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Increased Plasma GDF15 Is Associated with Altered Levels of Soluble VEGF Receptors 1 and 2 in Symptomatic Multiple Myeloma

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Keywords
Multiple myeloma · Growth differentiation factor-15 · Soluble vascular endothelial growth factor receptors

Abstract
Introduction: In multiple myeloma, there is an increase in bone marrow microvascular density and enhanced renal lymphangiogenesis. Increased levels of the proangiogenic protein growth differentiation factor-15 (GDF15) have previously been reported to be associated with poor prognosis in myeloma. A possible association between GDF15 and the soluble forms of vascular endothelial growth factor receptors (sVEGFR) 1 and 2 has not yet been investigated, and a role for these receptors in pathological angiogenesis in myeloma is still to be defined. ***Methods:*** Plasma levels of GDF15 and sVEGFR1 and 2 were determined by ELISA in patients with smouldering multiple myeloma (sMM), patients with symptomatic multiple myeloma (abbreviated as MM), and healthy controls. The levels were compared between the three groups, and correlation coefficients were calculated, as were Kaplan-Meier curves for GDF15 and sVEGFR1 and sVEGFR2. ***Results:*** Levels of GDF15 were significantly higher in MM than in both patients with sMM and controls. A gradual decrease in mean sVEGFR1 concentration was observed, with MM > sMM > controls. Mean sVEGFR2 was lower in patients with MM than in controls. There was a positive correlation between GDF15 and sVEGFR1, and GDF15 correlated negatively with sVEGFR2. High GDF15 (>3 ng/mL) was associated with poor prognosis. ***Conclusion:*** In multiple myeloma, increased expression of GDF15 correlates positively with sVEGFR1 and negatively with sVEGFR2. It is possible that the altered levels of sVEGFR1 and 2 contribute to the increased angio- and lymphangiogenesis observed in myeloma.

Introduction

Multiple myeloma accounts for 1% of all cancers and is the second most common haematological malignancy. The disease is characterized by the accumulation of malignant clonal plasma cells in the bone marrow compartment, which eventually culminates in the clinical manifestations of the disease, that is, lytic bone lesions, anaemia, hypercalcaemia, and renal failure [1]. Probably all cases of multiple myeloma evolve from a pre-malignant, asymptomatic state known as "monoclonal gammopathy of undetermined significance." The prevalence of monoclonal gammopathy of undetermined significance in the population above the age of 50 is 3%. Smouldering multiple myeloma (sMM) is a more advanced, but still

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asymptomatic, stage of the disease. In 10% of patients per year over the first 5 years following diagnosis, sMM progresses to symptomatic multiple myeloma (herein abbreviated as simply "MM") [2].

Angiogenesis is of importance in the progression of the disease; in patients with myeloma, an increased bone marrow microvascular density is seen [3]. Lymphatic angiogenesis in the kidneys is also enhanced in myeloma [4]. Vascular endothelial growth factor (VEGF) is a family of proteins that regulate angiogenesis and lymphatic angiogenesis. These growth factors exert their effect on angiogenesis by binding and activating specific transmembrane receptor tyrosine kinases predominantly expressed on endothelial cells [5]. The VEGF-VEGF receptor interaction stimulates proliferation and migration of endothelial cells. Three different VEGF receptors (R1, R2, and R3) have been identified, and activation of VEGFR2 by VEGF-A is the most important ligand-receptor interaction in angiogenesis. The receptor VEGFR1, expressed as a soluble protein (sVEGFR1), has mainly been regarded as a negative regulator of VEGF signalling by acting as a decoy receptor for VEGF. Proliferation and migration of lymphatic endothelial cells depends on activation of VEGF receptors 2 and 3 by the growth factors VEGF-C and D. Alternative splicing of VEGF and VEGFR pre-mRNA gives rise to different VEGF and VEGFR protein isoforms that can have opposing functions in vessel formation [6, 7].

Vascular endothelial growth factor mRNA is overexpressed in the majority of tumours in humans [7], including myeloma [8], and its expression correlates positively with invasiveness, metastasis, and adverse prognosis [5]. Also, VEGFRs are expressed by malignant plasma cells [8]. There are currently several cancer therapies targeting the VEGF-VEGFR signalling pathway, including bevacizumab, ziv-aflibercept, and multiple tyrosine kinase inhibitors such as sorafenib and sunitinib [5].

The proangiogenic protein growth differentiation factor-15 (GDF15) is a member of the transforming growth factor β superfamily. It is highly expressed in the placenta, with increasing serum concentration during pregnancy [9], and it stimulates endothelial colony-forming cell migration and proliferation in a paracrine fashion [10]. High levels of circulating GDF15 are seen in patients with various types of cancer such as prostate and colorectal cancer [9]. An increased concentration of GDF15 has also been observed and is associated with poor prognosis, in patients with myeloma [11, 12]. The poor prognosis can at least partly be explained by the finding that bone marrow mesenchymal stem cell-derived GDF15 confers resistance to chemotherapeutic drugs commonly used for treatment of multiple myeloma, such as melphalan, bortezomib, and lenalidomide [12]. It has been reported that GDF15 stimulates VEGF expression in vitro [13], but whether it exerts its effect on angiogenesis any further by modulation of the VEGF receptor expression is currently not known. A possible role for soluble VEGFR1 and 2 in pathological angiogenesis in multiple myeloma has not yet been investigated.

In this study, plasma levels of sVEGFR1 and 2, in relation to levels of GDF15, were determined by ELISA, in patients with MM, patients with sMM, and healthy controls. To our knowledge, this is the first study investigating levels of GDF15 in patients with MM, in comparison with patients with sMM.

Patients and Methods

Blood plasma from 142 newly diagnosed myeloma patients, stored in the Uppsala-Umeå Cancer Consortium (U-CAN) Biobank, was used for ELISA analyses of GDF15 and sVEGFR 1 and 2. The patients were divided into two groups, those with MM (n = 122) and those with sMM (n = 20). The International Myeloma Working Group 2014 diagnostic criteria for MM and sMM [14] were used, with the exception of serum-free light chain analysis and skeletal MRI which were performed in a minority of the patients only. Laboratory and radiographic data from the time of the myeloma diagnosis were obtained from the individual patient charts. Serum creatinine and haemoglobin levels were available for all subjects in the two patient groups. Serum calcium levels were available for all sMM patients and for 91 (75%) of the patients in the MM group. A skeletal CT had been performed in all sMM patients and in 118 of the 122 (97%) patients in the MM group. Data for staging according to the International Staging System (ISS) [15] were available in 17 (85%) and 107 (88%) of the patients in the sMM and MM groups, respectively. Since lactate dehydrogenase was analysed in very few patients, we did not categorize the patients according to the revised ISS (R-ISS) [16].

Results of cytogenetic analyses were available for 17 (85%) of the sMM patients and 107 (88%) of the patients with MM. The presence of a least one of the cytogenetic abnormalities 1q gain, t(4;14), t(14;16) or deletion 17p in bone marrow plasma cells was defined as high-risk cytogenetics. Blood plasma from 71 age- and sex-matched individuals was used as control.

Written informed consent was obtained from all study participants. This study was approved by the Research Ethics Committee of Uppsala University (Epn 2010/98, 2014/233, and Ups 01-367).

The ELISA analyses were performed using commercial sandwich kits (R&D Systems, Minneapolis, MN, USA) for GDF15 (DY957) and for sVEGFR1 (DY321B) and sVEGFR2 (DY357). Absorbances were measured using a SpectraMax 250 analyser (Molecular Devices, Sunnyvale, CA, USA). The total coefficient of variations of the assays was approximately 6%. The assays were performed blinded without knowledge of the clinical diagnosis. All samples were analysed in the same run using two microtiter plates to reduce plate-to-plate variation.

Table 1. Clinical and laboratory characteristics of patients with smouldering myeloma, patients with symptomatic myeloma, and of controls

Variable	Smouldering myeloma (n = 20)	Symptomatic myeloma (n = 122)	Controls (n = 71)
Age, years	77 (45–91)	71 (43–90)	67 (43–79)
Renal insufficiency, %	5	25	n/a
Anaemia, %	5	34	n/a
Bone lesions, %	0	78	n/a
Hypercalcaemia, %	0	11	n/a
High-risk cytogenetics, %	0	15	n/a
ISS I, %	29	19	n/a
ISS II, %	65	44	n/a
ISS III, %	6	37	n/a
IgA myeloma, %	10	19	n/a
IgG myeloma, %	85	58	n/a
Bence Jones myeloma, %	5	20	n/a
Non-secretory myeloma, %	0	3	n/a
Hb, g/L	122 (92–147)	106 (72–162)	143 (113–175)
β2-Microglobulin, mg/L	2.8 (1.7–8.7)	4.1 (1.4–46)	2.8 (1.0–5.0)
GDF15, ng/mL	1.0 (0.3–2.9)	1.7 (0.3–10)	0.8 (0.4–2.4)
sVEGFR1, ng/mL	2.1 (0.2–5.0)	3.4 (0.2–18)	0.4 (0.2–4.5)
sVEGFR2, ng/mL	3.6 (2.0–8.2)	3.7 (1.7–7.9)	4.3 (2.4–6.9)

Age, Hb, β2-microglobulin, GDF15, sVEGFR1, and sVEGFR2 are expressed as median, range. n/a, not applicable; GDF15, growth differentiation factor-15; sVEGFR, soluble forms of vascular endothelial growth factor receptor.

Statistical analyses were performed using the software R 3.6.3 (R Foundation for Statistical Computing, Vienna, Austria). To assess the relative differences in biomarker levels between groups, linear models adjusted for age were used on log-transformed data (because of non-normality). Correlations were computed and tested using the Spearman rank correlation. Survival rates were assessed graphically using Kaplan-Meier curves and formally tested using a proportional hazards regression model adjusted for age. Throughout, the p values were adjusted for multiplicity using the Benjamini-Hochberg procedure. Cutoff value for GDF15 was chosen using a visual inspection of the GDF15 values (without looking at survival), in which it was observed that the values were clustered in two groups: a larger group with GDF15 ≤3 and a smaller group with GDF15 >3. We hypothesized that if GDF15 was correlated with survival, then a difference between these two groups would be seen in plots of Kaplan-Meier curves [17].

Results

In this study, we analysed blood plasma levels of GDF15 and sVEGFR1 and 2, by means of ELISA, in 142 newly diagnosed multiple myeloma patients. Twenty (14%) of the patients were diagnosed with sMM while 122 (86%) had symptomatic disease. The median age for patients with sMM and symptomatic MM was 77 and 71 years, respectively. The control population consisted of 71 individuals with a median age of 67 years. In the MM group, 11% had hypercalcaemia while 25% and 34% suffered from renal failure and anaemia, respectively. One patient with sMM had anaemia and renal failure not related to the haematological disease. The CT examination revealed myeloma bone disease in 78% of the patients in the MM group. Thirty-seven percent of the patients with symptomatic disease had ISS stage III. The corresponding number for sMM patients was 6%. High-risk cytogenetics was found in 15% of the MM patients. None of the sMM patients exhibited high-risk cytogenetic markers.

Forty-two percent (51/122) of the patients in need of treatment at the time of diagnosis were subjected to high-dose melphalan treatment and subsequent autologous haematopoietic stem cell transplantation while 58% (71/122) received conventional anti-myeloma therapy. Clinical and laboratory characteristics of patients with sMM, patients with MM, and controls, respectively, are summarized in Table 1.

The mean β2-microglobulin (B2M) level was significantly higher in patients with symptomatic MM than in patients with sMM ($p < 0.01$) and controls ($p < 0.001$), but there was no significant difference between sMM patients and controls (Table 2). Plasma levels of GDF15 were

Table 2. Relative difference between Hb, β2-microglobulin, GDF15, sVEGFR1, and sVEGFR2, between the different groups

Variable	Relative difference	±SE	*p* value
Symptomatic myeloma versus smouldering myeloma			
Hb	1.138436	0.001941	<0.01
β2-Microglobulin	0.567609	0.010693	<0.01
GDF15	0.582870	0.009072	<0.01
sVEGFR1	0.520962	0.018023	<0.05
sVEGFR2	1.047452	0.004436	ns
Symptomatic myeloma versus controls			
Hb	1.331054	0.000813	<0.001
β2-Microglobulin	0.565460	0.002661	<0.001
GDF15	0.476743	0.001687	<0.001
sVEGFR1	0.165185	0.000470	<0.001
sVEGFR2	1.136726	0.001900	<0.01
Smouldering myeloma versus controls			
Hb	1.177891	0.000708	<0.001
β2-Microglobulin	0.967769	0.005875	ns
GDF15	0.819418	0.006003	0.05
sVEGFR1	0.348627	0.004554	<0.001
sVEGFR2	1.123126	0.005200	ns

ns, not significant; GDF15, growth differentiation factor-15; sVEGFR, soluble forms of vascular endothelial growth factor receptor; SE, standard error. Relative difference (RD): 1 means no difference in levels between the two groups; RD <1 means that the first group has a higher level; RD >1 means that the first group has a lower level.

significantly higher in patients with symptomatic MM than in patients with sMM ($p < 0.01$) and controls ($p < 0.001$) (shown in Fig. 1a; Table 2). The difference in GDF15 levels between patients with sMM and healthy controls reached borderline significance ($p = 0.05$). Mean plasma levels of sVEGFR1 were significantly higher in patients with symptomatic MM than in sMM patients ($p < 0.05$) and controls ($p < 0.001$). Additionally, there was a significant difference in sVEGFR1 between the sMM and control groups ($p < 0.001$), with the latter group showing a lower mean level (shown in Fig. 1b; Table 2). Patients with MM had a lower mean plasma level of sVEGFR2 than controls ($p < 0.01$) (shown in Fig. 1c; Table 2).

There was a positive correlation between plasma levels of GDF15 and sVEGFR1, as well as between GDF15 and B2M. However, GDF15 correlated negatively with sVEGFR2 and haemoglobin levels (Table 3).

Twenty-three percent (32 of 142) of the patients had GDF15 levels >3 ng/mL, and all of them had symptomatic myeloma. Myeloma patients with GDF15 >3 ng/mL had a significantly ($p < 0.001$) reduced overall survival (OS) compared to those with GDF ≤3 ng/mL, with median survival from diagnosis to death being 36 and 59 months, respectively, for these two groups (shown in Fig. 2a). No correlation was found between either sVEGFR1 or sVEGFR2 and OS. The median survival for all patients with symptomatic myeloma was 50 months (shown in Fig. 2b).

Discussion

In an attempt to elucidate a possible association between expression of the proangiogenic protein GDF15 and proteins involved in the VEGF-VEGFR signalling pathways in multiple myeloma, we compared the plasma levels of GDF15 and soluble VEGF receptors 1 and 2 in patients with MM, patients with sMM, and healthy controls. Our results showing increased circulating GDF15 in patients with multiple myeloma are consistent with findings in previous studies [11, 12, 18]. In contrast to these three previous studies, we compared GDF15 levels between patients with symptomatic MM, sMM patients, and controls, and not only between patients with symptomatic disease and healthy controls. Our finding that there is a strong trend toward a significantly increased mean GDF15 in sMM compared with controls indicates that elevated GDF15 is an early event in myeloma progression. This has, to our knowledge, not previously been shown. Furthermore, we confirm previously published

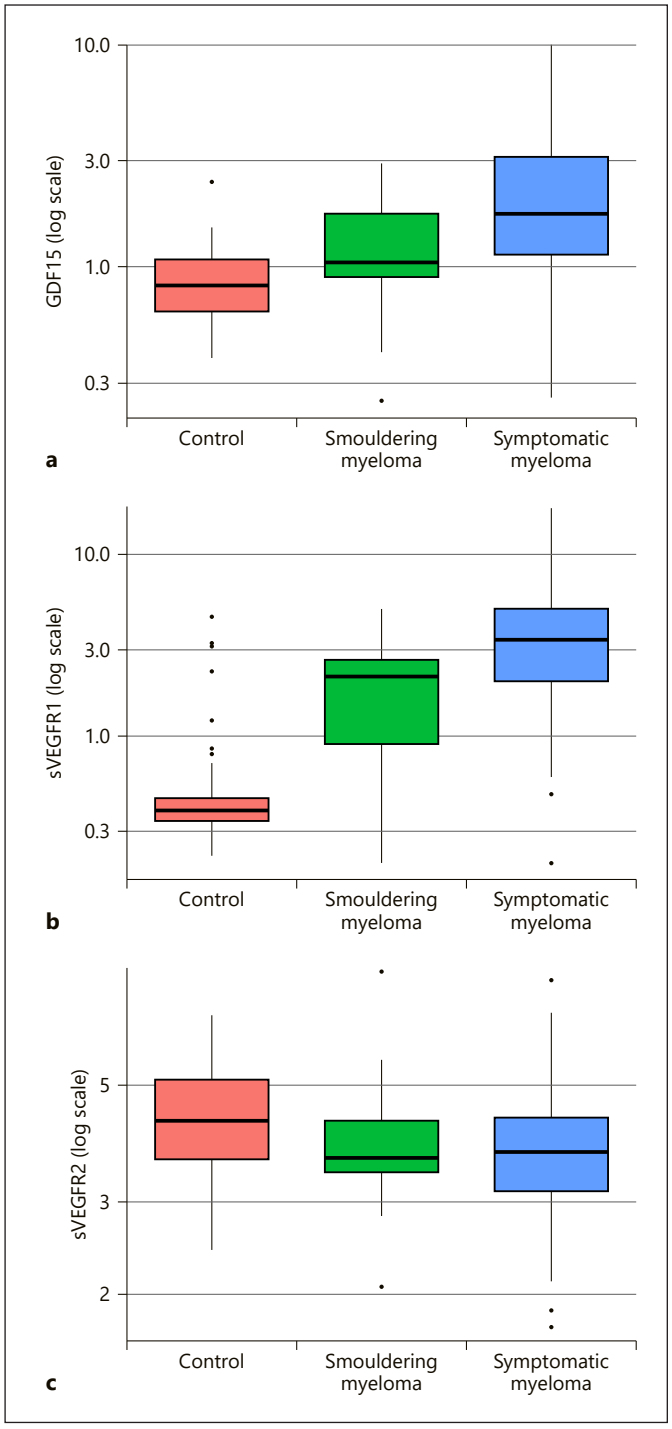

Fig. 1. Boxplots visualizing levels of GDF15 and sVEGR1 and 2 in nanogram per millilitre, in controls and in patients with smouldering myeloma and symptomatic myeloma. Medians are shown in thick lines. The bottom and top of the boxes represent the first and third quartiles. The whiskers show the smallest and highest non-outliers. Outliers are shown as circles. **a** Results for GDF15. **b** Results for sVEGFR1. **c** Results for sVEGFR2. GDF15, growth differentiation factor-15; sVEGFR, soluble forms of vascular endothelial growth factor receptor.

Table 3. Correlation coefficients (Spearman rank order correlation) of GDF15 with Hb, β2-microgobulin, sVEGFR1, and sVEGFR2

Variable	r_s (variable vs. GDF15)	*p* value
Hb	−0.53	<0.001
β2-Microglobulin	0.64	<0.001
sVEGFR1	0.36	<0.001
sVEGFR2	−0.23	<0.01

GDF15, growth differentiation factor-15; sVEGFR, soluble forms of vascular endothelial growth factor receptor.

findings [11, 18] that GDF15 correlates positively with B2M and negatively with haemoglobin, suggesting that high GDF15 is associated with a more advanced MM disease stage.

There are conflicting data published regarding a possible correlation between GDF15 expression and survival in multiple myeloma. In the present study, a population-based myeloma cohort was used in contrast to previous studies which have used myeloma patient cohorts from clinical trials [11, 12] or a smaller number of younger patients [18]. Our finding that myeloma patients with GDF15 levels >3 ng/mL have a significantly reduced mean OS (36 months vs. 59 months for GDF15 ≤3 ng/mL) is in line with the results published by Westhrin et al. [11] and Corre et al. [12]. The results in these 3 studies ([11, 12] and the present study) contrast with the results of a Turkish study in which GDF15 levels did not correlate with OS [18].

GDF15 was first discovered as a protein overexpressed in activated macrophages to regulate inflammation [19]. Increased plasma levels of GDF15 are associated with cancer and cardiovascular disease [9]. The role of GDF15 in cancer is contradictory: GDF15 has been found to act both as tumour suppressive and pro-tumoural, and it has been suggested that GDF15 acts as a tumour suppressor in the early stages of tumour growth, while in the later stages it becomes a promoter of cancer development and progression [20]. Also, GDF15 is known to act as a pro-angiogenic factor. Song et al. [13] have shown that GDF15 promotes angiogenesis in hypoxic human umbilical vein endothelial cells via hypoxia-inducible factor 1 alpha/VEGF-dependent signalling pathways. Expression of GDF15 increases in hepatocellular cancer cell lines in response to damage by chemotherapeutic agents, which in vitro enhances angiogenesis [21]. There is increased microvascular density in multiple myeloma bone marrow, with a higher density in the more advanced stages of the

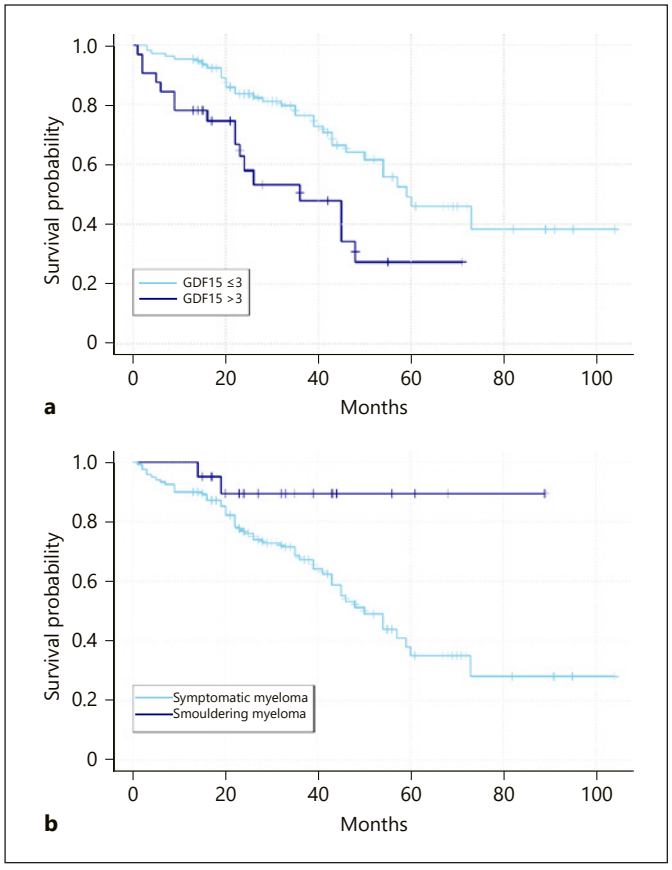

Fig. 2. Kaplan-Meier curves illustrating the difference in overall survival in patients with GDF15 levels >3 ng/mL in comparison with those with GDF15 levels ≤3 ng/mL (**a**) and in patients with smouldering myeloma in comparison with those with symptomatic disease (**b**). GDF15, growth differentiation factor-15.

disease [3]. This suggests an important role for angiogenesis in myeloma progression.

We found increased plasma levels of sVEGFR1 in patients with MM, in comparison with patients with sMM and controls. These results may seem contradictory, as sVEGFR1 is commonly regarded as a decoy receptor for VEGF-A, hence acting as an antiangiogenic agent. For example, sVEGFR1 is known to maintain corneal avascularity [22]. A study using murine embryonic stem cells with targeted disruption of the VEGF receptor 1 encoding gene, flt-1, revealed that this receptor is an important player in modulating early vascular development and vessel formation. In Flt-1 −/− embryos, endothelial cell proliferation is increased, and there is an abnormal vessel branching [23]. Reintroducing sVEGFR1 to this phenotype normalizes vascularization. In one study, when the transmembrane form of VEGFR1 was reintroduced, endothelial proliferation was restored, with no reduction in vessel branching [24]. Soluble VEGFR1, released from endothelial cells adjacent to vascular sprouts, is proposed to act as a guide for vascular sprouting by binding VEGF on the one side of the sprout, hence creating a VEGF-rich corridor, for the sprouting vessel on the other side, guiding it in the proper direction [25]. Huh et al. [26] have shown that GDF15 is expressed by malignant melanoma tumour cells, which promotes tumour growth by increasing angiogenesis, and that increased levels of VEGF further enhance angiogenesis. We therefore hypothesize that an increased local concentration of GDF15 and sVEGFR1 stimulates capillary growth, which results in the increased bone marrow microvascular density observed in multiple myeloma. Accordingly, the increased plasma levels of GDF15 and sVEGFR1 we observed in patients with multiple myeloma probably reflect a high bone marrow concentration of these two pro-angiogenic molecules. B2M correlates positively with multiple myeloma disease stage [14, 16]. Our finding that there is a positive correlation between B2M and sVEGFR1 levels suggests that elevated levels of sVEGFR1 are associated with a more advanced stage of the disease.

A previous study has shown that renal lymphangiogenesis is enhanced in patients with multiple myeloma [4]. Lymphangiogenesis, which is an important factor in tumour progression, is induced by VEGF-C binding to its transmembrane receptor VEGFR3. The receptor VEGFR2 exists in a soluble form, which is a result of alterative splicing. Soluble VEGFR2 is known to bind VEGF-C, thus inhibiting VEGF-C/VEGFR3-mediated signalling, which leads to decreased lymphatic endothelial cell proliferation [7, 27]. Shibata et al. [28] have shown that gene therapy with a vector expressing sVEGFR2 suppresses tumour growth and lymph node metastasis in a murine breast cancer model. Another study has shown that sVEGFR2 is downregulated in metastatic neuroblastoma [29]. In this study, we found that patients with MM have lower mean plasma levels of sVEGFR2 in comparison with controls, which can possibly explain the enhanced renal lymphangiogenesis seen in multiple myeloma. Medinger et al. [30] found that serum levels of sVEGFR2 in patients with multiple myeloma treated with immunomodulatory drugs, such as thalidomide and lenalidomide, significantly increased in responders, compared with non-responders. However, there are few studies published on lymphangiogenesis in multiple myeloma, and we currently do not know what role decreased sVEGFR2 expression plays in pathogenesis and progression of the disease.

The mechanisms by which GDF15 stimulates angiogenesis are to date not fully understood. We propose that

the increased levels of GDF15 seen in patients with multiple myeloma stimulate angiogenesis and lymphangiogenesis by modulating levels of sVEGFR1 and 2. Our finding that elevated levels of GDF15 and sVEGFR1 are early events in myeloma progression is a novel contribution to the myeloma research literature.

Statement of Ethics

The research was conducted ethically in accordance with the World Medical Association Declaration of Helsinki. Written informed consent was obtained from all study participants. This study was approved by the Research Ethics Committee of Uppsala University (Epn 2010/98, 2014/233, and Ups 01-367).

Conflict of Interest Statement

The authors have no conflicts of interest to declare.

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Author Contributions

A.L. and T.K. designed the study. A.L., J.H., and T.K. analysed the data and wrote the manuscript. M.T. performed the statistical analyses. All authors revised the manuscript and gave final approval for submission.

Data Availability Statement

All data generated or analysed during this study are included in this article. Further enquiries can be directed to the corresponding author.

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