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Chapter 6

Short-term effect of estrogen on human bone marrow fat.

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ABSTRACT

Bone marrow fat, a unique component of the bone marrow cavity increases with aging and menopause and is inversely related to bone mass. Sex steroids may be involved in the regulation of bone marrow fat, because men have higher bone marrow fat than women and clinical observations have suggested that the variation in bone marrow fat fraction is greater in premenopausal compared to postmenopausal women and men. We hypothesized that the menstrual cycle and/or estrogen affects the bone marrow fat fraction. First, we measured vertebral bone marrow fat fraction with Dixon Quantitative Chemical Shift MRI (QCSI) twice a week during one month in 10 regularly ovulating women.

The vertebral bone marrow fat fraction increased 0.02 (95% CI 0.00 to 0.03) during the follicular phase (p=0.033), and showed a non-significant decrease of 0.02 (95% CI -0.01 to 0.04) during the luteal phase (p=0.091). To determine the effect of estrogen on bone marrow fat, we measured vertebral bone marrow fat fraction every week for 6 consecutive weeks in 6 postmenopausal women before, during and after two weeks of oral 17-β estradiol treatment (2 mg/day). Bone marrow fat fraction decreased by 0.05 (95% CI 0.01 to 0.09) from 0.48 (95% CI 0.42 to 0.53) to 0.43 (95% CI 0.34 to 0.51) during 17-β estradiol administration (p<0.001) and increased again after cessation. During 17-β estradiol administration the bone formation marker procollagen type I N propeptide (P1NP) increased (p=0.034) and the bone resorption marker C-terminal crosslinking telopeptides of collagen type I (CTx) decreased (p<0.001).

In conclusion, we described the variation in vertebral bone marrow fat fraction among ovulating premenopausal women. And among postmenopausal women, we demonstrated that 17-β estradiol rapidly reduces the marrow fat fraction, suggesting that 17-β estradiol regulates bone marrow fat independent of bone mass.

INTRODUCTION

Bone marrow fat is functionally distinct from and not subject to the same regulation as subcutaneous and visceral fat depots.[1] This is best exemplified by caloric restriction, which increases bone marrow adipose tissue despite marked reductions in body fat in mice.[2] Osteoblasts and adipocytes arise from the same mesenchymal precursor cell present in bone marrow stroma.[3] Consequently, within the marrow cavity a balance exists between adipose tissue and bone. Previous observations indicate that men have higher bone marrow fat than women.[4] During aging, there is a progressive fatty infiltration of bone marrow.[5] Conditions associated with bone loss, like osteoporosis[6], are also characterized by an increase in bone marrow adiposity and these changes are associated with increased fracture risk.[7] This inverse relationship with bone mineral density is well documented and potentially makes bone marrow fat a therapeutic target for osteoporosis.[8,9]

While the quantification of bone marrow fat previously required invasive bone biopsies, it can now reliably be quantified by non-invasive techniques including magnetic resonance imaging (MRI) and spectroscopy.[10] To date, only long-term changes in bone marrow fat have been investigated. [11-13] A reciprocal relation between marrow adiposity and the amount of bone was observed in women aged 15-20 at baseline and after 18-24 months.[11] In addition, an increase in bone marrow fat was observed in ten out of 11 healthy volunteers that were re-evaluated after 15 years.[12] A recent study showed that bone mass was lower six months after gastric bypass surgery, whereas bone marrow fat did not change in non-diabetic (n=5) and decreased in diabetic subjects (n=6).[13] In this present study we determined short-term changes, i.e. within 2-4 weeks, which allows the study of bone marrow fat independent of (detectable) changes in bone mass.

In our clinic, we routinely measure vertebral bone marrow fat fractions in patients with type 1 Gaucher disease by Dixon quantitative chemical shift MRI (QCSI) to assess bone marrow involvement and response to therapy.[14] We observed considerable variation in the vertebral bone marrow fat fraction in premenopausal female Gaucher disease patients. This variation was not related to disease activity and was substantially less in men and postmenopausal women. We hypothesized that the menstrual cycle and/or estrogen affects the bone marrow fat fraction. First, we determined vertebral bone marrow fat fraction as a function of the phase of the menstrual cycle in healthy young women during one menstrual cycle. Next, we determined the effect of 17-β estradiol administration on bone marrow fat in estrogen deficient postmenopausal women.

MATERIALS AND METHODS

Subjects

First we included healthy female subjects aged 18-50 years with self-reported regular menstrual cycles between 25 and 35 days in the preceding year. An ovulatory menstrual cycle was verified by serum progesterone concentrations above 12 nmol/L during the luteal phase. For the second study we included healthy postmenopausal women between 50 and 60 years of age, who had their last menstrual cycle more than 12 months prior to inclusion. Postmenopausal status was confirmed by an elevated follicle stimulating-hormone (FSH) concentration (> 20 E/L) and 17-β estradiol level below the detection limit (0.04 nmol/L). Exclusion criteria for participation were contra-indications to magnetic resonance imaging (MRI), use of bone-modifying or adipose-tissue modifying drugs and use of hormonal contraception or contraindications to 17-β estradiol administration. Subjects were not allowed to have a bone, adipose tissue or bone marrow disease, or a T-score larger than -3.5. Subjects were recruited from the general population by advertisements in public spaces.

Study design

The study was performed at the department of Endocrinology and Metabolism of the Academic Medical Center of the University of Amsterdam in the Netherlands. At baseline we took a complete (family) history, measured weight and height and performed dual energy X-ray absorptiometry (DXA) scanning (Hologic Discovery, Bedford, MA, USA; APEX system software version 3.3) to measure bone mineral density (BMD). The premenopausal subjects were studied twice a week for 4 consecutive weeks (8 times in total) and the postmenopausal subjects once a week for 6 consecutive weeks (7 times in total). During this study period vertebral (L3-5) fat fraction and serum concentrations of FSH, LH, progesterone, 17-β estradiol, CBC, reticulocyte percentage, calcium, phosphate, alkaline phosphatase, PTH and markers of bone turnover (procollagen type I N propeptide (P1NP) and C-terminal crosslinking telopeptides of collagen type I (CTx)) were determined. CTx and P1NP are recommended by the International Osteoporosis Foundation and the International Federation of Clinical Chemistry and Laboratory Medicine as international reference markers for bone
resorption and formation. All postmenopausal subjects received no intervention day 0 to day 14, received oral 17-β estradiol administration 2 mg daily (Zumenon, Abbott Products BV, Weesp, Netherlands) day 14 to day 28 and again no treatment day 28 to day 42. The main outcome of the study was the variation in vertebral bone marrow fat fraction during the menstrual cycle and change in vertebral bone marrow fat fraction in response to 17-β estradiol administration.

Dixon QCSI vertebral bone marrow fat fraction measurement

Vertebral bone marrow fat fraction was measured on a 1.5 T, Magnetom Avanto (Siemens, Erlangen, Germany), using an in-house two-point Dixon spin-echo sequence. Dixon imaging uses the MR-frequency difference of fat and water to produce two types of images: in-phase images, which contain the sum of the water and fat signals, and opposed-phase images, containing the difference of these signals. Lines of the in-phase and opposed-phase image of the same slice are acquired in an interleaved fashion. Imaging parameters were: TR 2500 ms, TE 22.3 ms, slice thickness 4 mm, matrix 256x256, NEX 1, FOV 350x350 mm², resulting in an acquisition time of 21 min 20 s. A single slice was measured, which was positioned on a midsagittal localizer image, as illustrated in Figure 1. First, the slice was positioned, such that it passes through the posterior part of L4 and parallel to the spinal cord (Figure 1A). Next slight angulation adjustments were made to obtain similar cross sections through L3 and L5. In subsequent measurements of the same volunteer, the slice was positioned in exactly the same position. To avoid motion and respiration artifacts we used a small field-of-view surface coil (the "small flex coil") flattened out and placed underneath the body along the spine. From the acquired Dixon images a fat signal fraction image and a water only image were calculated, using an algorithm described in.[15] In the water only image, the contours of L3, L4 and L5 were manually drawn. The interior of these contours was subjected to four erosion operations, resulting in the regions of interest, which were used to calculate the average fat signal fraction in the three vertebrae. Details of the procedure are illustrated in Figure 1. Further details on the acquisition and post-processing procedures have been described previously.[16] A previous study by our department has shown good reproducibility of vertebral fat fraction measured by QCSI, with a coefficient of variation of 6.0%[16] Briefly, 16 healthy male and female volunteers underwent QCSI bone marrow fat fraction measurements on three different occasions. The coefficient of variation was computed as the ratio of the average standard deviation and the average mean.

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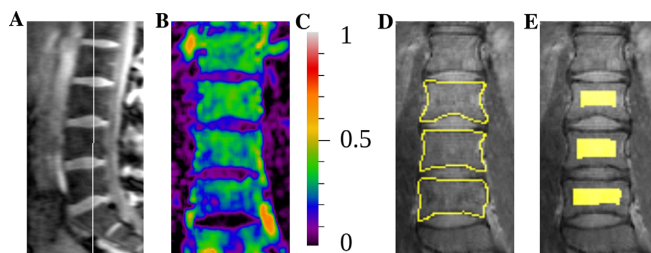
Figure 1 Fat signal fraction measurement details. a) The QCSI slice, indicated by the white line, positioned on a midsagittal localizer image, b) Fat signal fraction image, with c) color coding: from black (no fat), to white (all fat), d) contours of L3, L4 and L5, e) regions of interest.

Biochemical assessment

Serum concentrations of calcium, albumin, phosphate, alkaline phosphatase, creatinine and PTH were measured on a Hitachi Modular 8000 (C602 and C702) (Roche Diagnostics, Mannheim, Germany). 25(OH)D was measured using an automated immunoassay (Liaison, Diasorin, Stillwater, MN, USA). 17-β-estradiol was measured by radioimmunoassay (Siemens, Breda, the Netherlands). Hematologic parameters were measured on a Sysmex XE 500 (Sysmex Corporation, Kobo, Japan). Serum concentrations of CTx and P1NP were measured using an immunoassay (Modular Analytics E 170; Roche Diagnostics; and Orion Diagnostica, Espoo, Finland, respectively). Interassay coefficients of variation were as follows: 25-hydroxyvitamin D 7.2%, LH 1.7%, FSH 1.2%, progesterone 1.6%, 17-β estradiol 5.4%, CTx 3% and P1NP 8%. Serum samples for CTx and P1NP measurements were stored at -20°C until analysis and performed in duplicate at the end of the study period using one lot number reagents. All blood samples were collected between 7:00 and 9:00 h after an overnight fast.

Statistical analysis

The statistical analysis was carried out with SPSS for Windows (version 22.0; SPSS Inc., Chicago, IL, USA). The mean and 95% confidence interval (95% CI) or the median and range are reported depending on the distribution. Each menstrual cycle was normalized to 28 days and the day of the onset of menstruation was defined as day one. Since the measurements were obtained twice a week, the interval between the measurements was either three or four days. To examine summary values, the visits were assigned to 3.5-day bins. Cycles were divided into the follicular and luteal phase depending on serum progesterone and LH concentrations (progesterone >3 nmol/L or LH >25 E/L). To determine the effect of time on BM fat fraction linear mixed model (LMM) analysis with correction for repeated measurements (compound symmetry) was used. To meet the assumption of linearity, the effects of the follicular and luteal phase were analyzed separately. LMM analyses were also performed to assess the association between fat fraction, CTx or P1NP and 17-β estradiol administration in the postmenopausal group. The (absolute) residuals were used to test the assumptions of 1) normality (QQ plot and Shapiro-Wilk test) and 2) equal variances (Levene's test). When either or both assumptions were violated, the dependent variable was rank transformed (replacing the values by their ranks, or average ranks in case of ties) and effects were re-estimated. All statistical tests were two-sided and a P-value of 0.05 was considered significant.

Study approval

This study was approved by the Institutional Review Board of the Academic Medical Center, and was carried out in compliance with the Helsinki Declaration.

The intervention part of the study was registered in the Netherlands Trial Register (NTR4521) before start of the study. All subjects agreed to participate in the study and provided written informed consent.

RESULTS

Vertebral bone marrow fat fraction and the menstrual cycle

We included 10 subjects with a regular menstrual cycle with a median cycle length of 28 (range 24-30) days (Table 1). None of the subjects smoked or used more than 7 units of alcohol per week. One subject used a low-dose SSRI for obsessive compulsive disorder. All subjects had glucose, calcium, phosphate, alkaline phosphatase and PTH concentrations within the reference range. Median 25-hydroxyvitamin D concentration was 58 (range 34-112) nmol/L. Vertebral bone marrow fat fraction ranged from 0.18 to 0.42. There was a small but consistent pattern of variation in the vertebral bone marrow fat fraction during the menstrual cycle. Bone marrow fat fraction increased 0.02 (95% CI 0.00 to 0.03) during the follicular phase (p=0.033), and showed a non-significant decrease of 0.02 (95% CI -0.01 to 0.04) during the luteal phase (p=0.091) (Figure 2). Baseline BMD of the femoral neck and vertebral fat fraction were inversely correlated (r=-0.84, p=0.002), however there was no significant correlation between BMD of the lumbar spine and vertebral fat fraction (r=-0.37, p=0.293). Serum concentrations of bone formation marker P1NP were higher during the luteal phase compared to the follicular phase (p=0.002) of the menstrual cycle (Figure 3A). Serum concentrations of the bone resorption marker CTX during the luteal and follicular phase did not differ (p=0.171) (Figure 3B). Hemoglobin concentrations, leucocyte and reticulocyte counts did not show variation during the menstrual cycle (data not shown).

TABLE 1 Characteristics of women with a regular menstrual cycle

Subject	Age (years)	BMI (kg/m2)	Fat fraction	BMD spine (Z-score)	BMD FN (Z-score)
1	29	25.3	0.30	0,7	1,7
2	24	23.4	0.31	0,6	0,8
3	25	19.5	0.39	-1,8	-0,9
4	25	22.0	0.18	0,4	0,4
5	30	22.9	0.32	-0,1	-0,1
6	28	24.4	0.34	1,1	0
7	29	22.4	0.42	-0,7	-0,4
8	33	19.4	0.31	-0,3	0
9	38	18.9	0.24	2,1	0,8
10	46	24.9	0.40	1,8	0,1

BMI=body mass index, BMD=bone mineral density, FN=femoral neck
Fat fraction value listed is from day one of the menstrual cycle
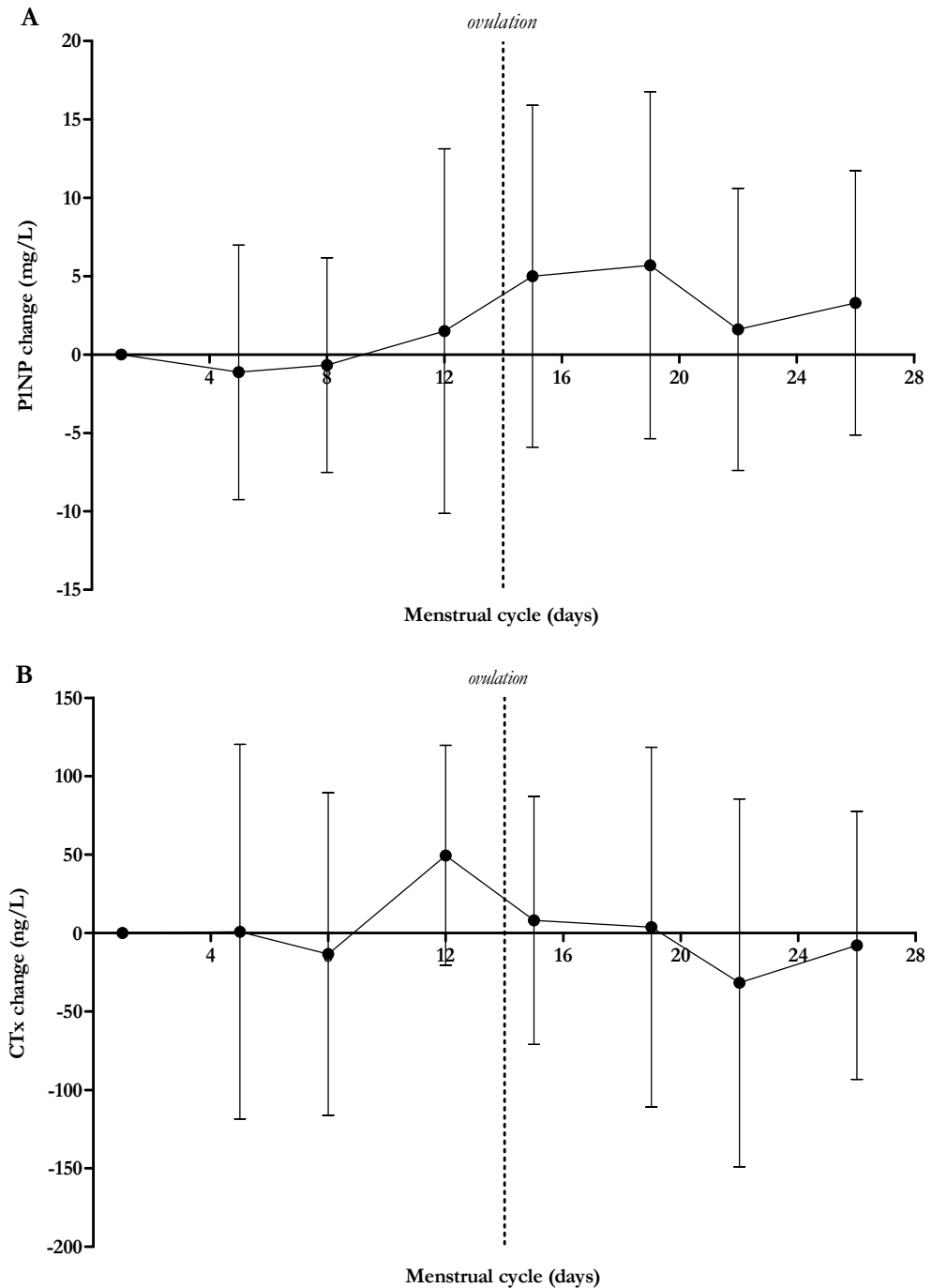
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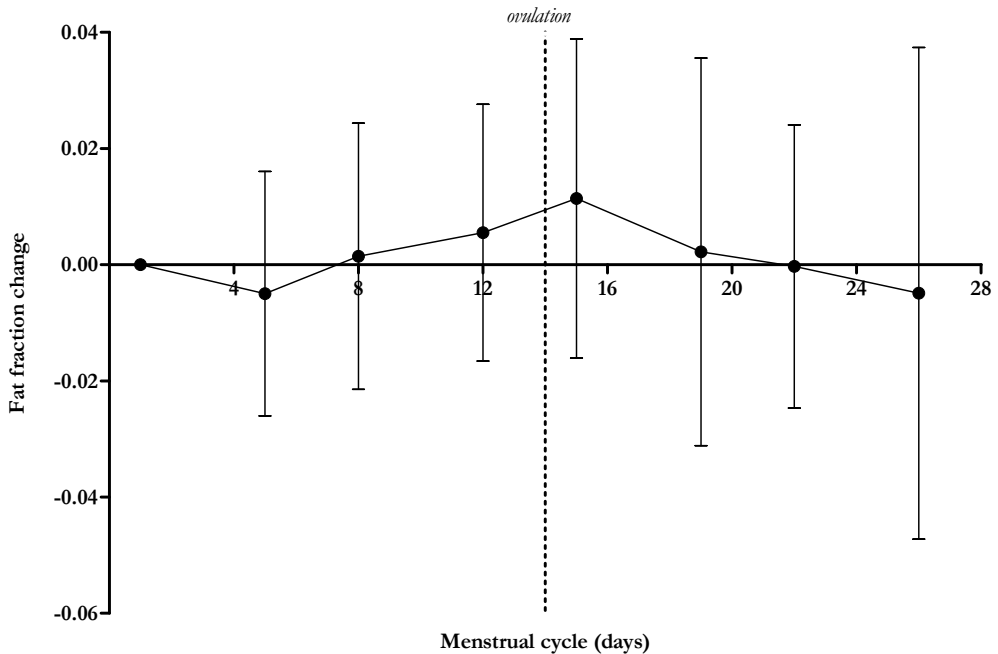
Figure 2 Change in vertebral bone marrow fat fraction from baseline (onset of menstruation = day one) during the menstrual cycle (mean±SD) (follicular phase: p=0.033, luteal phase: p=0.091)**.**

Vertebral bone marrow fat fraction and 17β-estradiol administration

We included 6 healthy postmenopausal women with a median time since the last menstrual cycle of 4.5 (range 3-10) years (Table 2). Serum concentrations of 17-β estradiol increased in all subjects during 17-β estradiol administration (p<0.001). The increase in 17-β estradiol concentration was accompanied by a decrease in LH and FSH concentrations. 17-β estradiol concentration returned to baseline values within one week after discontinuation of treatment (Figure 4). None of the subjects smoked or used medication, or used more than 7 units of alcohol per week. All subjects had glucose, calcium, phosphate, alkaline phosphatase and PTH concentrations within the reference range. Median 25-hydroxyvitamin D concentration was 57 (range 40-95) nmol/L. No adverse events occurred during the intervention period. The mean baseline vertebral bone marrow fat fraction ranged from 0.39 to 0.55. The median variation in vertebral bone marrow fat fraction during the first two study weeks was 0.015 (range 0.003-0.041) (p=0.959). During two weeks of 17-β estradiol administration the mean decrease in vertebral bone marrow fat fraction was 0.05 (95% CI 0.01 to 0.09) (p<0.001) and after discontinuation of 17-β estradiol administration the vertebral bone marrow fat fraction increased again (p=0.002), although BM fat fraction did not return to baseline

A

B

Figure 3 Change in serum concentrations of A) P1NP (µg/L) and B) CTx (ng/L) from baseline (onset of menstruation = day one) during the menstrual cycle (mean±SD). P1NP concentrations were higher during the luteal phase compared to the follicular phase (p=0.002) of the menstrual cycle.

completely (Figure 5). In the postmenopausal women baseline vertebral fat fraction was not correlated with BMD of the femoral neck (r=-0.31, p=0.564) or the lumbar spine (r=-0.31, p=0.564). During 17-β estradiol administration P1NP increased, and returned to baseline concentration following discontinuation (p=0.034) (Figure 6A), whereas CTx decreased during 17-β estradiol administration and increased after 17-β estradiol was stopped (p<0.001) (Figure 6B).

TABLE 2 Characteristics of postmenopausal women

| Subject	Age (years)	Years since menopause	BMI (kg/m2)	Fat fraction	BMD spine (T-score)	BMD FN (T-score)
1	54	3	24.3	0.50	-2.9	-2.0
2	59	5	18.1	0.45	0.0	-1.3
3	54	4	21.7	0.49	0.3	0.8
4	56	3	27.9	0.55	-0.7	-0.6
5	59	10	26.2	0.49	-0.8	-1.1
6	58	5	24.1	0.39	-0.8	-0.3

BMI=body mass index, BMD=bone mineral density, FN=femoral neck
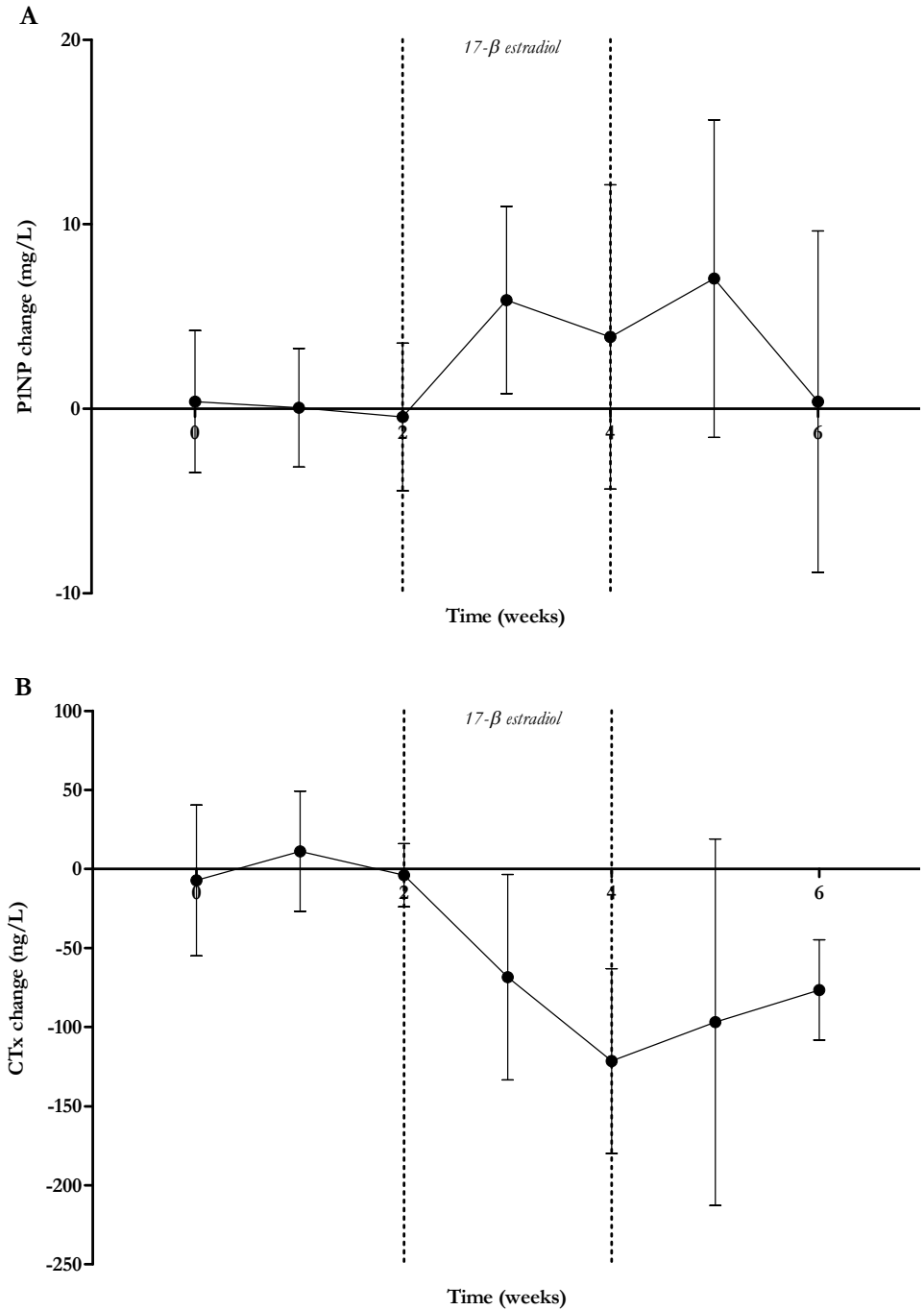
Fat fraction value listed is the mean of the three baseline measurements
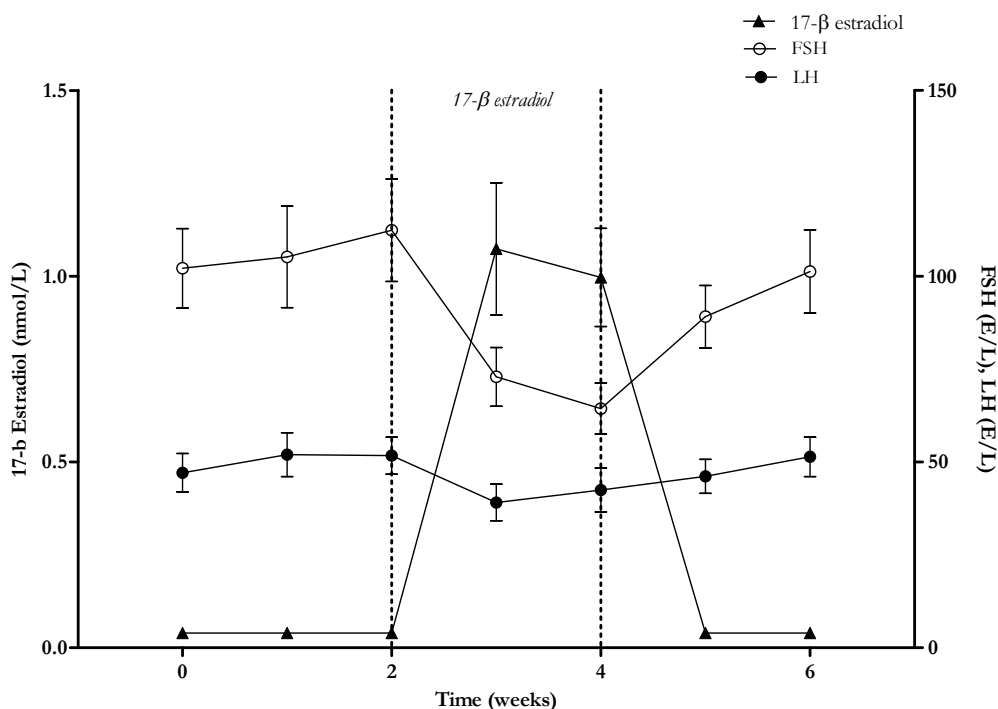
Figure 4 Serum concentrations of 17-β estradiol (nmol/L), FSH (E/L) and LH (E/L) before, during and after 17-β estradiol administration (mean±SD).
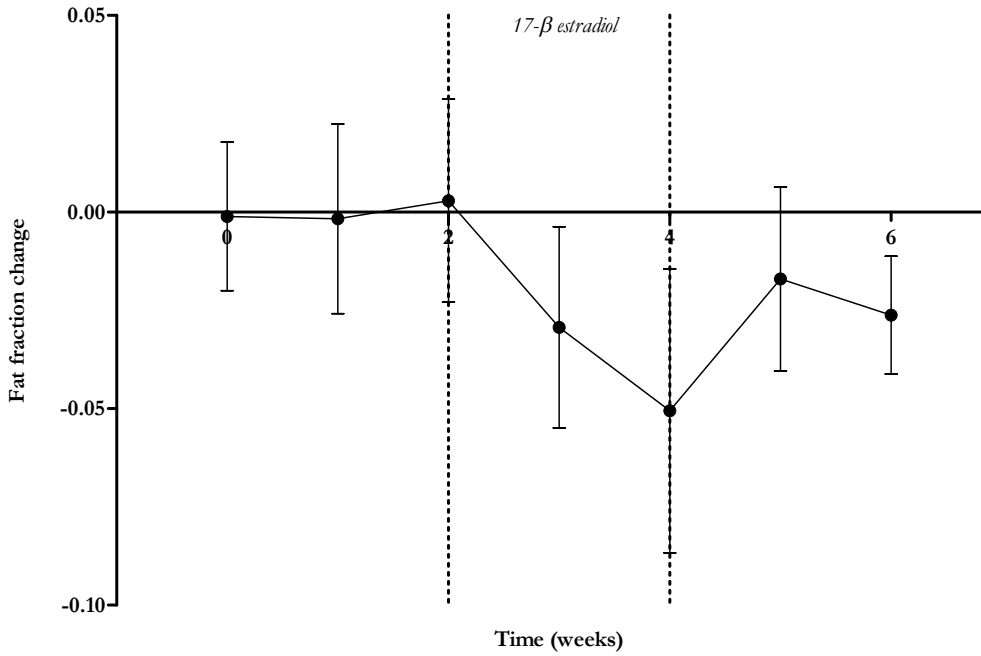
Figure 5 Change in vertebral bone marrow fat fraction before, during and after 17-β estradiol administration (mean±SD) (p<0.001).

A

B

Figure 6 Change in serum concentrations of A) P1NP (μg/L) and B) CTx (ng/L) before, during and after 17-β estradiol administration (mean±SD). P1NP concentrations increased (p=0.034) and CTx concentrations decreased (p<0.001) during 17-β estradiol administration.

DISCUSSION

In this study we observed a consistent increase in vertebral bone marrow fat fraction during the follicular phase of the menstrual cycle, but the size of this effect was small. We demonstrated for the first time that 17-β estradiol rapidly decreases the bone marrow fat fraction in postmenopausal women.

To date, only one study investigated the effect of estrogen on bone marrow adiposity in humans.[17] In bone biopsies of 56 postmenopausal women, decreased adipocyte number and size was seen following one year of transdermal estradiol (n=29) compared to placebo (n=27). This study however did not investigate short-term changes in adipocyte parameters. In cross-sectional studies, postmenopausal estrogen deficiency was found to be associated with an increase in bone marrow fat of approximately 6% per decade.[5] Similar effects were seen in ovariectomized rodents[18], and these effects could be reversed by estrogen replacement.[18]

The rapid changes in BM fat indicate a change in adipocyte number, size or both. The marrow cavity houses a complex environment of several cell populations, including hematopoietic cells, bone cells, adipocytes and their progenitors. Osteoblasts and marrow adipocytes share a common precursor cell, the mesenchymal stem cell (MSC).[3] Consequently, the number of mature osteoblasts and marrow adipocytes is influenced by differentiation of the common progenitor cell towards one phenotype and away from the other.[19] Since the estrogen depletion of menopause coincides with a decrease in bone tissue and an increase in bone marrow fat, there has been a growing interest in the precise role of estrogen in determining mesenchymal stem cell faith. In in vitro studies, estrogen stimulated differentiation of human and murine MSCs to osteoblasts and not to adipocytes.[20,21] These observations suggest an active role of estrogen in the differentiation of the mesenchymal stem cell. A recent study also showed that estrogen suppresses the murine 'osteo-adipogenic transdifferentiation', the ability of a fully differentiated osteoblast to first dedifferentiate and then transdifferentiate into an adipocyte [22]. This indicates that short-term variation in bone marrow fat is possible, similar to our findings.

While we observed a rapid decrease in bone marrow fat fraction during two weeks of 17-β estradiol administration, the bone marrow fat fraction did not return to baseline completely within two weeks after discontinuation. The half-life of 17-β estradiol is approximately 24 hours. As expected, within one week after discontinuation plasma 17-β estradiol concentrations had returned to baseline. This suggests that part of the effect of 17-β estradiol on bone marrow fat persists after 17-β estradiol concentrations have returned to baseline. Estrogen signals mainly through estrogen receptors which have

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been identified in cells of the osteoblast lineage,[23] as well as mesenchymal stem cells.[24] Although purely speculative, the persisting effect of 17-β estradiol after discontinuation could indicate irreversible differentiation towards osteoblasts, which have a typical lifespan of approximately three months.

The effects of estrogen replacement on bone metabolism have long been recognized and are well-studied. Numerous studies indicate that long-term estrogen replacement lowers bone formation and bone resorption rates.[25] Surprisingly, we observed an increase instead of decrease in P1NP during two weeks of 17-β estradiol, indicating increased bone formation. Hannon et al. also observed increased P1NP concentrations after one week of hormone replacement therapy.[25] Together these observations suggest that the effect of estradiol on bone formation is biphasic, with an initial increase followed by decrease. The question remains whether long-term effects of estrogen on BM fat reflect a direct effect of estrogen or a reciprocal effect that is secondary to changes in bone mass. Although not demonstrated directly, the rapid simultaneous decrease in bone marrow fat fraction and increase in P1NP supports the above mentioned in vitro data on the reciprocal relationship between osteoblasts and adipocytes with lineage differentiation of the common progenitor cell towards one cell type at the expense of the other.

The biological relevance of the short-term variation in BM fat fraction needs to be considered. For a long time, marrow adipocytes were regarded as passive fillers of space not occupied by other tissue.[26] More recently it has been recognized that the number of mature osteoblasts is not only determined by progenitor cell faith, but also by an active role of marrow adipocytes in bone physiology. Marrow adipocytes secrete adipokines and fatty acids in the local microenvironment suspected to have a negative effect on osteoblast proliferation and function[27,28], also known as lipotoxicity. Lipotoxicity can be one of the reasons that marrow fat accumulation is negatively associated with measures of bone integrity and fracture risk.[29,30] Therefore, marrow adipose tissue should be regarded as an important indicator of bone integrity, and could possibly serve as an additional predictor for fracture risk in the future. Although we observed significant changes in the bone marrow fat fraction during the menstrual cycle and in particular after 17-β estradiol administration, the clinical relevance of these changes remains to be determined.

Besides estrogen deficiency, starvation has a pronounced influence on both bone marrow adiposity and bone mass. In mice, 30% caloric restriction leads to high bone marrow adiposity.[2] And despite marked reductions in body fat, bone marrow fat increases extremely in patients with anorexia nervosa compared to age-matched normal-weight controls.[31] Because hypoestrogenism is a characteristic of anorexia nervosa, it could be possible that this contributes to the increase in bone marrow adiposity seen in these

patients. In line with this concept, increased lumbar bone marrow fat is seen in anorexia nervosa subjects with amenorrhea compared with anorexia nervosa subjects with a regular menses.[31]

We observed a significant, but small increase in bone marrow fat fraction during the follicular phase of the menstrual cycle, but the decrease during the luteal phase did not reach statistical significance, suggesting that the effect of the menstrual cycle on bone marrow fat fraction is limited. Considering that the coefficient of variation for the bone marrow fat fraction measurement is 6%, detection of significant changes of 1-2% would have required a larger sample size. The size of the effect of 17-β estradiol on the bone marrow fat fraction in postmenopausal women was well above the coefficient of variation. Although we included only 6 postmenopausal women, the paired design resulted in sufficient power to detect an effect. It is unlikely that a larger sample size would have changed the results.

To determine the vertebral BM fat fraction we performed Dixon QCSI, which uses the difference in resonant frequencies between fat and water in bone marrow to compute a fat fraction. The technique cannot determine an absolute signal for water and/or fat. However, a decrease in the fat signal fraction by definition represents a decrease in fat <u>and</u> an increase in water, i.e. a change in bone marrow fat content as expressed by its fat signal fraction reflects the replacement of water inside the bone marrow cavity by fatty tissue or vice versa. In addition, we measured the fat signal fraction in subcutaneous fat (lower back region) as an internal reference standard. The subcutaneous fat fraction did not change during the study period (data not shown). Several studies have confirmed the validity of noninvasive investigation of bone marrow fat fraction.[10,32] MRI measurement of bone marrow fat does not provide information on the properties of the BM adipocytes. Therefore, it remains to be determined whether the changes in BM fat fraction are related to changes in adipocyte number, adipocyte volume or both.

In conclusion, we described the variation in vertebral bone marrow fat fraction among ovulating premenopausal women, and among postmenopausal women, we demonstrated that 17-β estradiol rapidly reduces the marrow fat fraction.

ACKNOWLEDGEMENTS

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Chapter 6