Endocrine
DOI 10.1007/s12020-016-1056-9

ORIGINAL ARTICLE

Changes of serum sclerostin and Dickkopf-1 levels during the menstrual cycle. A pilot study

Chrysoula G. Liakou[1] · George Mastorakos[2] · Konstantinos Makris[3] · Ioannis G. Fatouros[4] · Alexandra Avloniti[5] · Helen Marketos[3] · Julia D. Antoniou[1] · Antonios Galanos[1] · Ismene Dontas[1] · Demetrios Rizos[6] · Symeon Tournis[1]

Received: 12 April 2016 / Accepted: 6 July 2016

Abstract Studies in postmenopausal women have identified sclerostin as a strong candidate for mediating estrogen effects on the skeleton. The effects of estradiol on sclerostin and Dickkopf-1 in younger women remain unclear. The main purpose of this study is to investigate the impact of estradiol and gonadotrophins fluctuations during the menstrual cycle on circulating sclerostin and Dickkopf-1 levels and the possible relationship of sclerostin and Dickkopf-1 with changes in N-terminal propeptide of type 1 collagen and C-telopeptide of collagen cross-links. Fourteen healthy premenopausal Caucasian women, with regular menses, aged 33.6 ± 4.5 years participated. After the first day of menstruation and every-other-day up to the next menses, fasting serum estradiol, luteinizing hormone, follicle-stimulating hormone, sclerostin, Dickkopf-1, N-terminal propeptide of type 1 collagen, and C-telopeptide of collagen cross-links levels were measured in peripheral blood. Participants completed dietary questionnaires and the International physical activity questionnaire during the cycle. Neither sclerostin nor Dickkopf-1 levels changed significantly across the menstrual cycle ($p = 0.18$ and $p = 0.39$, respectively), while N-terminal propeptide of type 1 collagen and C-telopeptide of collagen cross-links levels presented cyclic variation ($p < 0.001$ and $p = 0.004$, respectively). Baseline sclerostin (29.23 ± 10.62 pmol/L) positively correlated with N-terminal propeptide of type 1 collagen ($r = 0.71$, $p < 0.01$) and C-telopeptide of collagen cross-links ($r = 0.63$, $p < 0.05$), while Dickkopf-1 (4.82 ± 2.23 pmol/L) correlated positively with N-terminal propeptide of type 1 collagen ($r = 0.56$, $p < 0.05$). Mid-cycle E2 levels presented significant negative association with the percent decrease of C-telopeptide of collagen cross-links at all-time points during the luteal period ($r = -0.60$ to -0.68, $p < 0.05$–0.01). Circulating sclerostin and Dickkopf-1 levels do not change across the menstrual cycle and do not demonstrate any relationship with estradiol in premenopausal women. Further investigation is needed concerning the role of sclerostin and Dickkopf-1 on bone turnover in young estrogen-sufficient women.

Keywords Sclerostin · Dkk-1 · Estradiol · Bone turnover markers

Abbreviations

BMD	bone mineral density
CTX	C-telopeptide of collagen cross-links
DeltaCTX	percent change of CTX
Dkk-1	Dickkopf-1
E2	estradiol
ERα	estrogen receptor-alpha
FEI	free estrogen index
FP	follicular phase

✉ Chrysoula G. Liakou
c_liakou@yahoo.com

[1] Laboratory for Research of the Musculoskeletal System "Th. Garofalidis", Medical School, Athens University, KAT Hospital, 10 Athinas Str., Kifissia, 14561 Athens, Greece

[2] Unit of Endocrinology, Diabetes and Metabolism, Aretaieion Hospital, Athens Medical School, Athens University, 76 Vas. Sofias Av., 11528 Athens, Greece

[3] Department of Biochemistry, KAT Hospital, 10 Athinas Str., Kifissia, 14561 Athens, Greece

[4] School of Physical Education and Sports Sciences, University of Thessaly, 42100 Karies, Trikala, Greece

[5] School of Physical Education and Sports Sciences, Democritus University of Thrace, 69100 Komotini, Greece

[6] Hormone Laboratory, Aretaieion Hospital, Medical School, Athens University, 76 Vas. Sofias Av., 11528 Athens, Greece

Published online: 06 September 2016

FSH	follicle-stimulating hormone
IPAQ	international physical activity questionnaire
LH	luteinizing hormone
LP	luteal phase
LRP	low density lipoprotein receptor-related protein
MET	metabolic equivalent of task
P1NP	N-terminal propeptide of type 1 collagen
PTH	parathyroid hormone
RANKL	receptor activator for nuclear factor-κB ligand
SHBG	sex hormone-binding globulin
TSH	thyroid-stimulating hormone

Introduction

Mature osteocytes play a major role in bone remodeling by secreting proteins, including sclerostin and Dickkopf-1 (Dkk-1) that serve as antagonists of the canonical Wnt signaling pathway [1]. The Wnt pathway is critical for the regulation of bone mass. Activation of the Wnt pathway promotes proliferation and differentiation of osteoblast precursors and averts apoptosis of mature osteoblasts [2]. Sclerostin and Dkk-1 antagonize its activation by binding to the Wnt co-receptor LRP5/6, thus preventing formation of the Wnt-Frizzled-LRP5/6 signaling complex [3]. Accordingly osteocytes, through the expression of Wnt antagonists, have the potential to regulate the formation and activity of osteoblasts.

In vitro studies have shown that sclerostin inhibits osteoblast proliferation and differentiation, promotes osteoblast apoptosis, suppresses mineralization of osteoblastic cells, and stimulates osteoclastic bone resorption [4]. Genetic conditions associated with defective sclerostin production result in diseases characterized by high-bone mass, such as sclerosteosis and Van Buchem's disease [5, 6]. Postmenopausal women treated with various doses of romososumab, a humanized antisclerostin monoclonal antibody, demonstrate a transient increase in bone formation markers, while resorption seems to decrease [7]. Moreover, downregulation of sclerostin seems to mediate in part the osteogenic response to systemic elevation of parathyroid hormone (PTH) or local mechanical loading thereby letting loose the Wnt signaling cascade [7, 8].

Dkk-1 has also been shown to be involved in the regulation of bone turnover. Alterations in Dkk-1 expression in mice led to reciprocal changes in bone mass, in a manner similar to sclerostin [9, 10]. In ovariectomized rats, attenuation of Dkk-1 expression alleviated loss of bone mass by increasing the number of osteoblasts and reducing receptor activator for nuclear factor-κB ligand expression, and thus decreasing osteoclastogenesis [11].

Estrogens are major regulators of sclerostin expression. By acting through the Estrogen receptor-alpha (ERα), estrogen decreases sclerostin expression [12]. Postmenopausal women have higher sclerostin levels as compared with premenopausal, with circulating sclerostin showing a negative correlation with free estradiol index in postmenopausal women [13]. Estrogen replacement reduces circulating sclerostin and bone sclerostin mRNA in postmenopausal women [14, 15]. Finally in postmenopausal women raloxifene administration results in reduction of circulating sclerostin [14].

These data indicate that sclerostin is a strong candidate for mediating estrogen effects on the skeleton. Data relating circulating sclerostin and Dkk-1 levels with the corresponding local bone production indicate that at least serum sclerostin is likely to reflect local bone production. Drake et al [16] in 38 postmenopausal women reported that circulating serum and bone marrow plasma sclerostin levels showed significant correlation ($r = 0.64$, $p < 0.001$), with the slope close to 1.0, while the corresponding Dkk-1 levels were not ($r = 0.14$, $p = 0.410$). Moreover, Fugita K, et al [17] reported that both serum ($r = 0.57$, $p = 0.008$) and plasma ($r = 0.48$, $p = 0.032$) circulating sclerostin levels correlated with sclerostin bone mRNA levels in estrogen treated postmenopausal women but not in control postmenopausal women. Despite the increased interest in sclerostin, there are not many studies examining the effects of sex steroids on sclerostin in premenopausal women and none to our knowledge on Dkk-1 [13, 18]. The present study examines the impact of estradiol and gonadotrophin fluctuations during the menstrual cycle on circulating sclerostin and Dkk-1 levels, and whether changes of the latter ones are coupled with variation of bone formation and resorption expressed in terms of serum N-terminal propeptide of type 1 collagen (P1NP) and serum C-telopeptide of collagen cross-links (CTX), respectively.

Methods

Subjects

Eighteen healthy premenopausal, Caucasian, women aged over 30 years (30–45 years) with regular (24–35 days) and spontaneous menstrual cycles were recruited. Subjects were hospital staff members recruited by word of mouth and advertisement in KAT hospital. Exclusion criteria included a history of endocrine, heart, renal, gastrointestinal, liver, or metabolic diseases, prior cancer diagnosis, eating disorders, pregnancy, or breast-feeding during the previous year and the use of oral contraceptives during the 3 months prior to the study or of any medication known to affect bone metabolism. Vitamin D deficiency [25(OH) D levels

<20 ng/ml] was also an exclusion criterion. Written informed consent was given by each subject. The protocol was designed according to the Declaration of Helsinki and approved by the Ethics Committee of KAT Hospital.

Fourteen women completed the study. During the sampling period one subject withdrew her consent, one became pregnant diagnosed just after the completion of sampling period, while one did not have ovulation judged by the absence of progesterone rise above 5 ng/ml. Finally, 3 months after the study one subject having high normal corrected calcium levels (10.1 mg/dl) with PTH 62 pg/ml was diagnosed with primary hyperparathyroidism, thereby we decided to exclude the subject from the analysis. There was no significant difference concerning age and BMI between the final study group and the four excluded subjects

Study protocol

All subjects had a full medical history and physical examination at baseline along with laboratory fasting serum screening tests including calcium, phosphate, albumin, alkaline phosphatase and creatinine levels, PTH, 25(OH) D levels and thyroid function tests (TSH, FT4).

The dates of sample collection were preset according to each subject's menstrual cycle duration. Specifically, after the first day of menstruation, women underwent blood sampling every-other-day, with a ± 1 day window, up to the onset of the next menses, for the measurement of serum estradiol, LH, FSH, sclerostin, Dkk-1, P1NP, and CTX levels. In women with a history of menstrual cycles shorter than 26 days, sampling was performed every day for 3 days per week and then, every-other-day for the 4 days left, in order to obtain the desirable number of samples. Progesterone was measured in the sample corresponding to the middle of the luteal phase, while serum sex hormone-binding globulin (SHBG) levels, which are known to remain constant throughout the menstrual cycle, were measured at baseline. Ovulatory cycles were indicated by a rise in serum progesterone levels above 5 ng/ml.

All samples were collected in the fasting state after an overnight fast, at the same time of the day. They were centrifuged for 10 min at 1,000 *g* within 30 min of collection and were frozen and stored at −80 °C until assayed.

Physical activity and dietary assessment

Participants completed 24 h dietary questionnaires at four random days during the cycle (3 weekdays and 1 weekend day) to assess their nutrient intake and the International Physical Activity Questionnaire (IPAQ, long last 7 Days Self-administered format for use with young and middle-aged adults 15–69 years) at 2 different weeks for the assessment of their physical activity. The questionnaire measures daily habitual physical activity and not the specific type (eg., resistance vs. aerobic). Nutrient content of daily food intake was quantified using the computerized nutritional analysis system Science Fit Diet 200A (Science Technologies, Athens, Greece) [17]. Based on the 7-day physical activity questionnaires, the metabolic equivalent of task (MET) was calculated in order to assess the daily habitual activity energy cost of the participants at the first and third week of the menstrual cycle.

Biochemical analysis

FSH, LH, and E2 were measured with a chemiluminescent immunoassay on Architect-i1000 automated analyzer (Abbott Diagnostics Division, Chicago, IL). The sensitivities of these assays is 0.05, 0.07 mIU/ml, and 10 pg/ml, respectively. The total imprecision (CV%) of these assays is <4.6 % for FSH, <4.1 % for LH, and <7.1 % for E2. SHBG was measured with an electrochemiluminescent immunoassay (ECLIA) on Cobas e-411 analyzer (Roche Diagnostics, Mannheim, Germany). The sensitivity of the assay is 0.35 nmol/l and the total imprecision <5.6 %. Free estradiol index was calculated using total E2 and SHBG values, by the following equations: FEI = E2 (pg/ml) × 0.367/SHBG (nmol/l) [16].

Serum 25(OH) D levels were determined by enzyme immunoassay (Immunodiagnostic systems, Boldon, UK). The sensitivity of this assay is 2 ng/ml and the total imprecision <5.6 %. Serum total calcium (corrected for albumin) and inorganic phosphate were measured by colorimetry using a Roche Hitachi 902 analyzer (Roche, Indianapolis, IN). The total imprecision of these assays is <1.5 % and <1.4 %, respectively.

Serum sclerostin and Dkk-1 levels were measured by enzyme immunoassays (Biomedica, Vienna, Austria). The sclerostin assay has a sensitivity of 2.6 pmol/L and total imprecision <6 %. The Dkk-1 assay has a sensitivity of 1.7 pmol/L and total imprecision <12 %.

P1NP and CTX levels were measured with an ECLIA on Cobas e-411 analyzer. The sensitivity of these assays is 5 ng/ml and 0.01 ng/ml, respectively. The total imprecision of these assays is <4.5 and <3.5 %, respectively. The percent change of CTX [DeltaCTX (%)] between time points ($t_{\pm x}$) and 0 was calculated as follows: DeltaCTX (%)={[CTX $t_{\pm x}$ − CTX t_0)]/CTX t_0} *100.

Statistical analysis

Each menstrual cycle was divided into follicular phase (FP) and luteal period (LP) by the serum LH surge (ovulation), which was defined as day 0. FP and LP were normalized by lengths to eliminate inter-individual variations in cycle and phase durations [19–21]. The first day of menstruation was

defined as day −14, while the last day before the start of the next was defined as day +14. Intermediate visits were defined as ±11, ±9, ±7, ±5, ±3. Samples corresponding to these time points, after the normalization of FP and LP, were selected and included in the study. Subphases of FP and LP were defined as early (±14 to ±11), middle (±9 to ±7), and late (±5 to ±3) [19, 22].

Fourteen women were included in the study and 13 serum samples were analyzed for each one of them. Posthoc power analysis using the G-power software indicated that the achieved power for sclerostin was 0.83, for Dkk-1 0.74, for CTX 0.99, and for P1NP 0.99. Normality was tested by the Kolmogorov-Smirnov test. Data were presented as mean ± SD. Differences during the observation period were analyzed using the one-way repeated measures analysis of variance (ANOVA) model, while corrections were made by Tukey test. Bivariate correlations were estimated by the Pearson correlation test and $p < 0.05$ was considered significant. Data analysis was performed using the Statistical Package for Social Sciences (version 20.0) software (SPSS Inc., Chicago, IL).

Results

Fourteen healthy premenopausal women completed the study. The clinical characteristics and baseline biochemical parameters are shown in Table 1. The mean age of women was 33.6 ± 4.5 years, while their mean body mass index (BMI) was 22.2 ± 2.5 kg/m^2. All subjects had 25(OH) D levels greater than 20 ng/ml (range: 22.4–34.0). All investigated cycles were ovulatory, as judged by the mid-luteal progesterone values (17.8 ± 7.2 ng/ml, range: 6.1–29.7). Mean serum levels SHBG levels were 86.9 ± 37.3 nmol/L.

According to the nutritional analysis (Table 1) mean calcium intake was 1093.4 ± 241.5 mg/day, while mean vitamin D intake was 139.2 ± 78.4 IU. Median energy cost per day did not differ between the first and third week of the menstrual cycle [812.8 (65–1440) kcal/day vs. 909.0 (69–4227) kcal/day, $p = 0.75$]. In terms of physical activity intensity (MET-hours/week), no differences were detected between the first and third week of the menstrual cycle (light: 11.68 ± 9.03 vs. 13.98 ± 13.39, moderate: 16.70 ± 9.58 vs. 16.79 ± 13.16, vigorous: 6.31 ± 6.66 vs. 15.54 ± 29.75; all $p > 0.1$).

Changes of FSH, LH, E2 during the menstrual cycle

(Figure 1) Serum LH and FSH levels were within normal limits. LH showed its well-known cyclic variation ($p < 0.001$), presenting mid-cyclic peak ($p < 0.001$ vs. all other time points). FSH also varied significantly ($p < 0.001$), showing a mid-cyclic rise ($p < 0.05$, $p < 0.001$ vs. all other time points). Serum estradiol levels also varied significantly

Table 1 Clinical characteristics and baseline biochemical and dietary parameters

Parameter	Mean ± SD	Range (min–max)	Reference values
Anthropometry/Menstrual cycle and Dietary history			
Age (years)	33.60 ± 4.50	30–45	–
Height (cm)	1.65 ± 0.05	1.58–1.73	–
Weight (kg)	60.10 ± 7.10	49.0–77.0	–
BMI (kg/m^2)	22.19 ± 2.48	18.87–25.73	–
Menarche (years)	12.82 ± 1.67	10–15	–
Menstrual cycle length (days)	26.79 ± 2.86	23–35	–
FP (days)	12.93 ± 3.02	8–20	–
LP (days)	12.79 ± 1.36	10–14	–
Dietary Calcium intake (mg/day)	1093.40 ± 241.60	756.6–1481.9	–
Dietary Vitamin D intake (IU/day)	139.20 ± 78.40	40.9–316.1	–
MET 1st week (h/week)	34.70 ± 20.88	2.40–64.32	–
MET 3rd week (h/week)	46.33 ± 52.13	2.55–188.71	–
Biochemical/hormonal variables			
Calcium (mg/dl)	8.77 ± 0.38	8.4–9.5	8.2–10.2
Phosphate (mg/dl)	3.60 ± 0.46	3.0–4.5	2.5–4.7
Creatinine (mg/dl)	0.69 ± 0.08	0.5–0.8	0.7–1.3
ALP (IU/L)	39.21 ± 11.68	21.0–69.0	40–150
SHBG (nmol/L)	86.85 ± 37.25	46.09–158.7	30–180
TSH (μU/ml)	1.90 ± 1.10	0.71–4.33	0.35–4.94
FT4 (ng/dl)	1.14 ± 0.10	0.95–1.29	0.8–1.8

MET metabolic equivalent of task

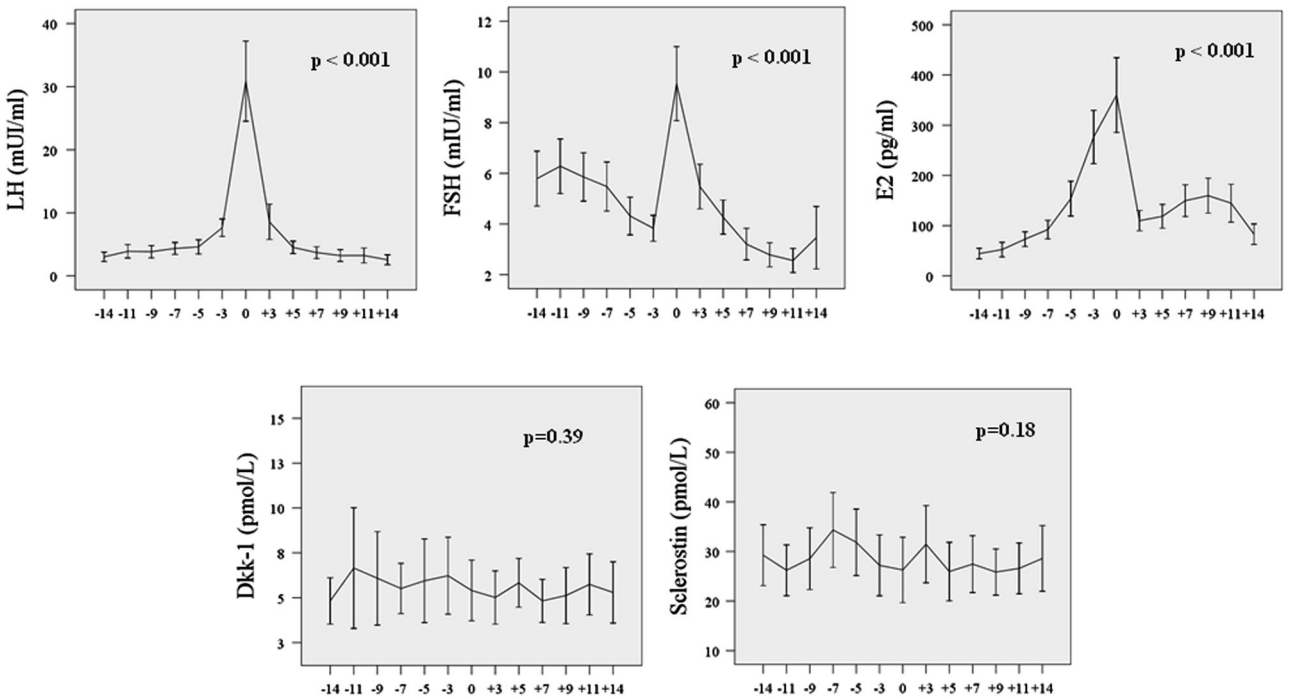

Fig. 1 Mean ± 95 % confidence intervals (CIs) of serum levels of LH, FSH, E2, Sclerostin, and Dkk-1 levels during the menstrual cycle. *p*: one-way repeated measures ANOVA

($p < 0.001$), showing the expected rise during the mid FP ($p = 0.002$ vs. baseline) to peak values at ovulation ($p < 0.001$ vs. almost all other time points), a rapid decrease after ovulation, and increased again during the mid LP. Estradiol levels were significantly lower at early and middle FP compared to late FP and LP ($p < 0.027$). Mean values for E2, LH, and FSH at the LH peak were 360.1 ± 128.7 pg/ml, 30.9 ± 11.0 mIU/ml, and 9.5 ± 2.5 mIU/ml, respectively.

Changes of serum sclerostin and Dkk-1 during the menstrual cycle

(Figure 1) Serum sclerostin levels did not present statistically significant variation across the menstrual cycle ($p = 0.18$). Mean serum sclerostin levels were 29.6 ± 6.4 pmol/L during the FP (day −14 to −3), 26.3 ± 11.4 pmol/L at the LH peak (day 0), and 27.6 ± 7.0 pmol/L during the LP (day +3 to +14). Similarly to sclerostin levels, Dkk-1 levels did not vary significantly during the menstrual cycle ($p = 0.39$). Mean Dkk-1 levels were 5.9 ± 3.4 pmol/L during the FP, 5.4 ± 2.9 pmol/L at the LH peak, and 5.3 ± 2.2 pmol/L during the LP.

Changes of serum P1NP and CTX during the menstrual cycle

(Figure 2) P1NP and CTX levels varied significantly across the cycle ($p < 0.001$ and $p = 0.004$, respectively). Mean serum P1NP levels were 36.9 ± 14.4 ng/ml during the FP, 41.1 ± 15.9 ng/ml at the LH peak, and 41.5 ± 14.9 ng/ml during the LP. When further broken down by subphases, P1NP levels were found statistically significantly lower between both early FP and middle FP in comparison to middle LP ($p = 0.007$ and $p = 0.006$, respectively). Mean CTX values were 0.26 ± 0.11 ng/ml during the FP, 0.24 ± 0.12 ng/ml at the LH peak, and 0.23 ± 0.10 ng/ml during the LP. Analysis of CTX levels among subphases, demonstrated higher mean CTX levels during middle FP relative to middle LP ($p = 0.047$).

Correlation analysis

At baseline (day −14) circulating sclerostin and Dkk-1 levels did not correlate with age, height, weight, BMI, and calcium intake (Table 2). Moreover, there was no association between calcium and phosphate levels with either sclerostin or Dkk-1 levels. On the contrary P1NP showed a positive correlation with both sclerostin ($r = 0.71$, $p < 0.01$) and Dkk-1 ($r = 0.56$, $p < 0.05$), while CTX had a positive correlation with circulating sclerostin ($r = 0.63$, $p < 0.05$) (Fig. 3). Baseline E2, FEI, FSH, and LH did not correlate with circulating sclerostin, Dkk-1, P1NP, and CTX levels. However, given the small sample size these findings should be interpreted with caution.

There was a significant positive correlation between P1NP and CTX values at all-time points during the menstrual cycle ($p < 0.01$). In order to examine the association between E2

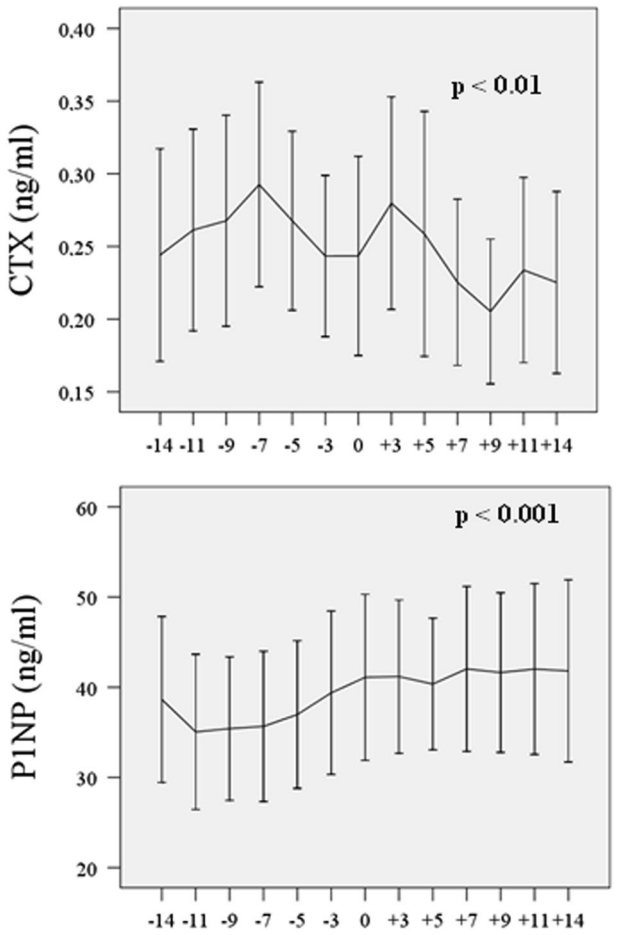

Fig. 2 Mean ± 95 % CIs of serum CTX and P1NP levels during the menstrual cycle. *p*: one-way repeated measures ANOVA

Table 2 Correlation (*r*) of baseline sclerostin and Dkk-1 levels with selected variables

	Sclerostin	Dkk-1
Age	−0.08	−0.28
Height	0.03	0.15
Weight	−0.27	−0.27
BMI	−0.30	−0.39
Calcium	0.38	0.39
Phosphate	0.47	0.39
Creatinine	0.05	−0.39
ALP	−0.21	−0.2
PTH	0.01	0.06
25(OH)D	−0.03	0.56*
E2	0.12	−0.6
FSH	0.15	0.12
LH	0.08	0.26
SHBG	−0.16	0.12
CTX	0.628*	0.38
P1NP	0.71**	0.56*
Dietary calcium intake	−0.15	−0.46
MET 1st week (h/week)	0.500	0.033

* $p < 0.05$, ** $p < 0.01$

and changes of CTX and P1NP during the menstrual cycle we examined the correlation between percent change of P1NP and CTX with mid-cycle E2 levels. There was no association between mid-cycle E2 and percent change of P1NP at all-time points during the luteal phase. Furthermore absolute P1NP levels did not correlate at any time point with either E2 or FEI. On the contrary there was significant negative association between E2 levels at ovulation and percent change of CTX at all-time points during the luteal period ($r = -0.60$ to -0.68, $p < 0.05$–0.01), compatible with the restraining effects of estradiol on bone resorption (Fig. 3). No association was observed between mid-cycle LH and FSH with percent changes of CTX and P1NP. Finally there was no consistent relationship between physical activity with sclerostin and Dkk-1 levels at any time point during the menstrual cycle.

Discussion

The aim of this study was to examine whether circulating sclerostin and Dkk-1 levels change during the menstrual cycle and if the cyclic changes of E2 and gonadotrophins during a menstrual period modulate sclerostin and Dkk-1 levels, attending in parallel P1NP and CTX variations. We did not observe any significant variation neither in sclerostin nor in Dkk-1 across the menstrual cycle, in spite of the normal menstrual pattern of serum female hormones. On the contrary, bone markers did vary during the cycle; CTX tended to be significantly higher in the FP relative to LP, while P1NP presented a reverse pattern. The latter finding is consistent with previous studies, implying that bone resorption is relatively increased during the "estrogen deficient" FP, while bone formation increases in the LP [19–24]. Although the observed changes were small and close to the the critical significance, given that we controlled for almost all known factors affecting biological variability, the observed changes probably represent a true change [25].

Regarding sclerostin our results are in agreement with Cidem et al. who examined sclerostin levels among three time points during the menstrual cycle; menstruation (days 2–5), late follicular (days 11–13), and mid-luteal phase (days 21–23), without correcting for FP duration variability [18]. However, the majority of the studies, investigating the effect of estrogens on sclerostin, including in vitro studies and animal models, elderly men and postmenopausal

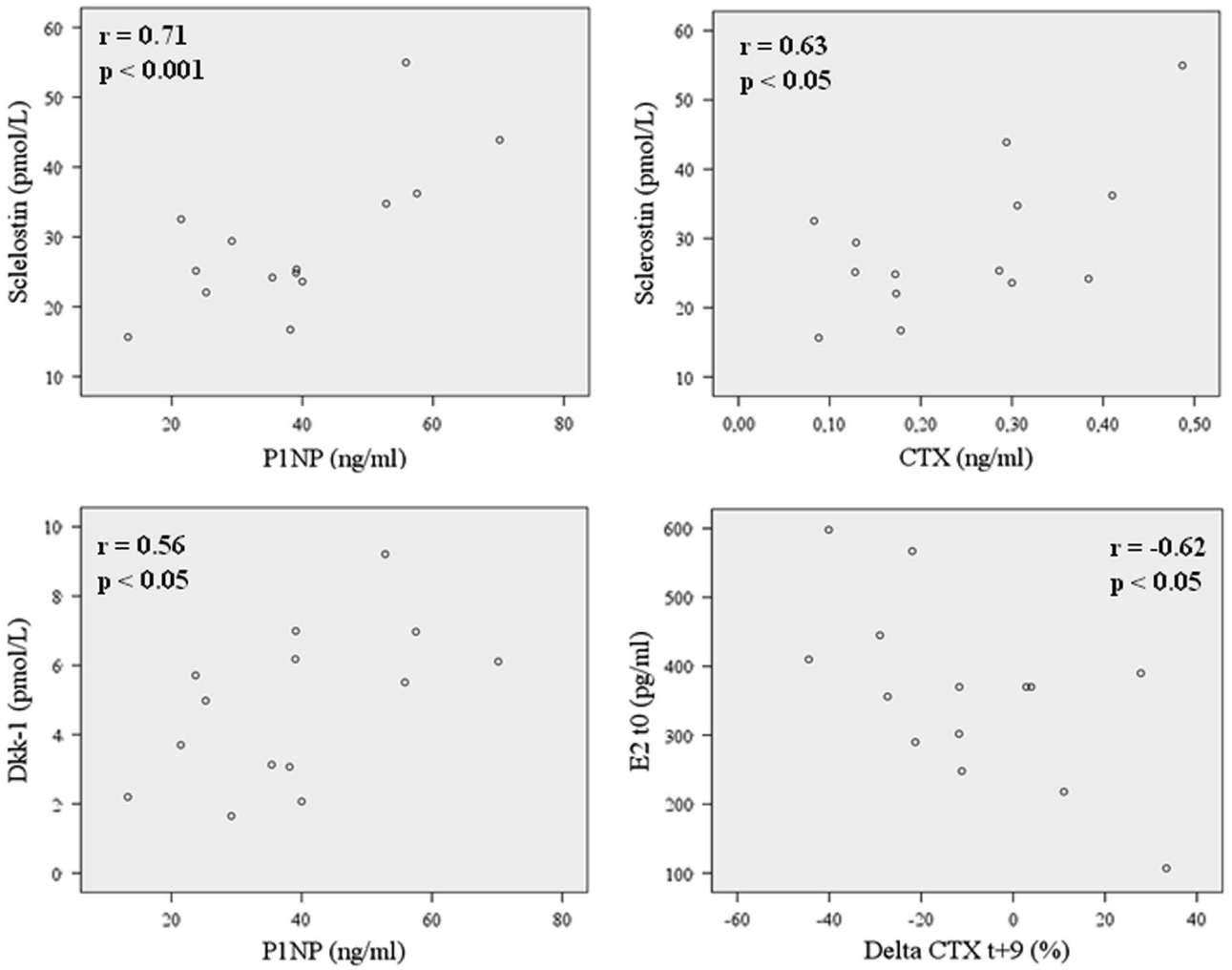

Fig. 3 Correlation between baseline (time −14) serum sclerostin and P1NP, sclerostin and CTX, Dkk-1, and P1NP, and E2 at mid-cycle with percent change of CTX at time +9 over mid-cycle value (delta $CTX_{time\ +9}$)

women, under estradiol treatment or not, suggest estrogens are negative modulators of sclerostin expression [13, 15, 26]. It has been demonstrated that postmenopausal women have substantially higher sclerostin levels (approximately double) compared to premenopausal [13, 27], while the administration of estrogens in postmenopausal women is accompanied by a decrease in circulating sclerostin levels [15, 28]. Specifically, 17β-estradiol (100 µg/d) administration by cutaneous patches for 3 [17] or 4 [29] weeks in postmenopausal women led to 28–27 % decrease in circulating sclerostin levels. Moreover, a strong negative correlation between serum sclerostin levels and free estrogen index ($r = -0.562$, $p = 0.01$) has been reported in postmenopausal women [13]. There are no reports examining the relationship between Dkk-1 and estrogens.

We did not find correlation between estradiol, FSH, or LH with sclerostin. Ardawi et al. in 1235 premenopausal Saudi women reported significant univariate negative association between sclerostin with E2 ($r = -0.519$) and positive with FSH ($r = 0.595$). However, following age-adjustment and BMI-adjustment these associations were attenuated and reversed [E2: $r = 0.091$, FSH: $r = -0.122$] [27]. In contrast, in the study of Mödder et al. in 123 premenopausal women, there was no association between E2 and sclerostin levels [28].

Concerning Dkk-1 we did not observe any correlation with E2 and gonadotrophins. The scarce data relating osteoblast and osteocyte Dkk-1 expression with circulating Dkk-1 levels [16], the multiple sources of Dkk-1 and certainly the small number of subjects in our study precludes any definite conclusion.

The association of circulating sclerostin levels with bone turnover markers has been studied by several groups, reporting conflicting results. The largest study by Ardawi et al. reported a negative association with P1NP and CTX [27], while there was no correlation in the studies of Mödder et al. (P1NP, CTX) [29] and Amrein et al. (CTX) [30, 29]. In contrast, Costa et al. [31] examining circulating

sclerostin levels measured by a different assay (TECOmedical group) in premenopausal and postmenopausal white and Chinese–American women reported a positive association between circulating sclerostin and CTX in whites, but no association in Chinese–American women. In our small study, not designed to examine the baseline association of bone markers with sclerostin or Dkk-1, we observed positive association between P1NP with sclerostin and Dkk-1 and between CTX with sclerostin. Although there is no clear explanation for these inconsistent results, it is possible that differences in the assays used, racial differences or other yet unknown factors underlie these contradictory data.

There are several possible explanations for our negative findings. First, given the possible limited association between circulating sclerostin and osteocyte sclerostin expression, it is possible that subtle changes in local sclerostin levels are not captured by serum sclerostin. Second, a threshold might exist between estrogens and sclerostin, concerning both duration and degree of estrogen "deficiency". It is possible that in the estrogen-replete premenopausal women short term, although relatively abrupt, fluctuations of estrogens during the menstrual cycle do not result in significant changes in circulating sclerostin or Dkk-1 levels. Another possible explanation relates to the specificity of Biomedica sclerostin assay for the intact molecule. It is recently reported that it also recognizes protein fragments similar to sclerostin, thus raising the possibility that a more specific assay might give different results [30, 32]. Finally, the small sample size of our study might precluded the ability to capture subtle changes in sclerostin levels among the time points of the cycle.

Our small sample size represents a limitation in terms of the evaluation of the association between baseline circulating sclerostin and Dkk-1 levels with bone turnover markers. Furthermore we did not measure bone mineral density (BMD) at baseline, although correction for BMD would probably not alter the results. Moreover, we did not evaluate the type of physical activity that might affect circulating scelostin levels [33]. However, none of the participants were involved in regular exercise or sports and most importantly there were no significant difference in the intensity of physical activity between the first and the third week of the menstrual cycle. Finally, we did not measure progesterone throughout the cycle. Although progesterone receptors are expressed in osteoblasts [34], the small number of studies examining the effect of progesterone changes during the menstrual cycle [24] or the effect of progesterone administration in postmenopausal women on bone turnover markers reported no [35, 36] or very small effect [37]. Moreover, we did not find any report concerning the association of progesterone with osteocytes or sclerostin and Dkk-1.

In conclusion, our study demonstrated a lack of variation of circulating sclerostin and Dkk-1 across the menstrual cycle and absence of any detectable effect of E2 or gonadotrophins in healthy premenopausal women. In accordance with previous studies P1NP and CTX levels presented cyclic variations. To our knowledge, this is the first study to investigate the regulation of sclerostin and Dkk-1 by E2 throughout the menstrual cycle. The physiological significance of sclerostin and Dkk-1 on bone turnover in healthy premenopausal women needs to be determined in further studies.

Author contribution Design and conduct of the study: Chrysoula G. Liakou, Julia D. Antoniou, George Mastorakos, Symeon Tournis. Biochemical analysis of the samples: Konstantinos Makris, Helen Marketos, Chrysoula G. Liakou, Dimitrios Rizos. Physical activity and dietary assessment: Ioannis G. Fatouros, Alexandra Avloniti. Data collection and analysis: Antonios Galanos, Chrysoula G. Liakou, George Mastorakos, Ioannis G. Fatouros, Alexandra Avloniti, Symeon, Tournis. Data interpretation: Chrysoula G. Liakou, George Mastorakos, Ioannis G. Fatouros, Konstantinos Makris, Symeon Tournis. Manuscript writing: Chrysoula G. Liakou, George Mastorakos, Ioannis G. Fatouros, Konstantinos Makris, Ismene Dontas, Symeon Tournis.

Compliance with ethical standards

Conflict of interest The authors declare that they have no competing interests.

References

1. K.E. Poole, R.L. van Bezooijen, N. Loveridge, H. Hamersma, S.E. Papapoulos, C.W. Lowik, J. Reeve, Sclerostin is a delayed secreted product of osteocytes that inhibits bone formation. FASEB J. **19**, 1842–1844 (2005)
2. R. Baron, G. Rawadi, S. Roman-Roman, Wnt signaling: a key regulator of bone mass. Curr. Top. Dev. Biol. **76**, 103–127 (2006)
3. R. Baron, G. Rawadi, Targeting the Wnt/beta-catenin pathway to regulate bone formation in the adult skeleton. Endocrinology **148**, 2635–2643 (2007)
4. M.K. Sutherland, J.C. Geoghegan, C. Yu, E. Turcott, J.E. Skonier, D.G. Winkler, J.A. Latham, Sclerostin promotes the apoptosis of human osteoblastic cells: a novel regulation of bone formation. Bone **35**, 828–835 (2004)
5. W. Balemans, M. Ebeling, N. Patel, E. Van Hul, P. Olson, M. Dioszegi, C. Lacza, W. Wuyts, J. Van Den Ende, P. Willems, A.F. Paes-Alves, S. Hill, M. Bueno, F.J. Ramos, P. Tacconi, F.G. Dikkers, C. Stratakis, K. Lindpaintner, B. Vickery, D. Foernzler, W. Van Hul, Increased bone density in sclerosteosis is due to the deficiency of a novel secreted protein (SOST). Hum. Mol. Genet. **10**, 537–543 (2001)
6. W. Balemans, N. Patel, M. Ebeling, E. Van Hul, W. Wuyts, C. Lacza, M. Dioszegi, F.G. Dikkers, P. Hildering, P.J. Willems, J.B. Verheij, K. Lindpaintner, B. Vickery, D. Foernzler, W. Van Hul, Identification of a 52 kb deletion downstream of the SOST gene in patients with van Buchem disease. J. Med. Genet. **39**, 91–97 (2002)
7. D.G. Winkler, M.K. Sutherland, J.C. Geoghegan, C. Yu, T. Hayes, J.E. Skonier, D. Shpektor, M. Jonas, B.R. Kovacevich, K. Staehling-Hampton, M. Appleby, M.E. Brunkow, J.A. Latham,

Osteocyte control of bone formation via sclerostin, a novel BMP antagonist. EMBO J. **22**, 6267–6276 (2003)
8. A.G. Robling, P.J. Niziolek, L.A. Baldridge, K.W. Condon, M.R. Allen, I. Alam, S.M. Mantila, J. Gluhak-Heinrich, T.M. Bellido, S.E. Harris, C.H. Turner, Mechanical stimulation of bone in vivo reduces osteocyte expression of Sost/sclerostin. J. Biol. Chem. **283**, 5866–5875 (2008)
9. F. Morvan, K. Boulukos, P. Clement-Lacroix, S. Roman Roman, I. Suc-Royer, B. Vayssiere, P. Ammann, P. Martin, S. Pinho, P. Pognonec, P. Mollat, C. Niehrs, R. Baron, G. Rawadi, Deletion of a single allele of the Dkk1 gene leads to an increase in bone formation and bone mass. J. Bone Miner. Res. **21**, 934–945 (2006)
10. J. Li, I. Sarosi, R.C. Cattley, J. Pretorius, F. Asuncion, M. Grisanti, S. Morony, S. Adamu, Z. Geng, W. Qiu, P. Kostenuik, D.L. Lacey, W.S. Simonet, B. Bolon, X. Qian, V. Shalhoub, M.S. Ominsky, H. Zhu Ke, X. Li, W.G. Richards, Dkk1-mediated inhibition of Wnt signaling in bone results in osteopenia. Bone **39**, 754–766 (2006)
11. F.S. Wang, J.Y. Ko, C.L. Lin, H.L. Wu, H.J. Ke, P.J. Tai, Knocking down dickkopf-1 alleviates estrogen deficiency induction of bone loss. A histomorphological study in ovariectomized rats. Bone **40**, 485–492 (2006)
12. G. Zaman, L.K. Saxon, A. Sunters, H. Hilton, P. Underhill, D. Williams, J.S. Price, L.E. Lanyon, Loading-related regulation of gene expression in bone in the contexts of estrogen deficiency, lack of estrogen receptor alpha and disuse. Bone **46**, 628–642 (2010)
13. F.S. Mirza, I.D. Padhi, L.G. Raisz, J.A. Lorenzo, Serum sclerostin levels negatively correlate with parathyroid hormone levels and free estrogen index in postmenopausal women. J. Clin. Endocrinol. Metab. **95**, 1991–1997 (2010)
14. Y.E. Chung, S.H. Lee, S.Y. Lee, S.Y. Kim, H.H. Kim, F.S. Mirza, S.K. Lee, J.A. Lorenzo, G.S. Kim, J.M. Koh, Long-term treatment with raloxifene, but not bisphosphonates, reduces circulating sclerostin levels in postmenopausal women. Osteoporos. Int. **23**, 1235–1243 (2012)
15. U.I. Mödder, J.A. Clowes, K. Hoey, J.M. Peterson, L. McCready, M.J. Oursler, B.L. Riggs, S. Khosla, Regulation of circulating sclerostin levels by sex steroids in women and in men. J. Bone Miner. Res. **26**, 27–34 (2011)
16. S. Rinaldi, A. Geay, H. Déchaud, C. Biessy, A. Zeleniuch-Jacquotte, A. Akhmedkhanov, R.E. Shore, E. Riboli, P. Toniolo, R. Kaaks, Validity of free testosterone and free estradiol determinations in serum samples from postmenopausal women by theoretical calculations. Cancer Epidemiol. Biomarkers Prev. **11**, 1065–1071 (2002)
17. S. Tournis, E. Michopoulou, I.G. Fatouros, I. Paspati, M. Michalopoulou, P. Raptou, D. Leontsini, A. Avloniti, M. Krekoukia, V. Zouvelou, A. Galanos, N. Aggelousis, A. Kambas, I. Douroudos, G.P. Lyritis, K. Taxildaris, N. Papaioannou, Effect of rhythmic gymnastics on volumetric bone mineral density and bone geometry in premenarcheal female athletes and controls. J. Clin. Endocrinol. Metab. **95**, 2755–2762 (2010)
18. M. Cidem, T.A. Usta, I. Karacan, S.H. Kucuk, M. Uludag, K. Gun, Effects of sex steroids on serum sclerostin levels during the menstrual cycle. Gynecol. Obstet. Invest. **75**, 179–184 (2013)
19. I. Gorai, Y. Taguchi, O. Chaki, R. Kikuchi, M. Nakayama, B.C. Yang, S. Yokota, H. Minaguchi, Serum soluble interleukin-6 receptor and biochemical markers of bone metabolism show significant variations during the menstrual cycle. J. Clin. Endocrinol. Metab. **83**, 326–332 (1998)
20. K.M. Chiu, J. Ju, D. Mayes, P. Bacchetti, S. Weitz, C.D. Arnaud, Changes in bone resorption during the menstrual cycle. J. Bone Miner. Res. **4**, 609–615 (1999)
21. H.K. Nielsen, K. Brixen, R. Bouillon, L. Mosekilde, Changes in biochemical markers of osteoblastic activity during the menstrual cycle. J. Clin. Endocrinol. Metab. **70**, 1431–1437 (1990)
22. I. Gorai, O. Chaki, M. Nakayama, H. Minaguchi, Urinary biochemical markers for bone resorption during the menstrual cycle. Calcif. Tissue Int. **57**, 100–104 (1995)
23. M.L. Gass, R. Kagan, J.D. Kohles, M.G. Martens, Bone turnover marker profile in relation to the menstrual cycle of premenopausal healthy women. Menopause **15**, 667–675 (2008)
24. B. Mozzanega, S. Gizzo, D. Bernardi, L. Salmaso, T.S. Patrelli, R. Mioni, L. Finos, G.B. Nardelli, Cyclic variations of bone resorption mediators and markers in the different phases of the menstrual cycle. J. Bone Miner. Metab. **31**, 461–467 (2013)
25. G. Lombardi, P. Lanteri, A. Colombini, G. Banfi, Blood biochemical markers of bone turnover: pre-analytical and technical aspects of sample collection and handling. Clin. Chem. Lab. Med. **50**, 771–789 (2012)
26. G. Mabilleau, A. Mieczkowska, M.E. Edmonds, Thiazolidinediones induce osteocyte apoptosis and increase sclerostin expression. Diabet. Med. **27**, 925–932 (2010)
27. M.S. Ardawi, H.A. Al-Kadi, A.A. Rouzi, M.H. Qari, Determinants of serum sclerostin in healthy pre- and postmenopausal women. J. Bone Miner. Res. **26**, 2812–2822 (2011)
28. U.I. Mödder, K.A. Hoey, S. Amin, L.K. McCready, S.J. Achenbach, B.L. Riggs, L.J. Melton III, S. Khosla, Relation of age, gender, and bone mass to circulating sclerostin levels in women and men. J. Bone Miner. Res. **26**, 373–379 (2011)
29. K. Amrein, S. Amrein, C. Drexler, H.P. Dimai, H. Dobnig, K. Pfeifer, A. Tomaschitz, T.R. Pieber, A. Fahrleitner-Pammer, Sclerostin and its association with physical activity, age, gender, body composition, and bone mineral content in healthy adults. J. Clin. Endocrinol. Metab. **97**, 148–154 (2012)
30. C. Durosier, A. van Lierop, S. Ferrari, T. Chevalley, S. Papapoulos, R. Rizzoli, Association of circulating sclerostin with bone mineral mass, microstructure, and turnover biochemical markers in healthy elderly men and women. J. Clin. Endocrinol. Metab. **98**, 3873–3883 (2013)
31. A.G. Costa, M.D. Walker, C.A. Zhang, S. Cremers, E. Dworakowski, D.J. McMahon, G. Liu, J.P. Bilezikian, Circulating sclerostin levels and markers of bone turnover in Chinese-American and white women. J. Clin. Endocrinol. Metab. **98**, 4736–4743 (2013)
32. B.L. Clarke, M.T. Drake, Clinical utility of serum sclerostin measurements. Bonekey Rep. **2**, 361 (2013)
33. G. Lombardi, P. Lanteri, A. Colombini, M. Mariotti, G. Banfi, Sclerostin concentrations in athletes: role of load and gender. J. Biol. Regul. Homeost. Agents. **26**, 157–163 (2012)
34. Z.A. Zhong, H. Sun WChen, H. Zhang, N.E. Lane, W. Yao, Inactivation of the progesterone receptor in Mx1+ cells potentiates osteogenesis in calvaria but not in long bone. PLoS One **10**, e0139490 (2015)
35. J.H. Liu, K.N. Muse, The effects of progestins on bone density and bone metabolism in postmenopausal women: a randomized controlled trial. Am. J. Obstet. Gynecol. **192**, 1316–1323 (2005)
36. Z. Ikram, L. Dulipsingh, K.M. Prestwood, Lack of effect of short-term micronized progesterone on bone turnover in postmenopausal women. J. Womens Health Gend. Based Med. **7**, 973–8 (1999)
37. G. Azizi, A. Hansen, K.M. Prestwood, Effect of micronized progesterone on bone turnover in postmenopausal women on estrogen replacement therapy. Endocr. Res. **29**, 133–140 (2003)