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26

Abstract

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

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

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

43 **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

49

Introduction

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

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

65 Therefore, the aim of the present study was to investigate the clinical significance of the Wnt
66 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.

71

Patients and Methods

Patients and study protocol

73 A prospective observational study was conducted in 91 patients with systemic autoimmune diseases,
74 comprising 29 patients with polymyositis/dermatomyositis (PM/DM), 28 patients with systemic lupus
75 erythematosus (SLE), 24 patients with vasculitis syndrome, 6 patients with adult onset Still's disease
76 (AOSD), 2 patients with systemic sclerosis (SSc), 1 patient with Sjögren's syndrome (SS), and 1
77 patient with IgG4-related disease (Table 1). The subjects were recruited at Toho University Omori
78 Hospital. All patients who commenced glucocorticoid therapy with prednisolone at doses from 30 to
79 60 mg daily [mean daily dose: 45.9 ± 1.0 mg (the mean \pm standard error of the mean (SEM))] according
80 to our standard therapeutic regimens were included in this study. Starting dose was given to all patients
81 for two weeks, and the dose was tapered by 5 mg/day every one or two weeks from an initial dose.

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

88 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 (PINP; 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.),

107 which automatically calculated BMD from the bone area (cm²) and bone mineral content (g) and
108 expressed the result in g/cm². BMD was measured again after 15.6 ± 1.4 months (the mean ± SEM)
109 of glucocorticoid treatment and the change of BMD was calculated.

110

111 *Cell culture*

112 Normal human osteoblasts (NHOst) originating from a 2-year-old boy were obtained from Lonza
113 Inc. (Williamsport, PA, U.S.A.), and were maintained at 37 °C in osteoblast growth medium (Lonza
114 Inc.) supplemented with 10 % fetal bovine serum, 50 µg/mL gentamycin sulphate, 2.5 µg/mL
115 amphotericin-B, and 50 mM L-ascorbic acid under a humidified atmosphere of 5 % CO₂ in air. Then
116 the cells were resuspended in 5 mL of osteoblast growth medium supplemented with 1 % (v/v) fetal
117 bovine serum at 1.0 × 10⁵ cells/mL cultured in 35-mm dishes. Cells between the 2nd and 3rd passages
118 were used for all experiments.

119

120 *Expression of mRNA and protein of sclerostin and Dkk-1*

121 NHOst were incubated for 6 hours with or without dexamethasone (0, 10⁻⁸, 10⁻⁷, or 10⁻⁶ M) (Wako,
122 Osaka, Japan), and RNA was extracted by using an RNeasy Mini kit (Qiagen GmbH, Hilden,
123 Germany). Then the reverse transcription polymerase chain reaction (RT-PCR) was performed with a
124 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

143 *Statistical analysis*

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

Results

150

151 *Serum levels of sclerostin, Dkk-1, and Wnt3a*

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

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

166 We divided the patients into two groups, whose serum Wnt3a level was over the 75th percentile
167 (0.51-1.26 ng/mL, High Wnt3a group) and less than the 75th percentile (0-0.49 ng/mL, Low Wnt3a

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

177

178 *Bone turnover markers*

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

184

185 *Correlations between Wnt signaling-related molecules and bone turnover markers*

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

191

192 *Changes of BMD*

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

199

200 *Fracture rate*

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

204

205 *Effect of dexamethasone on cultured NHOst*

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

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

212

Discussion

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

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

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

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

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

243 We observed that serum levels of sclerostin and Dkk-1 were decreased from the third week onward,
244 but were increased after one week. It is not clear why sclerostin and Dkk-1 decreased over time.
245 Another mechanism such as accelerated differentiation of osteoblast precursors to adipocytes is likely
246 to be involved in the inhibition of bone formation during long-term glucocorticoid therapy [29]. It has
247 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

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

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

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

283 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

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

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

310

Ethical standards

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

316

317

Conflict of Interest

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

322

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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 \pm

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

454 \blacktriangle , $P < 0.05$ 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 \pm

459 SEM. *, $P < 0.05$ versus baseline by Dunnett's multiple comparison test. \blacktriangle , $P < 0.05$ 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 \pm SEM. *, $P < 0.01$ versus baseline by Dunnett's multiple comparison test. \blacktriangle , $P < 0.05$ 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.

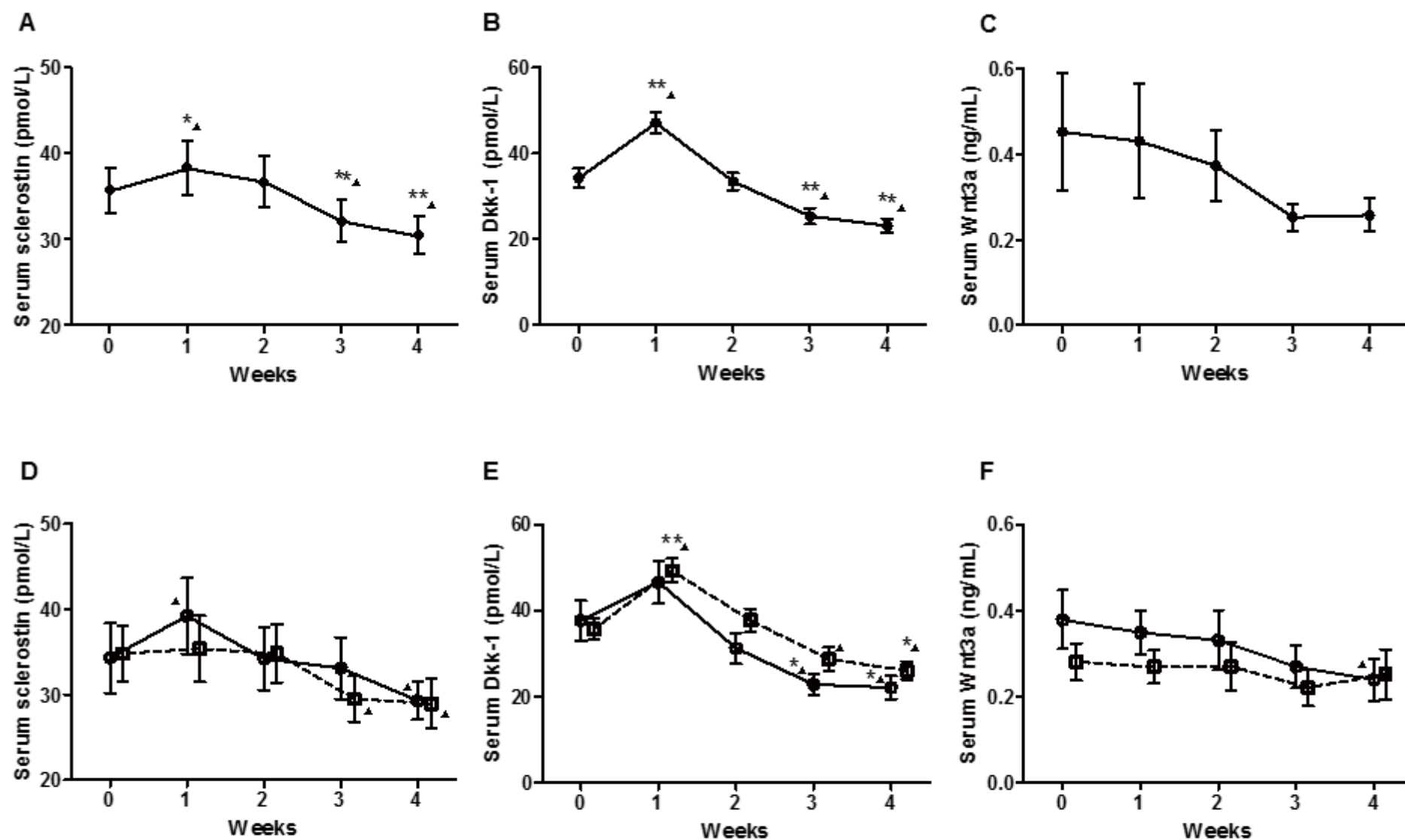
Table 1. Demographics and clinical data at baseline of the study population

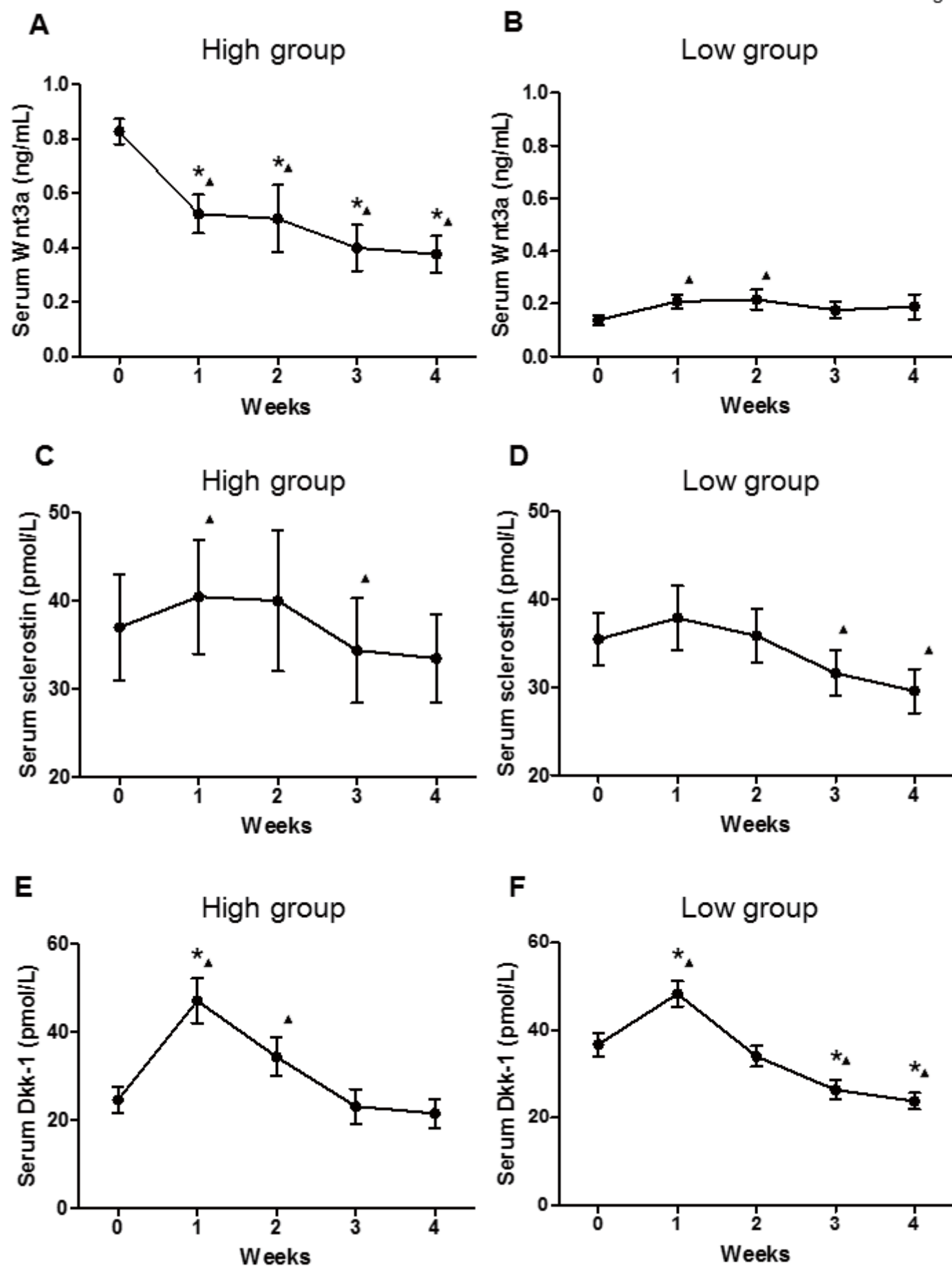
	Patients (n= 91)	High Wnt3a Group (n=22)	Low Wnt3a group (n=69)
Age (years)	56.9 ± 1.9	50.8 ± 4.5	59.0 ± 2.2
Male/female	37/54	10/12	27/42
Postmenopausal women (%)	32 (59.3)	7 (58)	25(60)
Body mass index (kg/m ²)	21.2 ± 0.4	21.9 ± 0.7	21.0 ± 0.8
Bone mineral density (g/cm ²)	0.95 ± 0.02	0.94 ± 0.05	0.94 ± 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 (SSc/SS/IgG4-related disease)	4(2/1/1)/91 (4.4%)	1(1/0/0)/22 (4.5%)	3(1/1/1)/69 (4.3%)
Serum markers			
CRP (mg/dL)	3.7 ± 0.5 1.5 [0.4-6.25]	2.7 ± 1.1 0.8 [0.4-2.2]	3.5 ± 2.2 1.6 [0.4-6.6]
P1NP (µg/L)	41.7 ± 2.6 35.3 [25.6-52.1]	36.0 ± 3.5 31.9 [26.3-41.7]	43.8 ± 3.2 36.4 [25.3-58.9]
BAP (µg/L)	12.9 ± 0.7 12.0 [8.6-15.2]	13.7 ± 1.1 13.9 [8.6-17.2]	12.3 ± 0.7 10.8 [8.4-14.6]
NTX (nmolBCE/L)	19.6 ± 1.4 16.4 [13.7-20.9]	18.8 ± 2.9 16.3 [13.5-18.9]	20.0 ± 1.7 16.5 [13.8-21.6]
TRACP-5b (mU/dL)	185.5 ± 14.0 157.5 [103.5-223.3]	183.8 ± 31.8 145.5 [111.8-218.3]	187.9 ± 15.6 165.0 [103.0-236.0]
sclerostin (pmol/L)	35.7 ± 2.7 28.3 [23.5-37.2]	37.0 ± 6.1 26.3 [21.7-37.1]	35.5 ± 3.0 29.5 [24.0-49.7]
Dkk-1 (pmol/L)	34.3 ± 2.2 29.8 [18.6-45.6]	24.6 ± 3.0 23.9 [13.6-33.6]*	36.8 ± 2.7 32.7 [21.0-49.7]
Wnt3a (ng/mL)	0.31 ± 0.04 0.20 [0.00-0.48]	0.83 ± 0.05 0.78 [0.66-0.97]**	0.14 ± 0.02 0.06 [0.00-0.26]

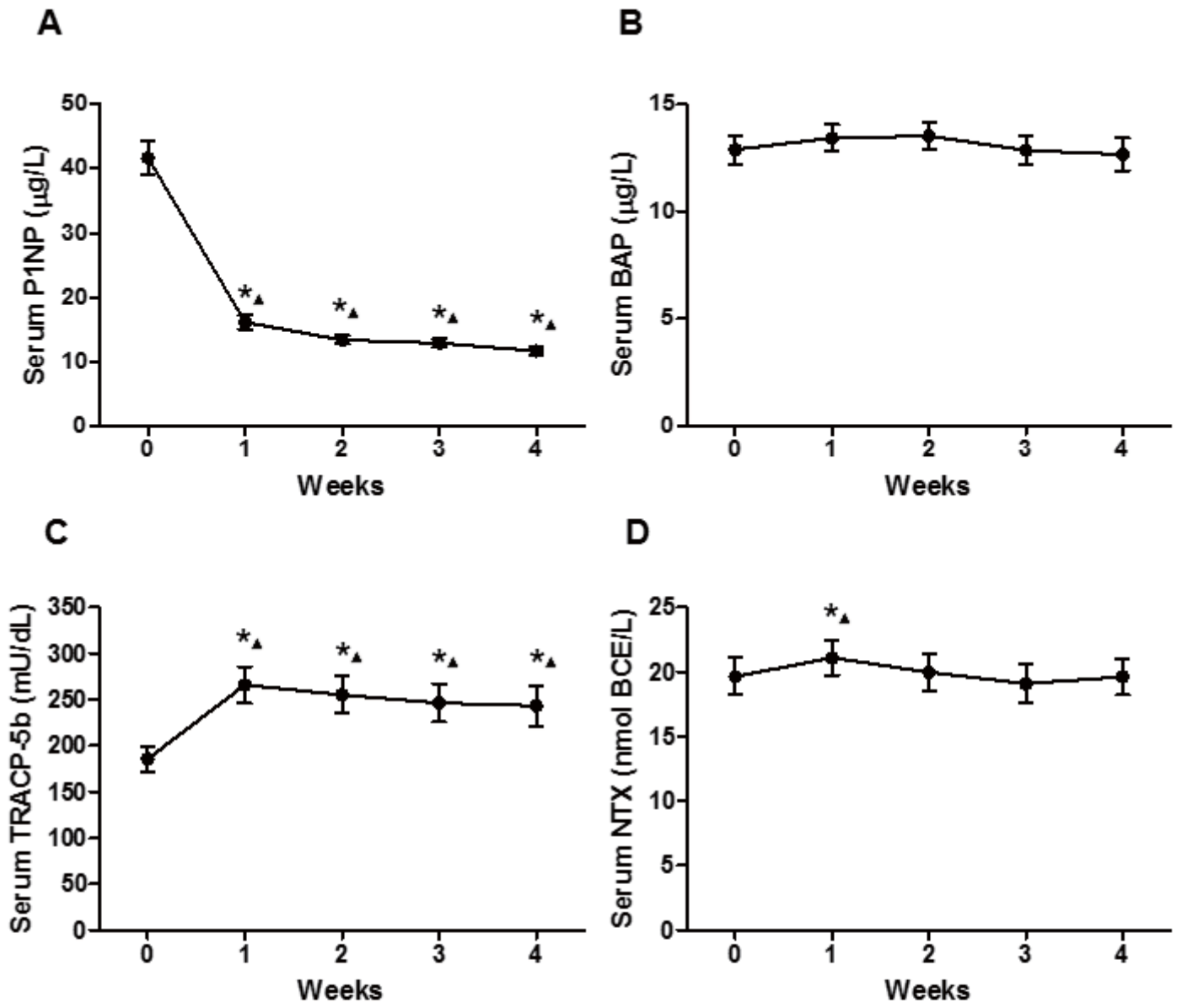
SSc, systemic sclerosis; SS, Sjögren syndrome; CRP, C-reactive protein; P1NP, N-terminal peptide of type I procollagen; BAP, bone alkaline phosphatase; NTX, N-telopeptide cross-linked type I collagen; TRACP-5b, tartrate-resistant acid phosphatase isoform 5b; Dkk-1, Dickkopf-1

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

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







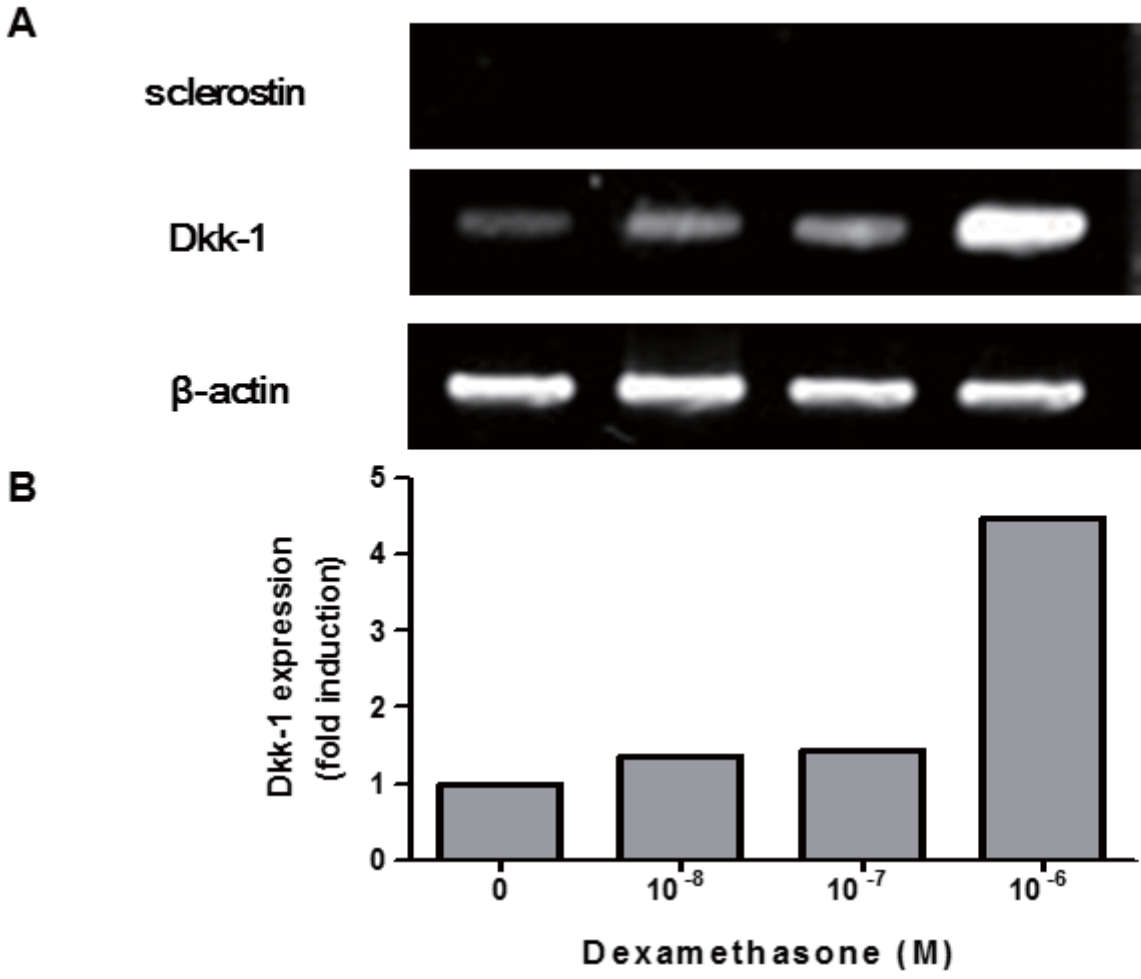


Fig. 5

