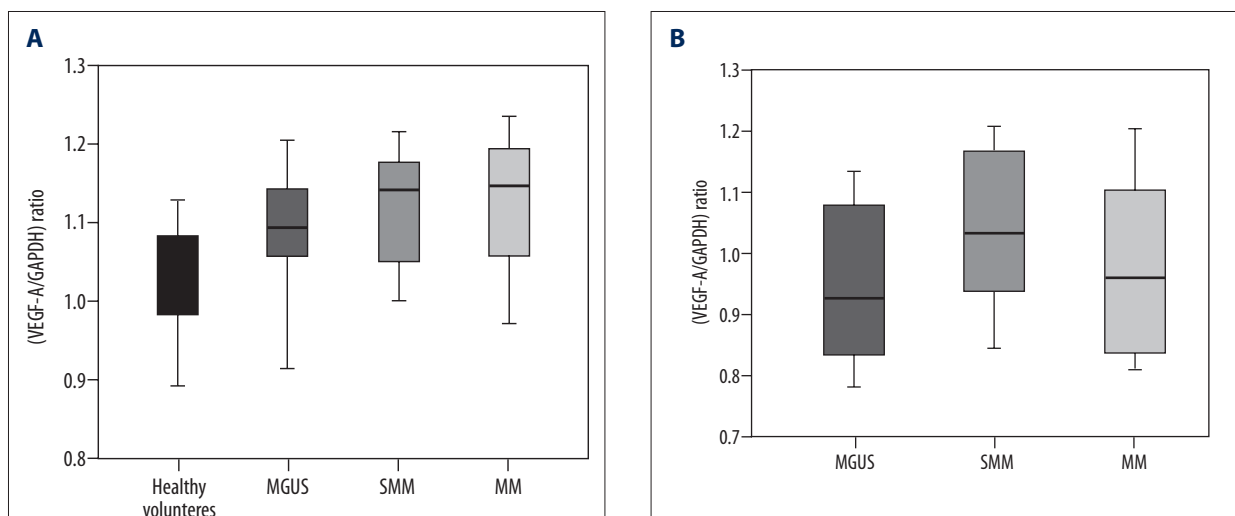
and MM patients, with median  $\pm$ SD VEGF-A/GAPDH ratios of  $1.019 \pm 0.081$ ,  $1.087 \pm 0.093$ ,  $1.116 \pm 0.091$  and  $1.119 \pm 0.109$ , respectively. Similarly, no difference in the levels of VEGF-A expression was observed in MM plasma cells among the 3 patients groups, with median  $\pm$ SD VEGF-A/GAPDH ratios of  $0.933 \pm 0.1322$ ,  $1.028 \pm 0.1279$ , and  $0.973 \pm 0.1406$ , respectively (Figure 3).

#### Discussion

Several cytokines are implicated in the angiogenesis process. The cross-talk among angiogenic and inflammatory cytokines plays an important role in the modulation of blood vessel growth in several pathological conditions, including cancer. In the abnormal tumor microenvironment, pro-angiogenic cytokines and inflammatory mediators prevail and promote structurally and functionally aberrant vessel formation [7]. During tumor angiogenesis, Ang-2 antagonizes Tie2 binding and destabilizes the vessel wall of mature vessels. Quiescent endothelial cells become sensitive to VEGF, then proliferate and migrate to form new vessels. Bone marrow-derived endothelial



**Figure 2.** Circulating VEGF (A) and angiogenin (B) in patients and controls.



**Figure 3.** VEGF-A expression by PBMCs (A) and plasma cells (B) in different patient groups.

cell progenitors are found in the peripheral blood and are recruited by angiogenin at sites of angiogenesis [20–23].

MM was the first hematologic malignancy in which angiogenesis were defined as a prognostic factor [24,25]. Vacca et al. first demonstrated that tumor growth is angiogenesis-dependent [26], by reporting a high correlation between the extent of BM angiogenesis (microvessel area) and the proliferating fraction of BM plasma cells (labeling index) in MGUS and MM patients. Angiogenesis itself was significantly associated with active, as opposed to non-active, MM and MGUS [26].

Angiogenesis is possibly important in the progression of MGUS to MM and in the progression of early-stage myeloma to advanced, refractory disease. Previous reports have shown that bone marrow angiogenesis progressively increases along the spectrum of plasma cell disorders, from the more benign stage

of MGUS to advanced myeloma, indicating that angiogenesis may be related to disease progression [8]. A proposed mechanism is that angiogenesis contributes to myeloma pathogenesis and progression by ensuring an adequate tumor oxygen and nutrient supply and by paracrine stimulation of tumor cell migration and proliferation [27]. However, the precise mechanism underlying the observed alterations of angiogenic cytokines in the evolution of MM has not been fully investigated; our study aimed to answer this critical question.

VEGF was increased in the serum of patients with MM as well as in patients with MGUS and SMM compared with healthy control samples, indicating that myeloma genesis, the transition process to symptomatic disease, occurs via mechanisms that are less VEGF-dependent. Nevertheless, patients with symptomatic disease had higher VEGF serum levels than other patients, perhaps due to greater tumor burden. However,

post-transcriptional modifications such as stabilization of Mrna, induction of translation and stabilization of final protein could be responsible, since we found no significant difference in the levels of VEGF-A mRNA expression from plasma cells of MGUS, SMM, and MM patients.

Reduced angiopoietin-1/angiopoietin-2 ratio has been previously correlated with advanced disease features, including international staging system (ISS)-3 stage, renal impairment,  $\beta$ 2-microglobulin levels, and extensive bone disease, as well as with inferior survival in newly diagnosed patients with MM who received therapy with novel antimyeloma agents [17,28]. In the present study, symptomatic MM patients, but not MGUS or SMM patients, had increased serum levels of ang-2 and decreased ang-1/ang-2 ratio compared with healthy controls. These results highlight the role of angiopoietins' pathway in the biology of symptomatic disease.

Interestingly, the patients with symptomatic MM exhibit decreased serum levels of angiogenin compared to MGUS and SMM patients, but had increased levels compared to healthy controls. The observed negative correlation between angiogenin and Ang-2 is a novel and intriguing finding.

Furthermore, it is well established that there is a widespread expression pattern of angiogenin, suggesting a physiological

function that is not restricted to the neovascularization process [29]. Further investigation is needed to unravel the complex network of angiogenic cytokines and their precise role in the biology of MM.

## Conclusions

Patients with SMM have similar levels of circulating angiopoietins, angiogenin, and VEGF compared with MGUS patients, but these levels are altered compared to symptomatic MM patients. Ang-1/Ang-2 and angiogenin is reduced in MM patients with symptomatic disease compared to MGUS or SMM. These results suggest that in the progression of MGUS to SMM, circulating angiogenic molecules appear to be the same. In contrast, in symptomatic disease the alterations of angiopoietins' pathway along with VEGF contribute to myeloma cell growth. Because there is great need for novel drugs active against myeloma cells, our data support that the Ang-1/Ang-2/Tie-2 axis may be an effective target for the development of novel antimyeloma agents.

## Conflicts of interest

The authors have no conflicts of interest to disclose regarding this paper.

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