タイトル	Glucocorticoid therapy causes contradictory changes of serum Wnt signaling-related molecules in systemic autoimmune diseases
別タイトル	膠原病患者においてWnt シグナル関連因子はステロイド治療により矛盾した変動をとる
作成者（著者）	川添, 麻衣
公開者	東邦大学
発行日	2018.03.14
掲載情報	東邦大学大学院医学研究科 博士論文. 15.
資料種別	学位論文
内容記述	主査 : 龍野一郎 / タイトル : Glucocorticoid therapy causes contradictory changes of serum Wnt signaling-related molecules in systemic autoimmune diseases / 著者 : Mai Kawazoe, Kaichi Kaneko, Kotaro Shikano, Natsuko Kusunoki, Toshihiro Nanki, Shinichi Kawai / 掲載誌 : Clinical Rheumatology / 巻号・発行年等 : 37(8):2169-2178, 2018 / 本文ファイル: 査読後原稿 / The final publication is available at Springer via http://dx.doi.org/10.1007/s10067-017-3689-3
著者版フラグ	ETD
報告番号	32661甲第863号
学位記番号	甲第586号
学位授与年月日	2018.03.14
学位授与機関	東邦大学
DOI	10.1007/s10067-017-3689-3
その他資源識別子	https://link.springer.com/article/10.1007/s10067-017-3689-3
メタデータのＵＲＬ	https://mylibrary.toho-u.ac.jp/webopac/TD74132625

Glucocorticoid therapy causes contradictory changes of serum Wnt signaling-related molecules in systemic autoimmune diseases

Mai Kawazoe [1,2], Kaichi Kaneko [2], Kotaro Shikano [2], Natsuko Kusunoki [2], Toshihiro Nanki [1,2], and Shinichi Kawai [3]

[1] Department of Internal Medicine, Toho University Graduate School of Medicine

[2] Division of Rheumatology, Department of Internal Medicine, Toho University School of Medicine

[3]Department of Inflammation & Pain Control Research, Toho University School of Medicine

Corresponding author: Shinichi Kawai, MD, PhD

Department of Inflammation & Pain Control Research, Toho University School of Medicine

5-21-16 Omori-Nishi, Ota-ku, Tokyo 143-8540, Japan

Phone: +81-3-3762-4151 (ext 2762), Fax: +81-3-5753-8513

e-mail: skawai@med.toho-u.ac.jp

Word count: 3415

Numbers of figures and tables: 6

19

20 Acknowledgements and Funding Information: We thank Sonoko Sakurai for secretarial assistance.

21 This study was partly supported by a Grant from the Strategic Research Foundation Project for Private

22 Schools in Heisei 23 (S1101016) from the Ministry of Education, Culture, Sports, Science and

23 Technology of Japan (2011-2015) to Toho University and a Grant from the Japan Agency for Medical

24 Research and Development on Regulatory Science of Pharmaceuticals and Medical Devices (2015-

25 2016) to SK.

Abstract

Objective: The objective of this study was to investigate the clinical significance of the Wnt/β-catenin signaling pathway in glucocorticoid-induced osteoporosis.

Methods: A total of 91 patients with systemic autoimmune diseases who received initial glucocorticoid therapy with prednisolone (30-60 mg daily) were prospectively enrolled. We measured serum levels of N-terminal peptide of type I procollagen (P1NP), bone alkaline phosphatase (BAP), tartrate-resistant acid phosphatase isoform 5b (TRACP-5b), N-telopeptide cross-linked type I collagen (NTX), sclerostin, Dickkopf-1 (Dkk-1), and Wnt3a before starting glucocorticoid therapy and every week for four weeks after its initiation. The effects of dexamethasone on expression of mRNA and protein of sclerostin and Dkk-1 by cultured normal human osteoblasts (NHOst) were evaluated by RT-PCR and ELISA, respectively.

Results: Serum levels of sclerostin and Dkk-1 increased significantly by one week of glucocorticoid therapy and then decreased from the second week onward. Serum Wnt3a tended to decrease and serum P1NP showed a significant decrease. However, TRACP-5b was significantly elevated from the first week of treatment onwards. *In vitro* study, dexamethasone increased Dkk-1 mRNA expression in cultured NHOst, but sclerostin mRNA was not detected. Dexamethasone also increased Dkk-1 protein production by osteoblasts, whereas sclerostin protein was not detected.

Conclusion: Bone formation might be impaired at least in the first week of the initiation of

44 glucocorticoid therapy by increase of the serum Wnt signaling inhibitors, however, their reductions in

45 the subsequent weeks were contradictory to the maintained suppression of the bone formation markers

46 after glucocorticoid therapy for patients with systemic autoimmune diseases.

47

48 **Keywords**: glucocorticoid, osteoporosis, sclerostin, Dickkopf-1, Wnt signaling

Introduction

Glucocorticoids are widely used to treat a variety of diseases, including systemic autoimmune diseases. Although glucocorticoids can improve the outcome of patients with these diseases, side effects of long-term treatment such as osteoporosis are important problems [1, 2]. Fractures tend to occur at a higher bone mineral density in glucocorticoid-treated patients than in patients with primary osteoporosis [3], and vertebral fracture may occur soon after exposure to glucocorticoids [4]. It has been reported that glucocorticoids decrease bone density by multiple mechanisms involving both inhibition of bone formation and enhancement of bone resorption. One of the mechanisms by which glucocorticoids suppress bone formation is *via* Wnt/β-catenin signaling [5-7].

The Wnt/β-catenin signaling pathway is one of the three major Wnt signaling pathways, and is the best characterized of these pathways. It plays a key role in regulating the differentiation and proliferation of osteoblasts. Binding of Wnt ligands to a specific receptor and its co-receptors is required for activation of the Wnt pathway, whereas this pathway is inactivated by binding to Wnt co-receptors of sclerostin and Dkk-1, which are negative regulators of Wnt signaling [5, 8-13]. However, the detailed changes of Wnt signaling in patients with glucocorticoid-induced osteoporosis have not been clarified.

Therefore, the aim of the present study was to investigate the clinical significance of the Wnt signaling pathway in patients receiving initial glucocorticoid therapy for autoimmune diseases by

67 measuring serum levels of three Wnt signaling-related molecules (sclerostin, Dkk-1, and Wnt3a).
68 Bone turnover markers and bone mineral density (BMD) were also measured in these patients.
69 Furthermore, we performed an *in vitro* study to assess the effects of glucocorticoids on primary
70 cultured human osteoblasts.

Patients and Methods

Patients and study protocol

A prospective observational study was conducted in 91 patients with systemic autoimmune diseases, comprising 29 patients with polymyositis/dermatomyositis (PM/DM), 28 patients with systemic lupus erythematosus (SLE), 24 patients with vasculitis syndrome, 6 patients with adult onset Still's disease (AOSD), 2 patients with systemic sclerosis (SSc), 1 patient with Sjögren's syndrome (SS), and 1 patient with IgG4-related disease (Table 1). The subjects were recruited at Toho University Omori Hospital. All patients who commenced glucocorticoid therapy with prednisolone at doses from 30 to 60 mg daily [mean daily dose: 45.9 ± 1.0 mg (the mean ± standard error of the mean (SEM))] according to our standard therapeutic regimens were included in this study. Starting dose was given to all patients for two weeks, and the dose was tapered by 5 mg/day every one or two weeks from an initial dose.

In the Japanese Society for Bone and Mineral Research guidelines on management and treatment of glucocorticoid-induced osteoporosis, bisphosphonates are recommended as first-line therapy for prevention of glucocorticoid-induced osteoporosis [14]. Accordingly, all of the patients treated with alendronate at the regular dose of 35 mg/week (Bonaron® 35 mg; Teijin Pharma Ltd., Tokyo, Japan) or risedronate at the regular dose of 17.5 mg/week (Actonel® 17.5 mg; Eisai Co., Ltd., Tokyo, Japan), both approved by Japanese Government, throughout this study.

Fasting morning blood samples were collected prospectively just before initiation of glucocorticoid

89 therapy and after the first, second, third, and fourth weeks of treatment. Serum samples were
90 immediately frozen at –80 °C until measurement of bone metabolism markers.
91
92 *Serum biochemical markers*
93 Serum levels of sclerostin (Biomedica, Vienna, Austria), Dkk-1 (Biomedica), and Wnt3a
94 (MyBioSource, San Diego, CA, U.S.A.) were determined with enzyme-linked immunosorbent assay
95 (ELISA) kits according to the manufacturer's instructions. As bone formation markers, the serum
96 levels of N-terminal peptide of type I procollagen (P1NP; Orion Diagnostica, Espoo, Finland) and
97 bone alkaline phosphatase (BAP; Quidel, San Diego, CA, U.S.A.) were assessed by
98 immunoradiometric assay and ELISA, respectively. As bone resorption markers, the serum levels of
99 N-telopeptide cross-linked type I collagen (NTX; Inverness, Princeton, NJ, U.S.A.) and tartrate-
100 resistant acid phosphatase isoform 5b (TRACP-5b, DS Pharma Biomedical Co., Ltd., Tokyo, Japan)
101 were measured by ELISA. As inflammatory markers, serum level of C-reactive protein (CRP, Sekisui
102 Medical, Tokyo, Japan) was measured by latex-enhanced nephelometry.
103
104 *Measurement of BMD*
105 Before starting glucocorticoid therapy, patients underwent measurement of BMD of the lumbar
106 spine (L2-4) by dual-energy X-ray absorptiometry using Discovery A (Hologic, Waltham, MA, U.S.A),

which automatically calculated BMD from the bone area (cm^2) and bone mineral content (g) and expressed the result in g/cm^2. BMD was measured again after 15.6 ± 1.4 months (the mean ± SEM) of glucocorticoid treatment and the change of BMD was calculated.

Cell culture

Normal human osteoblasts (NHOst) originating from a 2-year-old boy were obtained from Lonza Inc. (Williamsport, PA, U.S.A.), and were maintained at 37 °C in osteoblast growth medium (Lonza Inc.) supplemented with 10 % fetal bovine serum, 50 μg/mL gentamycin sulphate, 2.5 μg/mL amphotericin-B, and 50 mM L-ascorbic acid under a humidified atmosphere of 5 % CO_2 in air. Then the cells were resuspended in 5 mL of osteoblast growth medium supplemented with 1 % (v/v) fetal bovine serum at 1.0×10^5 cells/mL cultured in 35-mm dishes. Cells between the 2nd and 3rd passages were used for all experiments.

Expression of mRNA and protein of sclerostin and Dkk-1

NHOst were incubated for 6 hours with or without dexamethasone (0, 10^{-8}, 10^{-7}, or 10^{-6} M) (Wako, Osaka, Japan), and RNA was extracted by using an RNeasy Mini kit (Qiagen GmbH, Hilden, Germany). Then the reverse transcription polymerase chain reaction (RT-PCR) was performed with a SuperScript first-strand synthesis system (Invitrogen Corporation, Carlsbad, CA, U.S.A.) using 1 μg

125 of total cellular RNA as the template. Equal amounts of the reverse-transcribed products were
126 amplified by the polymerase chain reaction (PCR) with HotStarTaq polymerase (Qiagen GmbH) and
127 the following primers: sclerostin (sense 5'-CCGGAGCTGGAGAACAACAAG-3' and antisense 5'-
128 GCACTGGCCGGAGCACACC-3'), Dkk-1 (sense 5'-TGATGAGTACTGCGCTAGTC-3' and
129 antisense 5'-CTCCTATGCTTGGTACACAC-3'), and β-actin as the endogenous control (sense 5'-
130 CCTCGCCTTTGCCGATCC-3' and antisense 5'-GGATCTTCATGAGGTAGTCAGTC-3'). For
131 amplification of sclerostin (186 bp) and Dkk-1 cDNA (396 bp), PCR was done with 38 cycles of 94
132 °C for 30 sec, 55 °C for 30 sec, and 72 °C for 30 sec, while 28 cycles under the same conditions
133 were employed to amplify β-actin cDNA (626 bp). The amplified cDNA fragments were resolved by
134 electrophoresis on 2 % (w/v) agarose gel, and were detected under UV light using an LAS-3000
135 (Fujifilm Corp, Tokyo, Japan) after the gel was stained with ethidium bromide.
136 To evaluate the effect of dexamethasone on production of sclerostin and Dkk-1 by NHOst, the
137 cells were cultured for 24 hours under various conditions in medium containing 1 % (v/v) fetal
138 bovine serum. Then culture supernatants were collected, centrifuged, and stored at –80 °C until
139 analysis. The concentrations of sclerostin (Biomedica) and Dkk-1 (R&D System Inc., Minneapolis,
140 MN, U.S.A.) in the thawed culture supernatants were measured in duplicate by ELISA according to
141 the manufacturer's instructions.
142

Statistical analysis

Stastical analysis was performed with Prism ver. 5.0 software (Graphpad Software, San Diego, CA, U.S.A.). Numerical data were expressed as the mean ± SEM. Dunnett's multiple comparison test and paired *t*-test were used to assess differences of continuous variables. To compare two groups, the Mann-Whitney *U* test was applied for numerical data and the Fisher test was used for categorical data. We used the Kruskal-Wallis test to compare three groups. The level of significance was set at $P < 0.05$ in all analyses.

Results

Serum levels of sclerostin, Dkk-1, and Wnt3a

The serum sclerostin level increased significantly ($P < 0.05$) by one week after starting glucocorticoid therapy in comparison to before therapy, but then showed a significant decrease compared to before therapy in the third week and was even lower in the fourth week (Fig. 1A). Similarly, the serum Dkk-1 level showed a significant increase ($P < 0.01$) by one week, but was significantly lower than before therapy from the third week onward (Fig. 1B). As shown in Fig. 1C, the serum Wnt3a level tended to decrease from the first week onwards, but did not change significantly throughout the study period.

It is known that serum CRP tends to be low in patients with SLE [15] and PM/DM [16] regardless of disease activity, the diseases of our patients could be divided into inflammatory diseases (vasculitis syndrome [17, 18] and AOSD [19], n=30) and non-inflammatory diseases (SLE and PM/DM, n=57). In fact, serum CRP was lower in non-inflammatory diseases [inflammatory diseases: 7.1±1.0 mg/dL (mean±SEM), non-inflammatory diseases: 1.8±0.3 mg/dL, respectively, $P < 0.0001$]. We compared the changes of serum Wnt signaling-related molecules during glucocorticoid therapy between these two groups, and each molecules showed a similar change (Fig. 1D, E and F, respectively).

We divided the patients into two groups, whose serum Wnt3a level was over the 75th percentile (0.51-1.26 ng/mL, High Wnt3a group) and less than the 75th percentile (0-0.49 ng/mL, Low Wnt3a

group) of the cohort. There were no significant differences of baseline characteristics between the High Wnt3a group (n=22) and the Low Wnt3a group (n=69), including the age, sex, percentage of postmenopausal women, mean daily dose of prednisolone, and distribution of underlying diseases (Table 1). There were also no differences in baseline serum levels of bone metabolism markers, except for Dkk-1 and Wnt3a. As shown in Fig. 2 (A and B), the mean serum Wnt3a level was significantly decreased by glucocorticoid therapy in the High Wnt3a group, whereas it did not change significantly throughout the study in the Low Wnt3a group. On the other hand, there were no significant differences between these two groups with regard to the changes of serum sclerostin (Figs. 2C, D) and Dkk-1 (Figs. 2E and F).

Bone turnover markers

As serum bone formation markers, the P1NP level decreased significantly in the first week and then remained low, whereas the serum BAP level did not change throughout this study (Fig. 3A, B). As the serum bone resorption markers, the TRACP-5b level increased significantly in the first week and then remained high, while the serum NTX level increased significantly relative to baseline only in the first week (Fig. 3C, D).

Correlations between Wnt signaling-related molecules and bone turnover markers

We examined the correlations between serum Wnt signaling-related molecules (sclerostin, Dkk-1, Wnt3a) and bone turnover markers (P1NP, BAP, TRACP-5b, NTX) before initiation of glucocorticoid therapy. Univariate analysis revealed that only serum Dkk-1 was positively correlated with serum P1NP level ($R= 0.27$, $p< 0.01$). Neither serum sclerostin nor Wnt3a was correlated with any serum bone turnover markers.

Changes of BMD

Seventy-nine patients underwent measurement of BMD. Nine patients were withdrawn due to death (n=5) or hospital transfer (n=4). Seventy-eight patients continued to receive prednisolone at 15.6 months except for a patient with SS. The mean BMD of the patients decreased from 0.96 g/cm^2 to 0.93 g/cm^2 after they received a mean of 15.6 months of glucocorticoid treatment. The change of BMD in the High Wnt3a group [median (25th to 75th percentile range): -0.03 (-0.06 to 0.01)] was not significantly different ($P = 0.11$) from that in the Low Wnt3a group [0.02 (-0.01 to 0.03)].

Fracture rate

The new fracture rate was 1.0 % (1/79 patients) during glucocorticoid therapy for a mean of 15.6 months. The patient with a lumbar compression fracture was a 75-year-old postmenopausal woman with vasculitis syndrome taking prednisolone (initial dose: 30 mg/day) and alendronate (35 mg/week).

Effect of dexamethasone on cultured NHOst

We also investigated the effect of dexamethasone on sclerostin and Dkk-1 mRNA expression by cultured NHOst. As shown in Fig. 4, Dkk-1 mRNA expression was increased by addition of dexamethasone to the culture medium for 6 hours, whereas sclerostin mRNA was undetectable.

In addition, the Dkk-1 protein level in the culture medium was increased by addition of dexamethasone for 24 hours (Fig. 5), but sclerostin protein was not detected in the NHOst culture medium by ELISA.

Discussion

The present study demonstrated that the serum levels of sclerostin and Dkk-1 were increased significantly after one week of glucocorticoid therapy, while serum Wnt3a tended to decrease. These findings suggest that the decrease of Wnt3a and the increase of sclerostin and Dkk-1 in the early phase of glucocorticoid therapy suppressed Wnt signaling, which may result in impairment of bone formation. To our knowledge, this is the first prospective observational investigation of changes in the serum levels of Wnt signaling pathway antagonists and a ligand from before to after initiation of glucocorticoid therapy.

There have been a few previous reports about the effects of glucocorticoids on Wnt pathway antagonists, but the findings differed from our results. Brabnikova et al. [20] reported a significant reduction of the serum sclerostin level and an increase of serum Dkk-1 after 96 hours of glucocorticoid treatment in 17 patients with chronic rheumatic diseases. On the other hand, the serum sclerostin level was increased and serum Dkk-1 was decreased at 12 months compared with approximately 50 days after initiating glucocorticoid therapy in 25 patients with hematologic disorders [21]. However, neither of these studies investigated sclerostin and Dkk-1 levels before glucocorticoid therapy.

We also performed an *in vitro* study, which demonstrated that dexamethasone increased Dkk-1 mRNA expression in NHOst, as previously reported by Ohnaka et al. [22]. In addition, we found an increase of the Dkk-1 protein level in the culture medium after incubation of NHOst with

dexamethasone. The increase of Dkk-1 mRNA and protein after incubating these cells with dexamethasone might explain why glucocorticoid therapy increased the serum Dkk-1 concentration after 1 week in our clinical study. On the other hand, sclerostin mRNA and protein were undetectable in cultured NHOst. This finding is consistent with the reports that sclerostin is exclusively expressed by osteocytes and differentiated osteoblasts [23, 24].

Serum changes of bone formation markers indicate that suppression of Wnt signaling in the early phase of glucocorticoid therapy results in impairment of bone formation. The present study showed that, among serum bone formation markers, the serum P1NP level was significantly suppressed in the first week. Serum P1NP was also suppressed for the entire 4-week study period after initiation of glucocorticoid therapy, in agreement with the results of previous studies [25, 26]. On the other hand, the serum BAP level did not change. BAP has a longer half-life, and also no glucocorticoid-sensitive elements have been found in the BAP gene [27, 28]. These reports may explain the difference in the changes between P1NP and BAP.

We observed that serum levels of sclerostin and Dkk-1 were decreased from the third week onward, but were increased after one week. It is not clear why sclerostin and Dkk-1 decreased over time. Another mechanism such as accelerated differentiation of osteoblast precursors to adipocytes is likely to be involved in the inhibition of bone formation during long-term glucocorticoid therapy [29]. It has also been suggested that sclerostin [30] and Dkk-1 [31] enhance the apoptosis of osteoblasts. While

248 we did not examine these effects, reduction of the serum levels of sclerostin and Dkk-1 from the third
249 week of glucocorticoid therapy could possibly be explained by a decrease of osteoblasts in response
250 to elevation of sclerostin and Dkk-1 in the first week.

251 In the present study, serum Dkk-1 level had a positive correlation with serum P1NP. Dovjak et al.
252 [32] reported a positive correlation between serum Dkk-1 and BAP levels in young healthy controls.
253 These results suggest that increased Dkk-1 was associated with increased bone formation markers,
254 P1NP or BAP. The reasons for these contradictorily correlations remain to be studied.

255 We stratified the subjects into two groups according to the baseline serum Wnt3a level, and found
256 that the serum Wnt3a level of the High Wnt3a group, but not the Low Wnt3a group, was significantly
257 decreased by glucocorticoid therapy. The change of BMD after initiation of glucocorticoid therapy in
258 the High Wnt3a group have a trend to decrease in comparison to Low Wnt3a group, therefore a
259 decrease of the serum Wnt3a level might lead to a trend of BMD reduction. On the other hand, the
260 changes in serum sclerostin and Dkk-1 of these two groups were similar. In this study, the baseline
261 serum Dkk-1 level of the High Wnt3a group was lower. Recent studies have indicated that serum Dkk-
262 1 is increased by inflammation [33], but there were not significant differences in serum CRP and
263 distribution of diseases between these two groups. There were also no statistically significant
264 differences of the baseline and the changes in serum Wnt signaling-related molecules during
265 glucocorticoid therapy between inflammatory and non-inflammatory diseases. This implies that the

grade of inflammation and difference of diseases has no influence on Wnt signaling-related molecules.

In our study, we chose Wnt3a among several Wnt molecules. Wnt3a has been known as the ligand of Wnt/β-catenin signaling pathway and shown to stimulate the proliferation of osteoblasts [34]. Wnt10b has also been shown as the ligand of Wnt/β-catenin signaling pathway. Studies using mice revealed that Wnt10b enhance osteoblast differentiation and promotes bone formation [35, 36]. We tried to measure Wnt10b level in blood samples of fifteen patients, but serum Wnt10b was hardly detectable with an ELISA kit (data not shown).

All of our patients were concomitantly treated with bisphosphonates during glucocorticoid therapy. These drugs generally suppress serum levels of bone resorption markers in patients with postmenopausal osteoporosis [37, 38]. However, we observed a significant persistent increase of serum TRACP-5b and a significant increase of serum NTX in the first week. This finding suggests that high-dose glucocorticoid therapy has a strong effect on bone turnover and can counteract the suppression of bone resorption by bisphosphonates. In addition, our study showed that the mean BMD of the patients decreased after they received a mean of 15.6 months of glucocorticoid therapy. Hoes at al. [39] reported that patients with rheumatic diseases had a higher risk of vertebral fractures in spite of coadministration of glucocorticoids with alendronate. Thus, bisphosphonate monotherapy may be insufficient for the prevention and treatment of glucocorticoid-induced osteoporosis.

In this study, we could not clarify the effects of bisphosphonates themselves on Wnt signaling-

284 related molecules. It was reported that bisphosphonate monotherapy for more than 1 year did not affect
285 the serum sclerostin level in women with postmenopausal osteoporosis [40], while another study
286 showed that the serum level of sclerostin increased gradually and serum Dkk-1 did not change during
287 bisphosphonate treatment [41]. Thus, it is suggested that bisphosphonates themselves do not have an
288 obvious influence on inhibitors of Wnt/β-catenin signaling pathway.

289 Recent studies have shown that treatment with antibody against sclerostin increases trabecular bone
290 mass and cortical bone mass in postmenopausal women [42]. Antibody against Dkk-1 has a similar
291 effect in various animal models and is under development for clinical use as a bone anabolic agent [8].
292 The effect of sclerostin-antibody treatment in glucocorticoid-induced osteoporosis in mice was also
293 recently reported [43-45]. According to our findings, antibodies targeting sclerostin and Dkk-1 might
294 be an attractive therapeutic option for preventing glucocorticoid-induced bone loss, as well as in
295 human, if they are initiated as early as possible after the start of glucocorticoid therapy.

296 This study however has several limitations. First, we observed serum biochemical markers only for
297 four weeks' duration of glucocorticoid therapy and there are no subsequent data. Second, we measured
298 sclerostin, Dkk-1 and Wnt3a, but there are some other Wnt signaling-related molecules. Third, our
299 patients were comprised of various systemic autoimmune diseases. Although we analyzed the
300 differences between inflammatory diseases and non-inflammatory diseases, the studies in an
301 individual disease remain to be examined. Furthermore, we cannot rule out the effects of

bisphosphonates on changes of these biochemical markers since there was no control group because of ethical reasons.

In conclusion, the results of the present study suggest that bone formation may be impaired by the increases of the serum Wnt signaling inhibitors at least at the first week of the initiation of glucocorticoid therapy. Although the mechanisms of the significant reduction of the serum Wnt signaling inhibitors in the subsequent weeks remain to be studied, their changes were contradictory to the maintained suppression of the bone formation markers after glucocorticoid therapy for patients with systemic autoimmune diseases.

Ethical standards

This study was approved by the Ethics Committees of Toho University Omori Medical Center (approval number; 24-78, 25-215) and complied with the 1964 Declaration of Helsinki and its later amendments, and Ethical Guidelines for Medical and Health Research Involving Human Subjects by Ministries of Education, Culture, Sports, Science and Technology and Health, Labour and Welfare of the Japanese Government. All of the subjects gave written informed consent before enrollment.

Conflict of Interest

SK is affiliated with Department of Inflammation & Pain Control Research which is sponsored by Chugai Pharmaceutical Co., Ltd., Nippon Zoki Pharmaceutical Co., Ltd., Ono Pharmaceutical Co., Ltd., Ayumi Pharmaceutical Corporation, Eisai Co., Ltd., Hisamitsu Pharmaceutical Co., Inc., Japan Tobacco Inc., and Nippon Kayaku Co., Ltd. The other authors declare they have no conflicts of interest.

References

1. Van Staa TP, Leufkens HG, Abenhaim L, Zhang B, Cooper C (2000) Use of oral corticosteroids and risk of fractures. J Bone Miner Res 15: 993-1000. doi: 10.1359/jbmr.2000.15.6.993

2. Weinstein RS (2011) Clinical practice. Glucocorticoid-induced bone disease. N Engl J Med 365: 62-70. doi: 10.1056/NEJMcp1012926

3. Nawata H, Soen S, Takayanagi R, Tanaka I, Takaoka K, Fukunaga M, Matsumoto T, Suzuki Y, Tanaka H, Fujiwara S, Miki T, Sagawa A, Nishizawa Y, Seino Y (2005) Guidelines on the management and treatment of glucocorticoid-induced osteoporosis of the Japanese Society for Bone and Mineral Research (2004). J Bone Miner Metab 23: 105-109. doi: 10.1007/s00774-004-0596-x

4. Van Staa TP, Leufkens HG, Cooper C (2002) The epidemiology of corticosteroid-induced osteoporosis: a meta-analysis. Osteoporos Int 13: 777-787. doi: 10.1007/s001980200108

5. Canalis E, Mazziotti G, Giustina A, Bilezikian JP (2007) Glucocorticoid-induced osteoporosis: pathophysiology and therapy. Osteoporos Int 18: 1319-1328. doi: 10.1007/s00198-007-0394-0

6. Seibel MJ, Cooper MS, Zhou H (2013) Glucocorticoid-induced osteoporosis: mechanisms, management, and future perspectives. Lancet Diabetes Endocrinol 1: 59-70. doi: 10.1016/S2213-8587(13)70045-7

7. Guañabens N, Gifre L, Peris P (2014) The role of Wnt signaling and sclerostin in the pathogenesis

340 of glucocorticoid-induced osteoporosis. Curr Osteoporos Rep 12: 90-97. doi: 10.1007/s11914-
341 014-0197-0
342 8. Canalis E (2013) Wnt signalling in osteoporosis: mechanisms and novel therapeutic approaches.
343 Nat Rev Endocrinol 9: 575-583. doi: 10.1038/nrendo.2013.154
344 9. Baron R, Kneissel M (2013) WNT signaling in bone homeostasis and disease: from human
345 mutations to treatments. Nat Med 19: 179-192. doi: 10.1038/nm.3074
346 10. Monroe DG, McGee-Lawrence ME, Oursler MJ, Westendorf JJ (2012) Update on Wnt signaling
347 in bone cell biology and bone disease. Gene 492: 1-18. doi: 10.1016/j.gene.2011.10.044
348 11. Rossini M, Gatti D, Adami S (2013) Involvement of WNT/β-catenin signaling in the treatment of
349 osteoporosis. Calcif Tissue Int 93: 121-132. doi: 10.1007/s00223-013-9749-z
350 12. Ke HZ, Richards WG, Li X, Ominsky MS (2012) Sclerostin and Dickkopf-1 as therapeutic targets
351 in bone diseases. Endocr Rev 33: 747-783. doi: 10.1210/er.2011-1060
352 13. Moester MJ, Papapoulos SE, Löwik CW, van Bezooijen RL (2010) Sclerostin: current knowledge
353 and future perspectives. Calcif Tissue Int 87: 99-107. doi: 10.1007/s00223-010-9372-1
354 14. Suzuki Y, Nawata H, Soen S, Fujiwara S, Nakayama H, Tanaka I, Ozono K, Sagawa A, Takayanagi
355 R, Tanaka H, Miki T, Masunari N, Tanaka Y (2014) Guidelines on the management and treatment
356 of glucocorticoid-induced osteoporosis of the Japanese Society for Bone and Mineral Research:
357 2014 update. J Bone Miner Metab 32: 337-350. doi: 10.1007/s00774-014-0586-6

15. Bertouch JV, Roberts-Thompson PJ, Feng PH, Bradley J (1983) C-reactive protein and serological indices of disease activity in systemic lupus erythematosus. Ann Rheum Dis 42: 655-658.

16. Gabay C, Gay-Croisier F, Roux-Lombard P, Meyer O, Maineti C, Guerne PA, Vischer T, Dayer JM (1994) Elevated serum levels of interleukin-1 receptor antagonist in polymyositis/dermatomyositis. A biologic marker of disease activity with a possible role in the lack of acute-phase protein response. Arthritis Rheum 37: 1744-1751.

17. Kermani TA, Schmidt J, Crowson CS, Ytterberg SR, Hunder GG, Matteson EL, Warrington KJ (2012) Utility of erythrocyte sedimentation rate and C-reactive protein for the diagnosis of giant cell arteritis. Semin Arthritis Rheum 41: 866-71. doi: 10.1016/j.semarthrit.2011.10.005.

18. Tse WY, Cockwell P, Savage CO (1998) Assessment of disease activity in systemic vasculitis. Postgrad Med J 74: 1-6.

19. Bagnari V, Colina M, Ciancio G, Govoni M, Trotta F (2010) Adult-onset Still's disease. Rheumatol Int 30: 855-62. doi: 10.1007/s00296-009-1291-y.

20. Brabnikova Maresova K, Pavelka K, Stepan JJ (2013) Acute effects of glucocorticoids on serum markers of osteoclasts, osteoblasts, and osteocytes. Calcif Tissue Int 92: 354-361. doi: 10.1007/s00223-012-9684-4

21. Gifre L, Ruiz-Gaspà S, Monegal A, Nomdedeu B, Filella X, Guañabens N, Peris P (2013) Effect of glucocorticoid treatment on Wnt signalling antagonists (sclerostin and Dkk-1) and their

376 relationship with bone turnover. Bone 57: 272-276. doi: 10.1016/j.bone.2013.08.016

377 22. Ohnaka K, Taniguchi H, Kawate H, Nawata H, Takayanagi R (2004) Glucocorticoid enhances the

378 expression of dickkopf-1 in human osteoblasts: novel mechanism of glucocorticoid-induced

379 osteoporosis. Biochem Biophys Res Commun 318: 259-264. doi: 10.1016/j.bbrc.2004.04.025

380 23. Poole KE, van Bezooijen RL, Loveridge N, Hamersma H, Papapoulos SE, Löwik CW, Reeve J

381 (2005) Sclerostin is a delayed secreted product of osteocytes that inhibits bone formation. FASEB

382 J 19: 1842-1844. doi: 10.1096/fj.05-4221fje

383 24. Compton JT, Lee FY (2014) A review of osteocyte function and the emerging importance of

384 sclerostin. J Bone Joint Surg Am 96: 1659-1668. doi: 10.2106/JBJS.M.01096

385 25. Kuroki Y, Kaji H, Kawano S, Kanda F, Takai Y, Kajikawa M, Sugimoto T (2008) Short-term

386 effects of glucocorticoid therapy on biochemical markers of bone metabolism in Japanese patients:

387 a prospective study. J Bone Miner Metab 26: 271-278. doi: 10.1007/s00774-007-0821-5

388 26. Shikano K, Kaneko K, Kawazoe M, Kaburaki M, Hasunuma T, Kawai S (2016) Efficacy of

389 vitamin K2 for glucocorticoid-induced osteoporosis in patients with systemic autoimmune

390 diseases. Intern Med 55: 1997-2003. doi: 10.2169/internalmedicine.55.6230

391 27. Walton RJ, Preston CJ, Russell RG, Kanis JA (1975) An estimate of the turnover rate of bone-

392 derived plasma alkaline phosphatase in Paget's disease. Clin Chim Acta 63: 227-9.

393 28. Weiss MJ, Ray K, Henthorn PS, Lamb B, Kadesch T, Harris H (1988) Structure of the human

394 liver/bone/kidney alkaline phosphatase gene. J Biol Chem 25; 263(24):12002-10.

395 29. Delany AM, Durant D, Canalis E (2001) Glucocorticoid suppression of IGF I transcription in

396 osteoblasts. Mol Endocrinol 15: 1781-1789. doi: 10.1210/mend.15.10.0704

397 30. Sutherland MK, Geoghegan JC, Yu C, Turcott E, Skonier JE, Winkler DG, Latham JA (2004)

398 Sclerostin promotes the apoptosis of human osteoblastic cells: a novel regulation of bone

399 formation. Bone 35: 828-835. doi: 10.1016/j.bone.2004.05.023

400 31. Wang FS, Ko JY, Yeh DW, Ke HC, Wu HL (2008) Modulation of Dickkopf-1 attenuates

401 glucocorticoid induction of osteoblast apoptosis, adipocytic differentiation, and bone mass loss.

402 Endocrinology 149: 1793-1801. doi: 10.1210/en.2007-0910

403 32. Dovjak P, Dorfer S, Föger-Samwald U, Kudlacek S, Marculescu R, Pietschmann P (2014) Serum

404 levels of sclerostin and dickkopf-1: effects of age, gender and fracture status. Gerontology 60:

405 493-501. doi: 10.1159/000358303.

406 33. Wang SY, Liu YY, Ye H, Guo JP, Li R, Liu X, Li ZG (2011) Circulating Dickkopf-1 is correlated

407 with bone erosion and inflammation in rheumatoid arthritis. J Rheumatol 38: 821-7. doi:

408 10.3899/jrheum.100089.

409 34. Boland GM, Perkins G, Hall DJ, Tuan RS (2004) Wnt3a promotes proliferation and suppresses

410 osteogenic differentiation of adult human mesenchymal stem cells. J Cell Biochem 93: 1210-30.

411 35. Bennett CN, Ouyang H, Ma YL, Zeng Q, Gerin I, Sousa KM, Lane TF, Krishnan V, Hankenson

412 KD, MacDougald OA (2007) Wnt10b increases postnatal bone formation by enhancing osteoblast
413 differentiation. J Bone Miner Res 22: 1924-32.
414 36. Stevens JR, Miranda-Carboni GA, Singer MA, Brugger SM, Lyons KM, Lane TF (2010) Wnt10b
415 deficiency results in age-dependent loss of bone mass and progressive reduction of mesenchymal
416 progenitor cells. J Bone Miner Res 25: 2138-47. doi: 10.1002/jbmr.118.
417 37. Hochberg MC, Greenspan S, Wasnich RD, Miller P, Thompson DE, Ross PD (2002) Changes in
418 bone density and turnover explain the reductions in incidence of nonvertebral fractures that occur
419 during treatment with antiresorptive agents. J Clin Endocrinol Metab 87: 1586-92.
420 38. Matsumoto T, Hagino H, Shiraki M, Fukunaga M, Nakano T, Takaoka K, Morii H, Ohashi Y,
421 Nakamura T (2009) Effect of daily oral minodronate on vertebral fractures in Japanese
422 postmenopausal women with established osteoporosis: a randomized placebo-controlled double-
423 blind study. Osteoporos Int 20: 1429-1437. doi: 10.1007/s00198-008-0816-7
424 39. Hoes JN, Jacobs JW, Hulsmans HM, De Nijs RN, Lems WF, Bruyn GA, Geusens PP, Bijlsma JW
425 (2010) High incidence rate of vertebral fractures during chronic prednisone treatment, in spite of
426 bisphosphonate or alfacalcidol use. Extension of the alendronate or alfacalcidol in glucocorticoid-
427 induced osteoporosis-trial. Clin Exp Rheumatol 28: 354-9.
428 40. Chung YE, Lee SH, Lee SY, Kim SY, Kim HH, Mirza FS, Lee SK, Lorenzo JA, Kim GS, Koh
429 JM (2012) Long-term treatment with raloxifene, but not bisphosphonates, reduces circulating

sclerostin levels in postmenopausal women. Osteoporos Int 23: 1235-1243. doi: 10.1007/s00198-011-1675-1

41. Gatti D, Viapiana O, Adami S, Idolazzi L, Fracassi E, Rossini M (2012) Bisphosphonate treatment of postmenopausal osteoporosis is associated with a dose dependent increase in serum sclerostin. Bone 50: 739-742. doi: 10.1016/j.bone.2011.11.028

42. Cosman F, Crittenden DB, Adachi JD, Binkley N, Czerwinski E, Ferrari S, Hofbauer LC, Lau E, Lewiecki EM, Miyauchi A, Zerbini CA, Milmont CE, Chen L, Maddox J, Meisner PD, Libanati C, Grauer A (2016) Romosozumab treatment in postmenopausal women with osteoporosis. N Engl J Med 375: 1532-1543. doi: 10.1056/NEJMoa1607948

43. Marenzana M, Greenslade K, Eddleston A, Okoye R, Marshall D, Moore A, Robinson MK (2011) Sclerostin antibody treatment enhances bone strength but does not prevent growth retardation in young mice treated with dexamethasone. Arthritis Rheum 63: 2385-95. doi: 10.1002/art.30385.

44. Yao W, Dai W, Jiang L, Lay EY, Zhong Z, Ritchie RO, Li X, Ke H, Lane NE (2016) Sclerostin-antibody treatment of glucocorticoid-induced osteoporosis maintained bone mass and strength. Osteoporos Int 27: 283-94. doi: 10.1007/s00198-015-3308-6.

45. Sato AY, Cregor M, Delgado-Calle J, Condon KW, Allen MR, Peacock M, Plotkin LI, Bellido T (2016) Protection from glucocorticoid-induced osteoporosis by anti-catabolic signaling in the absence of Sost/sclerostin. J Bone Miner Res 31: 1791-802. doi: 10.1002/jbmr.2869.

448 **Figure Legends**

449 **Fig 1. Serum levels of sclerostin, Dkk-1 and Wnt3a in all patients during glucocorticoid therapy.**

450 Changes of serum sclerostin (A), Dkk-1 (B) and Wnt3a (C) in all patients (closed circle) and changes

451 of serum sclerostin (D), Dkk-1 (E) and Wnt3a (F) in patients with inflammatory diseases (empty

452 circles) and non-inflammatory diseases (empty squares) are shown. Data are expressed as the mean ±

453 SEM. *, $P < 0.05$ versus baseline; **, $P < 0.01$ versus baseline by Dunnett's multiple comparison test.

454 ▲, $P < 0.05$ versus baseline by paired *t*-test.

455

456 **Fig 2. Serum levels of Wnt3a, sclerostin, and Dkk-1 in the High and Low Wnt3a groups during**

457 **glucocorticoid therapy.** Changes of serum Wnt3a (A, B), sclerostin (C, D), and Dkk-1 (E, F) in High

458 Wnt3a group (A, C, E) and Low Wnt3a group (B, D, F) are shown. Data are expressed as the mean ±

459 SEM. *, $P < 0.05$ versus baseline by Dunnett's multiple comparison test. ▲, $P < 0.05$ versus baseline

460 by paired *t*-test.

461

462 **Fig 3. Serum levels of P1NP, BAP, TRACP-5b, and NTX during glucocorticoid therapy.** Changes

463 of serum P1NP (A), BAP (B), TRACP-5b (C), and NTX (D) are shown. Data are expressed as the

464 mean ± SEM. *, $P < 0.01$ versus baseline by Dunnett's multiple comparison test. ▲, $P < 0.05$ versus

465 baseline by paired *t*-test.

466

467 **Fig 4. Effect of dexamethasone on sclerostin and Dkk-1 mRNA expression in NHOst.** After NHOst

468 were treated with dexamethasone (0, 10^{-8}, 10^{-7}, or 10^{-6} M) for 6 hours, RT-PCR was performed to

469 detect the expression of sclerostin, Dkk-1 and β-actin mRNA (A). Sclerostin mRNA was undetectable.

470 Dkk-1 mRNA expression was normalized by that of β-actin mRNA (B).

471

472 **Fig 5. Effect of dexamethasone on Dkk-1 protein production by NHOst.** After NHOst were treated

473 with dexamethasone (0, 10^{-8}, or 10^{-7} M) for 24 hours, ELISA was performed to detect the expression

474 of Dkk-1 protein in the culture supernatants. *, $P < 0.05$ by the Kruskal-Wallis test.

Table 1. Demographics and clinical data at baseline of the study population

	Patients (n= 91)	High Wnt3a Group (n=22)	Low Wnt3a group (n=69)
Age (years)	56.9 ± 1.9	50.8 ± 4.5	59.0 ± 2.2
Male/female	37/54	10/12	27/42
Postmenopausal women (%)	32 (59.3)	7 (58)	25(60)
Body mass index (kg/m^2)	21.2 ± 0.4	21.9 ± 0.7	21.0 ± 0.8
Bone mineral density (g/cm^2)	0.95 ± 0.02	0.94 ± 0.05	0.94 ± 0.15
Mean daily prednisolone dose (mg)	45.8 ± 1.1	49.3 ± 2.4	45.0 ± 1.4
Diagnosis, No. (%)			
Polymyositis/Dermatomyositis	29/91 (31.9%)	8/22 (36.4%)	21/69 (30.4%)
Systemic lupus erythematosus	28/91 (30.8%)	5/22 (22.7%)	23/69 (33.3%)
Vasculitis syndrome	24/91 (26.4%)	5/22 (22.7%)	19/69 (27.5%)
Adult onset Still's disease	6/91 (6.6%)	3/22 (13.6%)	3/69 (4.3%)
Others	4(2/1/1)/91	1(1/0/0) /22	3(1/1/1)/69
(SSc/SS/IgG4-related disease)	(4.4%)	(4.5%)	(4.3%)
Serum markers			
CRP (mg/dL)	3.7 ± 0.5	2.7 ± 1.1	3.5 ± 2.2
	1.5 [0.4-6.25]	0.8 [0.4-2.2]	1.6 [0.4-6.6]
P1NP (μg/L)	41.7 ± 2.6	36.0 ± 3.5	43.8 ± 3.2
	35.3 [25.6-52.1]	31.9 [26.3-41.7]	36.4 [25.3-58.9]
BAP (μg/L)	12.9 ± 0.7	13.7 ± 1.1	12.3 ± 0.7
	12.0 [8.6-15.2]	13.9 [8.6-17.2]	10.8 [8.4-14.6]
NTX (nmolBCE/L)	19.6 ± 1.4	18.8 ± 2.9	20.0 ± 1.7
	16.4 [13.7-20.9]	16.3 [13.5-18.9]	16.5 [13.8-21.6]
TRACP-5b (mU/dL)	185.5 ± 14.0	183.8 ± 31.8	187.9 ± 15.6
	157.5	145.5	165.0
	[103.5-223.3]	[111.8-218.3]	[103.0-236.0]
sclerostin (pmol/L)	35.7 ± 2.7	37.0 ± 6.1	35.5 ± 3.0
	28.3 [23.5-37.2]	26.3 [21.7-37.1]	29.5 [24.0-49.7]
Dkk-1 (pmol/L)	34.3 ± 2.2	24.6 ± 3.0	36.8 ± 2.7
	29.8 [18.6-45.6]	23.9 [13.6-33.6]*	32.7 [21.0-49.7]
Wnt3a (ng/mL)	0.31 ± 0.04	0.83 ± 0.05	0.14 ± 0.02
	0.20 [0.00-0.48]	0.78 [0.66-0.97]**	0.06 [0.00-0.26]

SSc, systemic sclerosis; SS, Sjögren syndrome; CRP, C-reactive protein; P1NP, N-terminal peptide of type I procollagen; BAP, bone alkaline phosphatase; NTX, N-telopeptide cross-linked type I collagen; TRACP-5b, tartrate-resistant acid phosphatase isoform 5b; Dkk-1, Dickkopf-1

Data are expressed as the mean ± SEM and median [25th to 75th percentile range].

*P < 0.05 and **P < 0.01 for the High Wnt3a group (> 75th percentile) compared with the Low Wnt3a group (< 75th percentile) by Mann-Whitney *U* test.

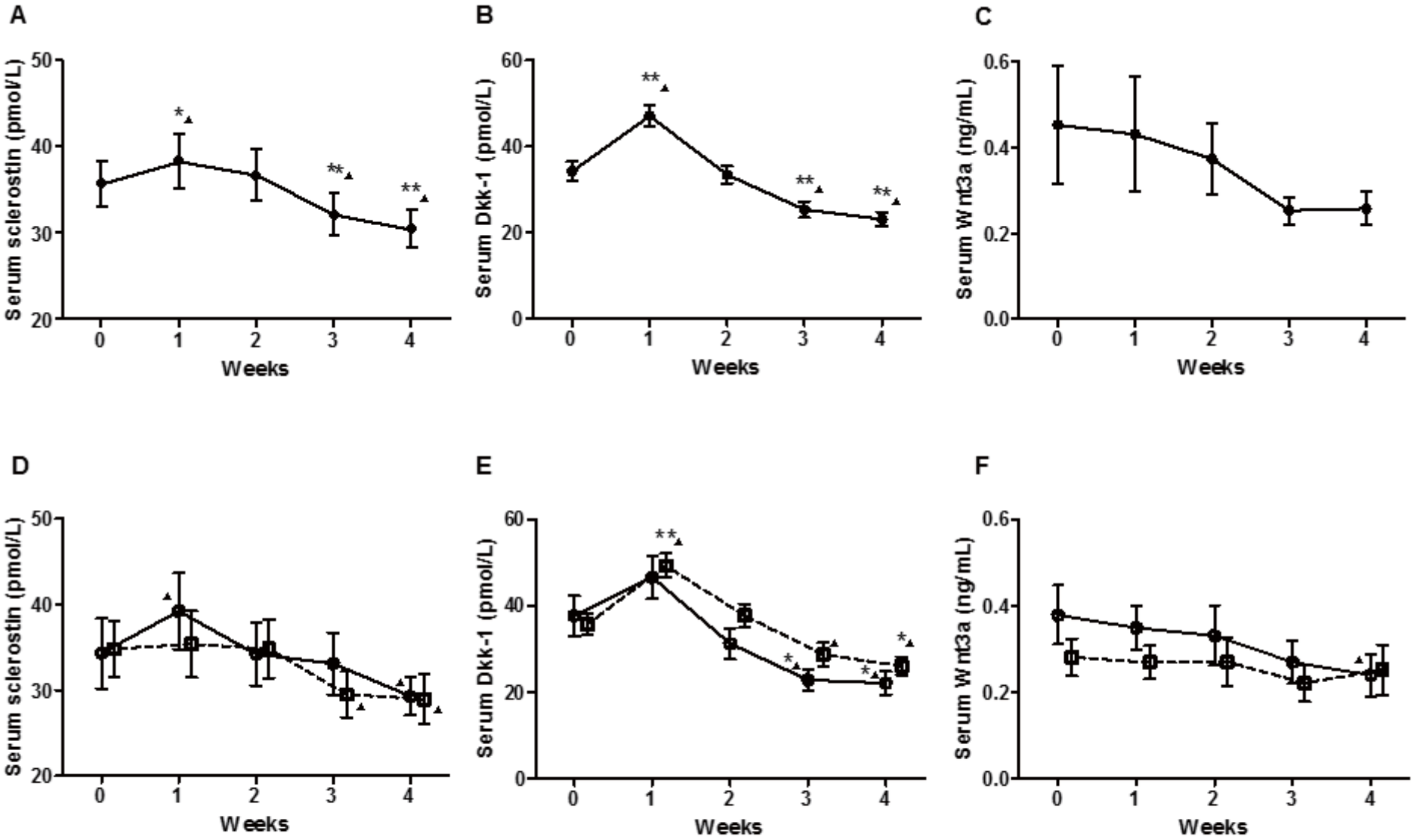
A
Serum sclerostin (pmol/L)
50
40
30
20
0
1
2
3
4
Weeks
*
**
**
B
Serum Dkk-1 (pmol/L)
60
40
20
0
Weeks
**
**
**
C
Serum Wnt3a (ng/mL)
0.6
0.4
0.2
0.0
Weeks
D
Serum sclerostin (pmol/L)
Weeks
E
Serum Dkk-1 (pmol/L)
Weeks
**
*
*
*
F
Serum Wnt3a (ng/mL)
Weeks

Fig. 2

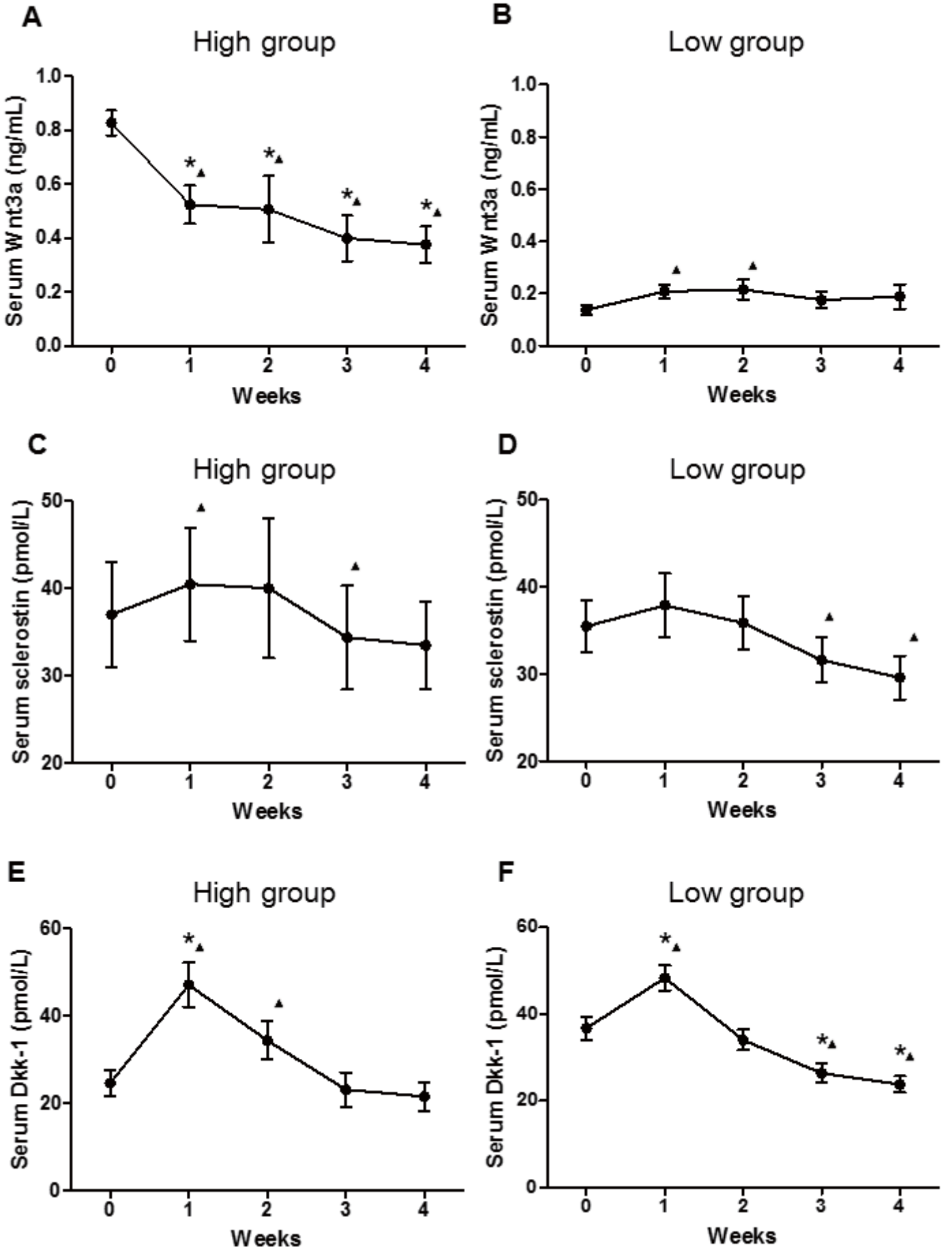
A
High group
Serum Wnt3a (ng/mL)
1.0
0.8
0.6
0.4
0.2
0.0
0
1
2
3
4
Weeks
B
Low group
Serum Wnt3a (ng/mL)
Weeks
C
High group
Serum sclerostin (pmol/L)
50
40
30
20
Weeks
D
Low group
Serum sclerostin (pmol/L)
Weeks
E
High group
Serum Dkk-1 (pmol/L)
60
40
20
0
Weeks
F
Low group
Serum Dkk-1 (pmol/L)
Weeks

Fig. 3

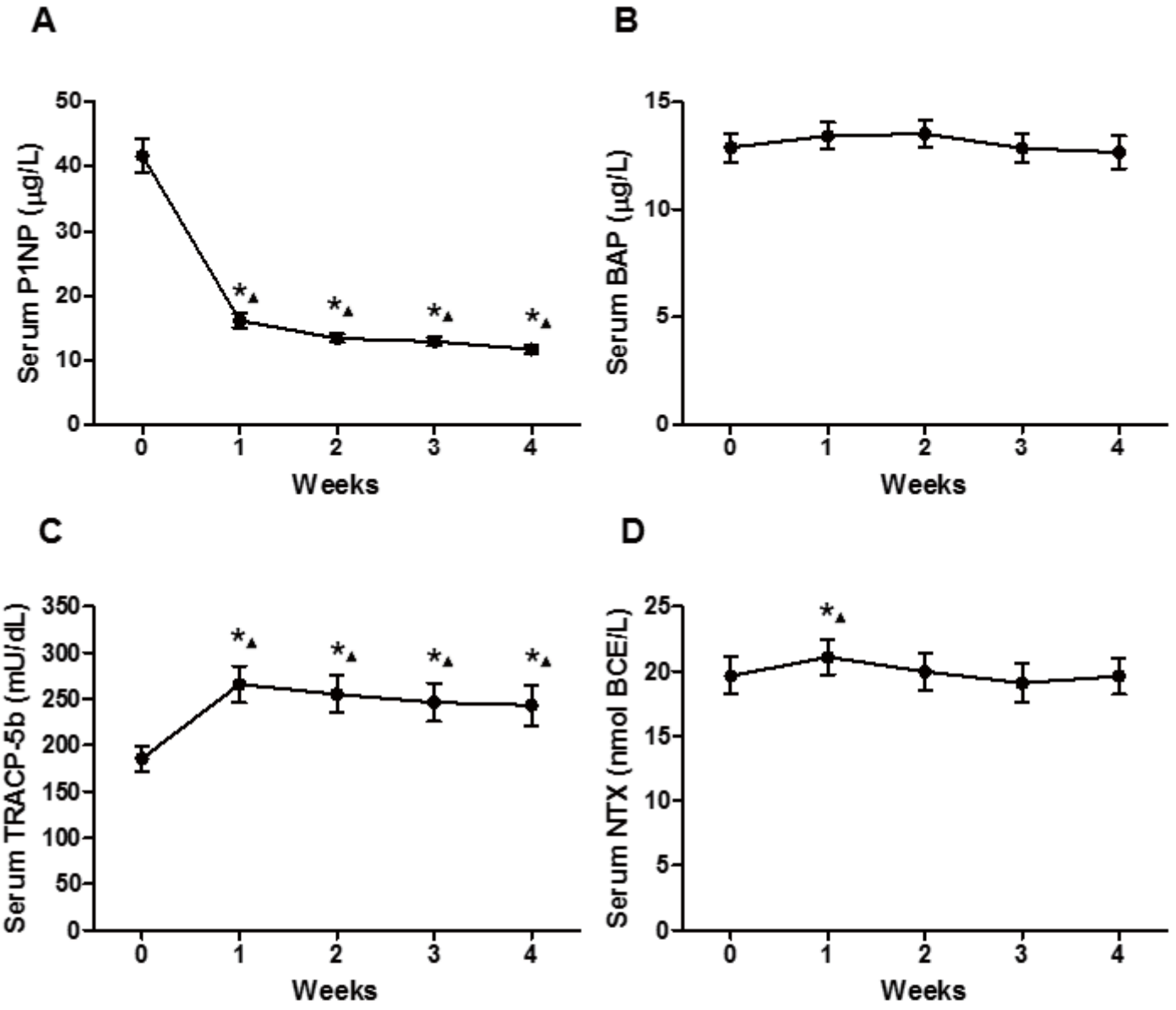
A
Serum P1NP (μg/L)
50
40
30
20
10
0
0
1
2
3
4
Weeks
B
Serum BAP (μg/L)
15
10
5
0
0
1
2
3
4
Weeks
C
Serum TRACP-5b (mU/dL)
350
300
250
200
150
100
50
0
0
1
2
3
4
Weeks
D
Serum NTX (nmol BCE/L)
25
20
15
10
5
0
0
1
2
3
4
Weeks

Fig. 4

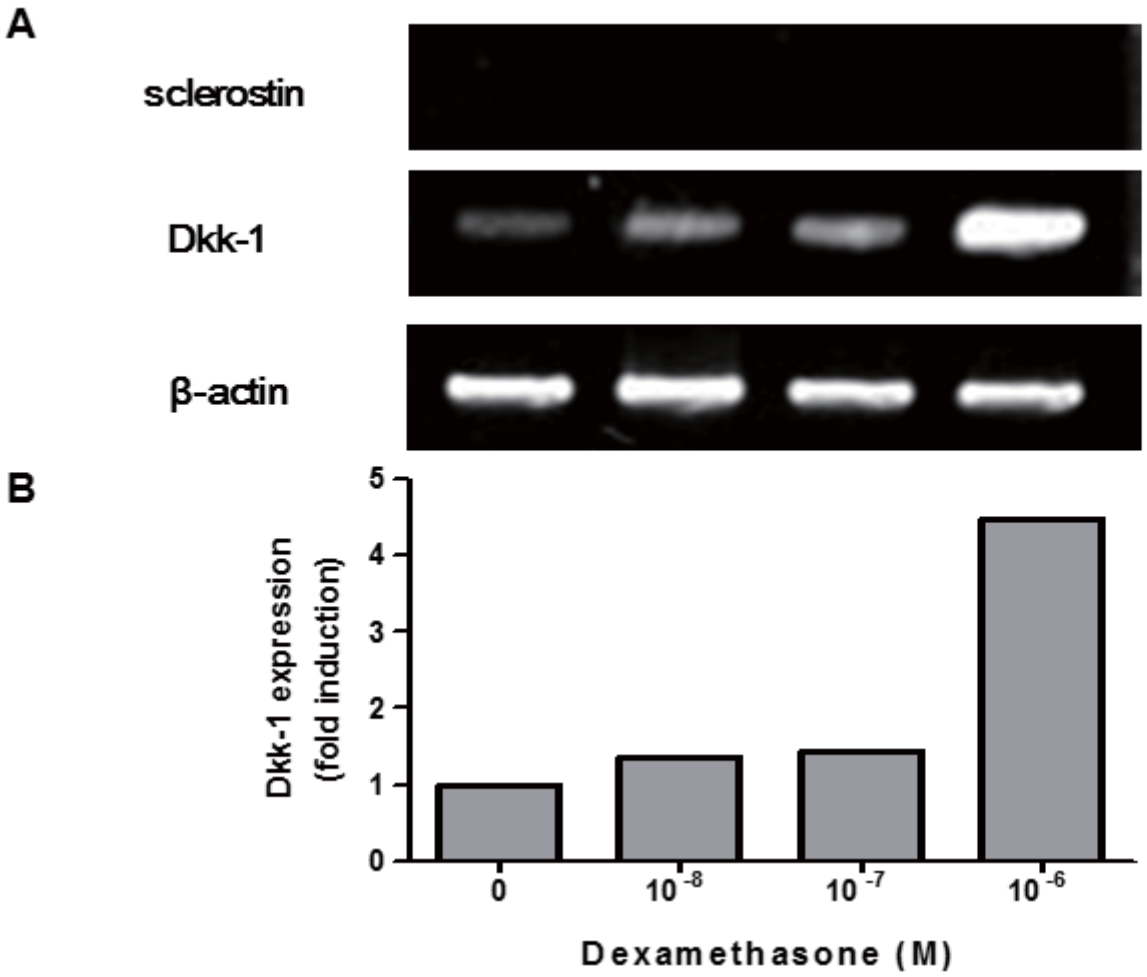
A
sclerostin
Dkk-1
β-actin
B
Dkk-1 expression
(fold induction)
0
1
2
3
4
5
0
10-8
10-7
10-6
Dexamethasone (M)

Fig. 5