

**Growth differentiation factor 15 (GDF15) promotes osteoclast differentiation and inhibits osteoblast differentiation and high serum GDF15 levels are associated with multiple myeloma bone disease**

---

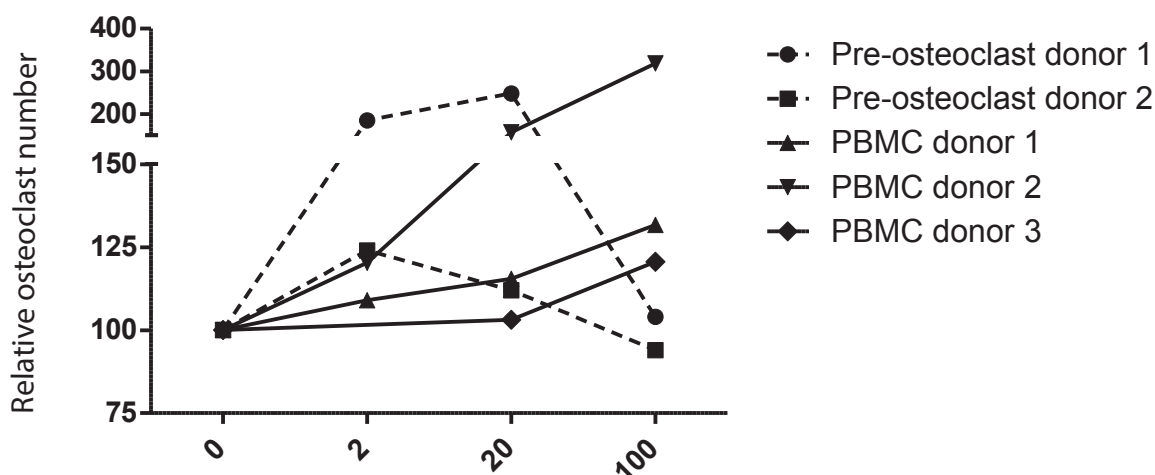
*Marita Westhrin,<sup>1</sup> Siv Helen Moen,<sup>1</sup> Toril Holien,<sup>1</sup> Anne Kærsgaard Mylin,<sup>2</sup> Lene Heickendorff,<sup>3</sup> Oddrun Elise Olsen,<sup>1</sup> Anders Sundan,<sup>1</sup> Ingegar Turesson,<sup>4</sup> Peter Gimsing,<sup>2</sup> Anders Waage,<sup>1</sup> and Therese Standal<sup>1,5,\*</sup>*

*<sup>1</sup>K.G. Jebsen Center for Myeloma Research, Department of Cancer Research and Molecular Medicine, Norwegian University of Science and Technology, Trondheim, Norway; <sup>2</sup>Department of Haematology, Rigshospitalet, University of Copenhagen, Denmark; <sup>3</sup>Department of Clinical Biochemistry, Aarhus University Hospital, Denmark; <sup>4</sup>Department of Hematology, Skane University Hospital, Malmo, Sweden; and <sup>5</sup>Centre of Molecular Inflammation Research, Norwegian University of Science and Technology, Trondheim, Norway*

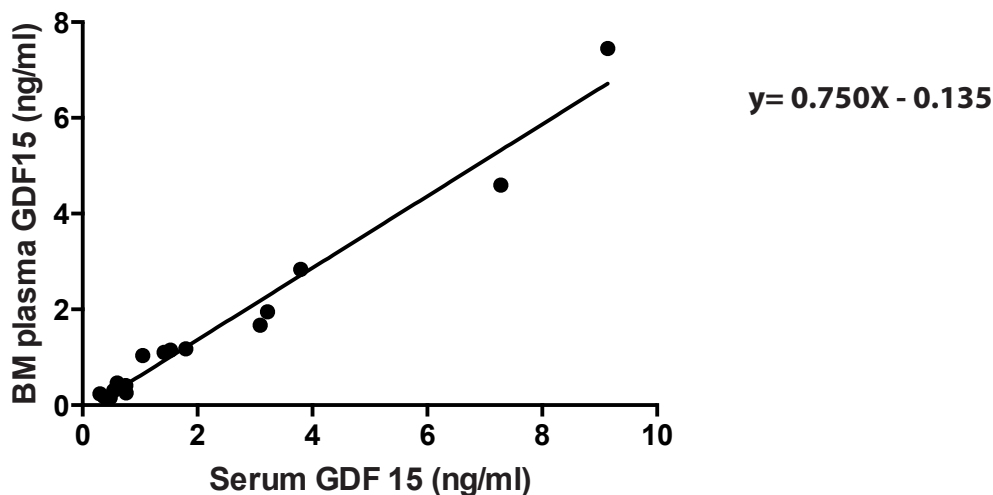
*Acknowledgments: we thank Berit Størdal for excellent technical assistance.*

*Funding: the work was supported by the Norwegian Cancer Society (project ID 450930), the K.G. Jebsen Foundation and the Cancer Foundation at St Olavs Hospital.*

*Correspondence: [therese.standal@ntnu.no](mailto:therese.standal@ntnu.no)  
doi:10.3324/haematol.2015.124511*



**Supplementary Figure 1. GDF15 promotes osteoclastogenesis in vitro.** Pre-osteoclasts (Lonza Inc) were treated with MCSF (33 ng/ml), RANKL (66 ng/ml) and GDF15 as indicated for 7 days before multinucleated (<3 nuclei), TRAP-positive cells were counted. Peripheral blood monocytes were treated with MCSF (30 ng/ml) for two days before the addition of RANKL (50 ng/ml) and various concentrations of GDF15 as indicated. At day 12-14 TRAP positive multinucleated cells were counted. Maximum numbers of osteoclasts varied between donors: 25-185 osteoclasts per well for PBMCs and 110-700 per well for pre-osteoclasts.



**Supplementary Figure 2. GDF15 levels in paired serum and bone marrow plasma samples correlate.** GDF15 was measured in paired bone marrow plasma and serum samples obtained from myeloma patients (n=16). Spearman's rho= 0.97, p < 0.0001.

## Supplementary results, materials and methods

### Serum analyses

In addition to GDF15, we measured 20 other cytokines in these samples. Briefly, of the 21 factors analyzed (GDF15, Nrp1, HGF, Leptin, Il1b, IL5, IL6, IL17, Eotaxin, GCSF, IP10, MCP1, MIP1a, MIPb, VEGF, GMCSF, BMP9, Follistatin, Dkk1, OPG and Endoglin) 12 cytokines were differently expressed between controls and patients: HGF, IL1b, IL5, IL6, Eotaxin, GCSF, MCP1, MIP1a, BMP9, Follistatin, GDF15, OPG (Independent samples Kruskal-Wallis test,  $p < 0.002$  was considered significant due to adjustment for multiple comparisons  $p = 0.05/21 = 0.002$ ).

We then examined whether there were differences in levels of these 12 cytokines when the patients were categorized according to degree of bone disease. Of the 12, 3 cytokines differed between the different bone status groups: HGF, BMP9 and GDF15. BMP9 levels were higher in the group of patients with limited bone disease compared with the group without bone disease, but there was no significance difference between the group with advanced bone disease compared with the ones without bone disease. The data on BMP9 (and Endoglin) has been previously published. (1) HGF was significantly higher in the group with advanced bone disease compared with the ones without bone disease, and significantly different between the group with osteopenia and the group without bone disease. (Table1). GDF15 results are presented in the main text.

**Table 1.** Median values of HGF and BMP9 in patients grouped according to skeletal disease. \*  $p < 0.05$ , \*\*  $p < 0.01$  as compared with "no lesion" group, Dunn's multiple comparisons test.

	No lesions	Limited	Advanced	Osteoporosis
HGF	362 pg/ml	637 pg/ml	816 pg/ml *	867 pg/ml *
BMP9	127 pg/ml	234 pg/ml **	161 pg/ml	237 pg/ml

## **Patient samples**

Patient serum samples were collected for the Nordic Myeloma Study Group during a randomized phase 3 clinical trial on pamidronate dosage in the years 2001–2005. Serum samples obtained at diagnosis were available from 138 patients, and these samples were included in the analyses. At inclusions the patients were defined as having skeletal disease according to the following criteria: advanced osteolytic bone disease ( $> 3$  osteolytic lesions,  $n= 51$ ), limited bone disease ( $\leq 3$  osteolytic lesions,  $n=51$ ), osteoporosis but no lesions ( $n=14$ ) and no bone disease ( $n=16$ ). Skeletal related events (SRE) were evaluated every third month and were defined as pathological fractures, radiotherapy or surgery to bone, new vertebral compression, symptomatic new or progressive osteolytic lesions and hypercalcemia.<sup>(2,3)</sup> Of the 138 patients, skeletal status was available at diagnosis for 132 patients. For comparison, serum was collected from 58 age- and gender-matched healthy volunteers. To examine levels of GDF15 in bone marrow versus blood serum, paired serum and bone marrow plasma samples ( $n=16$ ) were obtained from the Norwegian Myeloma Biobank, St. Olavs Hospital, Trondheim.

## **Serum assays**

GDF15, OPG and DKK1 were measured using a multiplex assay (The MILLIPLEX MAP Human Cancer/Metastasis Biomarker Panel 1, HCMBMAG-22K, Millipore Corporation, Billerica, MA, USA). BMP-9, Endoglin and Follistatin by MILLIPLEX MAP Human Angiogenesis/Growth Factor Magnetic Bead Panel - Cancer Multiplex Assay, Millipore). IL-1b, IL-5, IL-6, IL-17, IP-10, MCP-1, MIP-1a, MIP-1b, Eotaxin, G-CSF, GM-CSF, HGF, Leptin and VEGF were measured by a customized Bio-Plex Pro Assay, Bio-Rad. NRP1 were measured by ELISA (R&D, Minneapolis, MN, USA and Usen Life Science Inc, Houston, TX, USA). Markers for osteoclast activity (CTX1 and ICTP) and markers for osteoblast activity (bALP and PINP) were analyzed as previously described<sup>(3)</sup>.

## **Recombinant proteins**

MCSF, RANKL, OPG and GDF15 were obtained from R& D Systems (Minneapolis, MN, USA). All recombinant proteins were reconstituted and stored according to the manufacturer's instructions. In particular, GDF15 was reconstituted in 4mM HCL with 0.1% BSA, aliquoted and stored at  $-80^{\circ}\text{C}$  until used.

## **Osteoclast differentiation**

Isolation of PBMC from buffy coats obtained from the Blood bank, St. Olavs Hospital, Trondheim, was performed with density gradient (Lymphoprep<sup>TM</sup>, Axis-Shield PoC, Oslo, Norway). The mononuclear cells were incubated for 1 hour before non-adherent cells were washed away (3x) using Hanks Buffered Salt Solution (HBSS, Sigma-Aldrich, St Louis, MO). Isolated monocytes ( $n=3$  donors) were seeded  $1 \times 10^5$  cells/ml and cultured in  $\alpha$ MEM (without phenol red) with human serum (20%) and M-CSF (30 ng/ml) for two days. At that point RANKL (50 ng/ml), OPG (200 ng/ml) and GDF15 (5-200 ng/ml) were added (all R&D Systems). Medium was renewed every 4-5 days. Human pre-osteoclast precursors purchased from Lonza ( $n=2$ , Lonza Inc, Walkersville, MD, USA) and cultured according to the

manufacturer's instructions. To induce differentiation, M-CSF (33ng/ml) and RANKL (66ng/ml) with/without OPG (200ng/ml) and GDF15 (0.5 -100 ng/ml ) were added. Medium was renewed after 3 days.

### **Osteoblast differentiation**

Human mesenchymal stem cells (n=4, Lot numbers 0000318006, 0000307219, 0000351482 and 000374385) were purchased from Lonza and cultured in MSC growth medium (MSCGM, Lonza) and cultured at 37°C with 5% CO<sub>2</sub>. MSC were used before reaching passage 6 and were subcultivated at 80 % confluence. For differentiation towards osteoblasts, MSC were cultured in MSCGM containing ascorbic acid (50 uM), dexamethasone (10<sup>-8</sup>M) and β-glycerol phosphate (10 mM). The osteogenic medium was changed every 4-5 days, and the cells were differentiated for up to 17 days as indicated in the figure legends.

### **ALP assay**

Alkaline phosphatase (ALP) activity was quantified by ELF 97 Endogenous Phosphatase Detection Kit (Molecular Probes, Eugene, OR) as previously described (4) Briefly, MSCs were seeded at 3000 cells/well in 96-well plates, cultured for 7 days as indicated before fixation in 4% para-formaldehyde for 10 minutes at room temperature. To permeabilize the cells, 200 μL/well of phosphate-buffered saline (PBS)/0.2% Tween-20 was added and the plates were incubated for 15 minutes. The cells were briefly rinsed twice in water, and then left for 10 minutes in H<sub>2</sub>O. Substrate was diluted 1:20 in substrate buffer, 50 μL/well, and incubated for 5 minutes. Then, fluorescence was detected using a multilabel counter (Viktor 1420; Perkin Elmer, Wellesley, MA) with excitation filter at 355 nm and emission filter at 535 nm. To adjust for differences in cell number, cells were subsequently lysed in 100 μL 10% SDS and DNA were quantified using SybrGreen (Molecular Probes). All samples were run in triplicates.

### **Alizarin Red staining**

hMSCs were seeded at 9 000 cells/well in 24-well plates and cultured for 17 days. Media were renewed every 3 to 4 days. Cultures were terminated by fixing cells in 3-4 % para-formaldehyde for 10 minutes on ice. Fixed cultures were rinsed with PBS and then with water, thereafter stained with 500 μL/well 40 mM Alizarin Red-S (ARS; Sigma-Aldrich) at pH 4.2 and room temperature with gentle agitation. Cells were then washed 5 times with water followed by a 15-minute rinse with PBS under gentle rotation. Stained cultures were photographed, then destained by incubating in 10% (wt/vol) cetylpyridinium chloride (CPC) in 10 mM sodium phosphate buffer pH 7.0 for 1 hour at room temperature. ARS concentration in these extracts was determined by absorbance at 562 nm. A standard curve was obtained by diluting ARS in CPC.

### **TRAP staining**

TRAP staining was performed using the Tartrate-Resistant Acid Phosphatase, Leukocyte (*TRAP*) Kit (Sigma-Aldrich) according to the manufactures instructions, with the following modifications: Cells were fixed using glutaraldehyde (2.5%) and the samples were incubated in the staining solution for 2 hours for PBMC derived osteoclasts, and 1 hours for pre-osteoclasts. TRAP positive multinuclear cells with  $\geq 3$  nuclei were counted as osteoclasts. All samples were run in triplicates.

## PCR

For RNA isolation the High Pure Isolation Kit (Roche Applied Science, Indianapolis, IN) was used. Samples were frozen at  $-80^{\circ}\text{C}$  until used. For determination of RNA concentration and RNA quality the NanoDrop Spectrophotometer (ND-1000 Spectrophotometer, NanoDrop Technologies Inc.) was employed. For cDNA synthesis the High Capacity RNA-to-cDNA Kit (Applied Biosystems) was utilized. RT-PCR analysis was performed using TaqMan Gene Expression Array (Applied Biosystems). The comparative Ct method was used to estimate relative changes in gene expression using GAPDH as housekeeping gene. The following primers were used *RUNX2* (Hs00231692\_m1, lot 959159), *BGLAP* (Hs01587814\_g1, lot 969519) *COL1A1* (Hs 00164004\_m1, lot 963537). The housekeeping gene glyceraldehydes-3-phosphate dehydrogenase (GAPDH, Hs99999905\_m1, lot 853053) was used as an endogenous control. Genes with a Ct value  $\geq 35$  were considered as not detected. The Applied Step One Software 2.1 was used to analyze the samples.

## Statistics

Statistical analyses were performed with the SPSSX/PC computer program (SPSS, Chicago, IL, USA) or Prism version 6.0d (GraphPad Software, La Jolla, CA, USA). Independent samples Kruskal-Wallis test were performed to identify cytokines differently expressed between patients and controls. For this analysis, due to multiple comparisons, results were considered statistically significant when  $p < .002$ . Cytokines that were differently expressed between the two groups were further tested to find out whether they varied according to skeletal status. Kruskal-Wallis test followed by Dunn's multiple comparison test was used to test for significant differences in cytokine levels between the different skeletal disease groups. Spearman's test was used to test for correlations and Kaplan-Meier analyses followed by log-rank tests was used to test for differences in time to SRE or overall survival.

## References

1. Olsen OE, Wader KF, Misund K, Vatsveen TK, Ro TB, Mylin AK, et al. Bone morphogenetic protein-9 suppresses growth of myeloma cells by signaling through ALK2 but is inhibited by endoglin. *Blood cancer journal*. 2014;4:e196.
2. Gimsing P, Carlson K, Turesson I, Fayers P, Waage A, Vangsted A, et al. Effect of pamidronate 30 mg versus 90 mg on physical function in patients with newly diagnosed multiple myeloma (Nordic Myeloma Study Group): a double-blind, randomised controlled trial. *The Lancet Oncology*. 2010 Oct;11(10):973-82.
3. Mylin AK, Abildgaard N, Johansen JS, Heickendorff L, Kreiner S, Waage A, et al. Serum YKL-40: a new independent prognostic marker for skeletal complications in patients with multiple myeloma. *Leukemia & lymphoma*. 2015 Feb 11:1-10.

4. Standal T, Abildgaard N, Fagerli UM, Stordal B, Hjertner O, Borset M, et al. HGF inhibits BMP-induced osteoblastogenesis: possible implications for the bone disease of multiple myeloma. *Blood*. 2007;109(7):3024-30.