Mutual modulation of femarelle and vitamin D analog activities in human derived female cultured osteoblasts

D. Somjen*, S. Katzburg, O. Sharon, E. Knoll, D. Hendel and G.H. Posner

Abstract: Introduction: Pre-, post-menopausal, and male human osteoblasts (hObs) express estradiol-17β (E2) receptors α and β (ERα, ERβ), vitamin D receptor (VDR), and 25-hydroxy vitamin D3 1α-hydroxylase (1OHase) and produce 1,25 (OH)2D3 (1,25D). Pre-treatment with JKF (JKF 1624F2–2) up-regulated estrogenic responsiveness, via modulation of ERs expression and E2 binding. These estrogens induce VDR and 1OHase expression and 1,25D production. Purpose: Compared the effects of femarelle (F) to daidzein (D) and E2 by themselves and their modulation by JKF. Methods: hObs derived from human bones at different ages and sex were cultured, treated with different hormones and analyzed for the different parameters. Results: (1) F, D and E2 stimulated 3[H] thymidine incorporation (DNA) and creatine kinase specific activity (CK) in female but not in male hObs. The responses to E2 and to D unlike to F were up-regulated by JKF. (2) All hormones increased ERα and decreased ERβ mRNA in all hObs. (3) JKF modulated in different ways the expressions of ERα and ERβ mRNA in all hObs. (4) JKF did not significantly modulate the expressions of ERα and ERβ mRNA in all hObs. (5) JKF increased intracellular competitive binding of E2 by all hormones only in female hObs. (6) Pre-treatment with all hormones increased VDR and 1OHase expression and 1,25D formation in pre- and post-, but only JKF modulated it in male hObs. Conclusions: F, D and E2 increase different parameters in hObs. However, pre-treatment with JKF modulates the effect of E2 and D but not of F. F reciprocally modulates mRNA expression and activity of 1OHase.

ABOUT THE AUTHORS
The authors of the paper are members of the Tel-Aviv medical center and other institutions as reported. We are interested in bone and vascular endocrinology and especially in synthetic estrogens and vitamin D analogs as reported in here and in other previous studies and papers. We have shown their importance and their use with much less side effects as other ones, we believe in their use in human health and hope to promote it, without any commercial benefits to us.

PUBLIC INTEREST STATEMENT
In the last years a lot of interest is in the use of phytoestrogens and their derivatives in human biology and medicine. Since there are a lot of health problems in the use of either native estrogen or native phytoestrogens, it is important to use new compounds. We therefore decided to analyze our new compound femarelle which is a synthetic new phytoestrogens in our experiments. We used it before in animal models showing its importance, and now in human bone cells. We can see how unique it is in its estrogenic effects and its activity which is not affected by other hormones as the estrogen itself.
We therefore believe in its special importance for bone biology and other effects as shown before.
which in turn up-regulate ERs expression and activity. These finding may contribute to F’s beneficial role in treatment of post-menopausal bone loss even in vitamin D deficiency.

Subjects: Environment & Agriculture; Bioscience; Environmental Studies & Management

Keywords: human osteoblasts; femarelle; DNA; CK; phytoestrogens; JKF; ER; VDR

1. Introduction

Phytoestrogens are heterogeneous group of plant-derived compounds, some of which are selective estrogen receptor modulators (SERMs). They are all polyphenolic compounds with structural similarities to natural and synthetic estrogens and they bind to estrogen receptors (ERs) with much lower affinity compared to E₂ (Miksicek, 1994). Phytoestrogens have estrogenic activity in bone and in the cardiovascular system but have anti-estrogenic activity in the breast and in the uterus (Brzezinski & Debi, 1999). They have been proposed to prevent bone resorption and to promote bone formation and increased bone density (Satchell & Lydeking-Olsen, 2003; Tham, Gardner, & Haskell, 1998; Yoles et al., 2003). Femarelle (F), a chemical derivative of the phytoestrogen daidzein (D), which is a standardized extract derived from soybeans was shown to increase bone mineral density in post-menopausal women (Yoles et al., 2004) and to relieve vasomotor symptoms with no effect on sex hormone levels or endometrial thickness (Somjen & Yoles, 2003a). It has also properties as selective estrogen receptor modulator (SERM) as was shown previously both in rat in vivo and in different cultured cells in vitro (Eriksen & Glerup, 2002; Somjen, Katzburg, Lieberherr, Hendel, & Yoles, 2006; Somjen & Yoles, 2003b). It has an estrogen-like activity and activates human-derived female-cultured bone cells (hOBS) which express receptors for E₂ both ERα and ERβ and for vitamin D (VDR). Estrogens and Vitamin D metabolites and their analogs regulate cell proliferation (DNA) and energy metabolism through modulation of the specific activity of creatine kinase (CK). Pre-treatment with vitamin D less-calcemic analog: JKF 1624F2–2 (JKF) up-regulated responsiveness to E₂ and to different estrogens, via modulation of ERs mRNA expression. Estrogens, in turn, induce VDR and 25-hydroxy vitamin D₃ 1-α hydroxylase (1OHase) expression and 1,25(OH)₂D₃ (1,25D) synthesis.

Optimal bone growth and prevention of osteoporosis in post-menopausal women, requires adequate concentrations of vitamin D₃ as well as estrogens (Somjen, Waisman, Weisman, & Kaye, 2000). We have studied previously the interaction between the vitamin D₃ metabolite; 1,25D and its less-calcemic analogs with estrogens in a rat model (Reiss & Kaye, 1981; Somjen, Weisman, Lee, Posner, & Kaye, 2001) using the increase in CK as a response marker for hormonal treatment in cells containing biologically active ERs (Sömjen, Harell, Jaccard, Weisman, & Kaye, 1990; Sömjen, Weisman, Harell, Berger, & Kaye, 1989).

Moreover, pre-treatment with 1,25D up-regulated sex-specific responsiveness and sensitivity to E₂ and to several SERMs in osteoblast-like cell lines and rat bone, as measured by both the stimulation of CK and the increase in DNA synthesis (Fournier, Haring, Kaye, & Somjen, 1996; Reiss & Kaye, 1981; Sömjen, Waisman, Weisman, & Kaye, 1998; Sömjen et al., 1990). This mutual interaction between E₂ and vitamin D was also manifested by an increase in the expression of ERs after treatment with 1,25D (Kanis et al., 1979). However, the use of vitamin D₃ metabolites is restricted by their hypercalcemic effects (Posner et al., 1998). We reported that multiple treatments with “less-calcemic” analogs of vitamin D, particularly JKF (Posner, 2004), stimulated osteoblast-like cells (Katzburg et al., 2005; Posner, 2004) and pre-treatment of skeletal-derived cells with these analogs, up-regulated both responsiveness and sensitivity to E₂ (Fournier et al., 1996; Katzburg et al., 1999, 2005).

The present study was undertaken to measure age and sex specificities in the responses of cultured human female and male osteoblasts (hOBS) to E₂, to the phytoestrogen daidzein; D and to the synthetic phytoestrogen femarelle; F and their modulation by JKF. We sought to correlate these, VDR as β and ERαresponse changes with the expression of mRNA for ER well as 1OHase expression.
and activity resulting in the synthesis of the active metabolite 1,25D (Somjen et al., 2005), as well as the intracellular binding of E$_2$.

2. Materials and methods

2.1. Reagents
Estradiol-17$eta$ (E$_2$), Daidzein (D) and creatine kinase (CK) assay kit were purchased from Sigma Chemicals Co. (St. Louis, MO). Femarelle (F) was purchased from Se-Cure Pharmaceuticals (Dalton, Israel). JKF 1624F$_{3,4}$ (JKF) was synthesized and donated by Dr G.H. Posner et al. (1998). All other reagents were of analytical grade.

2.2. Cell Cultures
Human female osteoblasts (hObs) from pre- (age up to 45 years) and post-menopausal (age from 55 years) women or males (age 45–65 years) were prepared from bone explants of healthy individuals which were not taking any medicines, by a non-enzymatic method as described previously (Katzburg et al., 1999). Each culture was prepared from each donor separately. Shortly: samples of the trabecular surface of the iliac crest or long bones were cut into 1 mm$^3$ pieces and repeatedly washed with phosphate buffered saline (PBS) to remove blood components. The explants were incubated in DMEM medium without calcium (to avoid fibroblastic growth) containing 10% fetal calf serum (FCS) and antibiotics. First passage cells were seeded at a density of 3 × 105 cells/35 mm tissue culture dish, in phenol red- free DMEM with 10% charcoal stripped FCS, and incubated at 37°C in 5% CO$_2$.

2.3. Hormonal treatments
Cells were treated either for 24 h with the different hormones or daily with vehicle (0.01% ethanol in medium) or JKF at 1nM final concentration (Reiss & Kaye, 1981), for 3 days, starting on day 1 after seeding and on day 4 after seeding, the cultures were then treated for additional 24 h with 200 ng/ml F, 300nM D or 300nM E$_2$, followed by harvesting for CK assay or DNA synthesis. In some experiments, cells were pre-treated with the different estrogenic compounds in similar pattern and the changes in the vitamin D system was assayed (Somjen et al., 2005).

2.4. Creatine kinase extraction and assay in hObs
Cells were treated for 24 h with the various agents as specified, scraped off and homogenized by freezing and thawing three times in an extraction buffer, and CK was extracted and assayed as previously described (Reiss & Kaye, 1981; Somjen et al., 1989, 2001). Protein was determined by Coomassie blue dye binding, using bovine serum albumin (BSA) as the standard.

2.5. DNA synthesis assay in hObs
Cells were grown until sub-confluence and then treated with various hormones or agents as indicated. Twenty-two hours following the exposure to these agents, $^3$H]thymidine was added for 2 h and the $^3$H]thymidine incorporation into DNA was determined (Katzburg et al., 1999).

2.6. Determination of ER$_{\alpha}$ and ER$_{\beta}$ mRNA by real time PCR in hObs
RNA was extracted from cultured human bone cells, shown previously (Eriksen & Glerup, 2002; Somjen et al., 2005) and subjected to reverse transcription as previously described (Katzburg et al., 1999; Somjen et al., 2005). ER standard controls and compared to RNase P as internal control for mRNA.

2.7. Competitive binding assay for intracellular estrogenic binding sites in hObs
Cells with and without pre-treatment with JKF, were incubated for 60 min at 37°C with $^3$H]E$_2$ with and without excess of unlabelled compounds as described. Binding was terminated by four successive washes with ice-cold binding medium, and cellular content of $^3$H]E$_2$ was measured in a scintillation counter (Katzburg et al., 1999; Somjen et al., 2005).
2.8. Determination of vitamin D receptor (VDR) and 25-hydroxy vitamin D3 1-hydroxylase (1OHase) mRNA by real time PCR in hObs
RNA was extracted from cultured cells and subjected to reverse transcription as previously described (Katzburg et al., 1999; Somjen et al., 2005). RNAse P expression served as an internal control.

2.9. Assay of 1OHase activity in hObs
1OHase activity was measured by the level of 1,25D generated in hObs within 60 min after the addition of 25(OH)D3 (200 ng/ml) to culture, using the 1,25D 125I RIA kit from Dia Sorin, Mn, USA (Somjen et al., 2005). Protein of the cellular layer was assayed by the Bradford method.

2.10. Statistical analysis
Differences between the mean values of experimental and control groups were evaluated by analysis of variance (ANOVA); p values less than 0.05 were considered significant.

3. Results

3.1. The effects of F, D, or E2 with and without JKF on stimulation of DNA synthesis in hObs
All cells analyzed pre-, post- and male hObs have basal DNA synthesis with lower activity in post-hObs (4,250 ± 300 in pre- vs. 3,000 ± 375 in post- and 4,450 ± 400 dpm/well in male hObs) (Figure 1). Addition of different hormones except JKF stimulated significantly [3H] thymidine incorporation into all female but not in male hObs, with higher responses in pre-hObs compared to post-hObs 305 ± 11% vs. 155 ± 10% by E2, 167 ± 11% vs. 150 ± 14% by D and 173 ± 14% vs. 159 ± 12% by F (Figure 2). Pretreatment with JKF did not change the stimulation of DNA synthesis by F 159 ± 5% vs. 141 ± 12% in pre- and 159 ± 4% vs. 141 ± 5% in post-hObs, but did up-regulate the stimulation by E2 (165 ± 6% vs. 188 ± 12% in pre- and 135 ± 4% vs. 176 ± 12% in post-hObs) and by D (175 ± 12% vs. 200 ± 13% in pre- and 141 ± 12% vs. 176 ± 13% in post-hObs) (Figure 3). No significant changes in the age dependent responsiveness to F unlike to E2 or to D were observed (Figures 2 and 3).

Figure 1. Basal levels of DNA synthesis and CK specific activity in pre-menopausal (pre), in post-menopausal human osteoblasts (post) and male human cultured bone cells.

Notes: Bone cells were cultured, treated, and analyzed for DNA synthesis and CK specific activity as described in Materials. Results are means ± SEM for triplicate cultures from 5 humans/group. Experimental means were compared to control (vehicle alone) means: *p < 0.05, **p < 0.01.
3.2. The effects of F, D or E₂ with and without JKF on stimulation of creatine kinase (CK) specific activity in hO bs
All cells analyzed pre-, post- and male hO bs have basal CK specific activity with lower activity in post-hO bs (32.5 ± 0.5 in pre- vs. 24.5 ± 3.0 in post- and 32.5 ± 0.5 μmol/min/mg protein in male hO bs) (Figure 1). Addition of the hormones except JKF stimulated significantly CK in female hO bs at different ages but not in male, with higher responses in pre- compared to post-hO bs (155 ± 9% vs. 141 ± 9% by E₂, 155 ± 12% vs. 141 ± 9% by D and 161 ± 10% vs. 136 ± 11% by F) (Figure 2). Pre-treatment with JKF did not change the stimulation of CK by F (150 ± 6% vs. 138 ± 10% in pre- and 150 ± 5% vs. 144 ± 12% in post-hO bs) but did up-regulate the stimulation by E₂ (150 ± 5% vs. 169 ± 11% in pre- and 144 ± 5% vs. 200 ± 25% in post-hO bs) and by D (156 ± 11% vs. 175 ± 12% in pre- and 138 ± 5% vs. 175 ± 12% in post-hO bs) (Figure 3). No significant changes in the age dependent responsiveness to F unlike to E₂ or to D were observed (Figures 2 and 3).

3.3. The expression and its modulation by JKF, F, D or E₂ of ERα and ERβ in hO bs
All cells analyzed; pre-, post- and male hO bs expressed mRNA for both ERα and ERβ as measured by real time PCR, corrected for RNase P mRNA. In pre-ERα expression was 0.094 ± 0.007 vs. 0.076 ± 0.006 in post- and 0.100 ± 0.008 2-ΔCT in male hO bs, whereas ERβ in pre- 0.00085 ± 0.00012 vs. 0.00098 ± 0.00011 in post- and 0.00094 ± 0.00008 2-ΔCT in male hO bs. The ratio of ERα to ERβ was 121:1 in pre-, 78:1 in post- and 105:1 in male hO bs (Figure 4). Pre-treatment with the different hormones as well as JKF modulated the expression of ERα and ERβ to different extents in both female age groups and to less extent in male hO bs (in ERα 117 ± 20% in pre- and 167 ± 10% in post-hO bs and 117 ± 20% in male hO bs and in ERβ 50 ± 9% in pre-, 49 ± 15% in post- and 67 ± 17% in male hO bs) (Figure 5). On the other hand E₂ increased ERα by 156 ± 11 in pre- but not in post- and in male hO bs (67 ± 15% and 99 ± 12% respectively). D increased ERα (by 167 ± 12% in pre-, by 133 ± 5% in post- and 101 ± 21% in male hO bs). F increased ERα by 178 ± 22% in pre-, but not in post- (78 ± 5%)
and in male (106 + 10%) hOBS. D increased ERβ by 267 + 30% in pre-, by 167 + 18% in post- and by 100 + 10% in male hOBS. E2 did not increase ERβ in pre- (50 + 15%), or in post- (33 + 5%) and in male (67 + 10%) hOBS, JKF increased ERα in pre- and post-hOBS and decreased ERβ in pre- and post-hOBS with no effect on male hOBS (Figure 5).
3.4. The intracellular binding of E\textsubscript{2} and its modulation by JKF, F, D or E\textsubscript{2} in hObs

Both pre- and post-menopausal female but not male-derived hObs demonstrated E\textsubscript{2} specific binding of \textsuperscript{3}H\textsubscript{E}\textsubscript{2} (Figure 6), presumably predominantly nuclear under these conditions (37\textdegree for 60 min). All compounds tested for competition with \textsuperscript{3}H\textsubscript{E}\textsubscript{2} i.e. E\textsubscript{2}, F and D, but not JKF showed significant binding in both age groups but not in male hObs (Figure 6). Pre-treatment of hObs with JKF increased only slightly non significantly the specific binding of \textsuperscript{3}H\textsubscript{E}\textsubscript{2} in female cells from both age groups by all hormones studied (Figure 7).

Figure 6. The effect of E\textsubscript{2}, D, F or JKF on intracellular binding of E\textsubscript{2} in pre- or post-menopausal female hObs, as measured by competition of the binding of \textsuperscript{3}H\textsubscript{E}\textsubscript{2}. The effects of treatment with JKF (3 days with 1nM) or E\textsubscript{2}, D and F on intracellular binding of E\textsubscript{2} in pre- or post-menopausal female hObs, as measured by competition of the binding of \textsuperscript{3}H\textsubscript{E}\textsubscript{2}.

Notes: Results are means of 4 donors for each group, and are expressed as % modulation of the binding in hormone treated cells compared to untreated cells. **p < 0.01.
3.5. The modulation by JKF, F, D or E₂ of VDR and 1OHase mRNA expression in hObS

Female-derived bone cells from both ages and male hObS expressed mRNA for VDR and 1OHase as measured by real time PCR, corrected for RNase P mRNA (VDR 0.8 ± 0.033 in pre-, 0.41 ± 0.067 in post- and 0.50 ± 0.133 2−∆CT in male hObS, 1OHase 0.188 ± 0.025 in pre-, 0.25 ± 0.02 in post- and 0.075 ± 0.002 2−∆CT in male hObS) (Figure 8). Treatment with all hormones tested increased the expression of 1OHase and VDR by about 35–165%, in both age groups but only JKF increased the expressions in male hObS by about 50% (Figure 9).

3.6. The production of 1,25D and its modulation by JKF, F, D or E₂ in hObS

Female-derived bone cells from both ages as well as male hObS produced 1,25D as measured by radio-immunoassay 53.7 ± 7.1 in pre-, 72.8 ± 14.3 in post- and 35.7 ± 7.1 pg/ml in male hObS (Figure 8). F, D and E₂ treatment increased the activity of 1OHase by increasing the production of 1,25D by about 80–150%, in both age groups but not in male hObS (Figure 9). JKF increased 1,25D production also in male hObS by about 40% as well as by about 180% in pre- and about 160% in post-hObS (Figure 9).
4. Discussion

In our previous studies as well as in the present one, we found that F similar to D (its precursor) or E<sub>2</sub> stimulates—age- and sex-dependently—both DNA synthesis and CK specific activity in primary cultures of human female hObs but not in human male hObs (Eriksen & Glerup, 2002). Pre-treatment with JKF emphasizes the difference between F and D or E<sub>2</sub>, whereas the stimulation of D or E<sub>2</sub> on both parameters and in both female age groups were up-regulated by the vitamin D analog JKF, the effects of F were not significantly changed in either age groups by JKF pre-treatment (Figures 3, 7 and 9). We showed that up-regulation of the stimulation by vitamin D<sub>3</sub> less-calcemic analogs occurs by modulation of ER<sub>α</sub> and ER<sub>β</sub> mRNA in these cells. We have previously demonstrated, using western blot analysis (Kanis et al., 1979) that JKF increased the protein levels of ER<sub>α</sub> and ER<sub>β</sub> with a greater increase of ER<sub>α</sub> than ER<sub>β</sub> in pre-menopausal-derived cells and the opposite occurred in post-menopausal-derived cells (Sömjen, Weissman, & Kaye, 1995; Sömjen et al., 1990). The effects of JKF on nuclear binding (Katzburg et al., 1999; Somjen et al., 2005) are consistent with the increased responsiveness to E<sub>2</sub> after JKF pre-treatment as well as changes in ER proteins (Fournier et al., 1996). In contrast, the membranal binding of E<sub>2</sub> after JKF pre-treatment was abolished by JKF pre-treatment (Katzburg et al., 1999). Membranal binding is therefore not correlated with the up-regulation of DNA synthesis or CK specific activity stimulated by estrogens after JKF pre-treatment. This indicates that membranal processes are not involved in CK stimulation by estrogenic compounds tested in this study or others (Katzburg et al., 1999; Somjen & Yoles, 2003b; Somjen et al., 2006).

It is important to notice that these results are seen in vitamin D deficient rats in which the response of bone to E<sub>2</sub> is attenuated and is restored after treatment with sufficient amounts of vitamin D (Somjen et al., 2007).

ER<sub>α</sub> and ER<sub>β</sub> mRNA were found in both ages of female-derived osteoblasts but while JKF increased ER<sub>α</sub> expression and decreased ER<sub>β</sub> expression, E<sub>2</sub> and D significantly down regulated ER<sub>β</sub> without affecting ER<sub>α</sub>s suggesting a negative effect of E<sub>2</sub> on estrogenic responsiveness in cells from both age groups. On the other hand, F increased both ERs suggesting its positive effect on estrogenic responsiveness in human female-derived osteoblasts from both age groups.
The synthesis of 1,25D from its precursor 25-hydroxyvitamin D₃ (25(OH) D₃), is catalyzed by 1OHase in epithelial cells comprising various parts of the human nephron (Sömjen, Kaye, Harell, & Weisman, 1989; Zehnder, Bland, Walker, et al., 2001). Renal 1OHase is subject to tight systemic metabolic control by parathyroid hormone (PTH), calcium, phosphate and vitamin D₃ metabolites, predominantly 1,25D itself (Reichel, Koeffler, & Norman, 1987). Renal 1OHase is the major source of circulating 1,25D, which controls systemic calcium homeostasis; nevertheless, external-renal expression of 1OHase and 1,25D is now well documented in various tissues and cell types such as prostate cells (Reichel et al., 1987; Sömjen et al., 1989; Zehnder, Bland, Walker, et al., 2001). Molecular studies indicate that the enzyme is expressed in both the kidney and non-renal tissues (Sömjen et al., 1989; Zehnder, Bland, Walker, et al., 2001) and is differentially regulated at least in some of these tissues (Feldman, Malloy, Goldschmidt, & Gross, 2001; Pryke, Duggan, White, Posen, & Mason, 1990; Zehnder, Bland, Williams, et al., 2001). In contrast to circulating 1,25D which controls systemic calcium homeostasis through its action on the intestinal mucosa, bone and kidney, accumulating data indicate that the 1,25D produced by extra-renal 1OHase in various tissues does not contribute to circulatory levels but rather appears to act in an autocrine and/or paracrine fashion by modulating cell proliferation, differentiation, apoptosis, immunoregulation and other functions at a local level (Holick, 2003; Walters, 1992).

We have shown that human female osteoblasts express 1OHase mRNA and produces 1,25D both of which are modulated by different hormones including estrogenic compounds (Somjen et al., 2007). The synthesis of 1,25D in hObs is quantitatively significant at basal production rate of ~1.5 pmol/mg protein/hr, reaching ~4 pmol/mg protein/hr under saturating concentrations of its substrate 25(OH)D₃ (Somjen et al., 2005), which might be important for the physiology of bone as was shown for other parameters.

The effects of F as well as D and E₂ on 1OHase expression and activity remain obscure. The current study demonstrates that F modulates local 1OHase expression in hObs which might, in turn, modulate the effects of other estrogenic compounds on the cells. It is not known, however, whether this happens also in vivo. If it does, it might be used as a mixed new treatment for bone formation in post-menopausal osteoporosis.

The effect of the locally formed 1,25D might be involved in the modulation by vitamin D of the hormonal responses of hObs to the different estrogenic compounds. This might be due to the changes in the expression of estrogen receptors, which were reported to be changed with external vitamin D treatments as described previously (Holick, 2003; Somjen et al., 2005, 2007). This might be a defense mechanism in the pathophysiological conditions of absence of vitamin D in the body. The different parameters changed in hObs response to estrogens by vitamin D analogs show a high increase in CK, and a slight increase in intracellular estrogen binding as well as increased mRNA for ERα. On the other hand, both binding of membranal receptors and mRNA for ERβ were reduced. Whether this indicates a differential interaction of F with the different binding sites through which it exerts its biological activity, needs to be studied.

In conclusion, the present study provides evidence for the mutual interaction between F or other estrogenic compounds and the vitamin D system. The potential role of this system as an autocrine/paracrine mechanism to modulate bone cell metabolism and physiology warrants further investigation. Of importance is the fact that since F is not up-regulated by vitamin D-like estrogens, it is also active in vitamin D deficient conditions, unlike estrogens. This might add to its advantage in hormone replacement therapy treatment over the use of E₂ and other estrogens.
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Competing Interests
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Author details
D. Somjen1
E-mail: disomjen10@gmail.com
S. Katzburg1
E-mail: skatzburg@gmail.com
O. Sharon1
E-mail: osharon@gmail.com
D. Hendl1
E-mail: eknoll@gmail.com

1 The Sackler Faculty of Medicine, Tel-Aviv Sourasky Medical Center, Institute of Endocrinology, Metabolism and Hypertension and Bone Diseases Unit, Tel-Aviv University, 6 Weizman Street, Tel-Aviv 66239, Israel.

2 Department of Orthopedic Surgery, Shearei-Zedek Medical Center, Jerusalem, Israel.

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