Physiol. Res. 67 (Suppl. 3): S455-S464, 2018

Endocrine Disruptors of the Bisphenol and Paraben Families and Bone Metabolism

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Received June 15, 2018
Accepted August 8, 2018

Summary

After menopause, when estrogen levels decrease, there is room for the activity of anthropogenic substances with estrogenic properties – endocrine disruptors (EDs) – that can interfere with bone remodeling and changes in calcium-phosphate metabolism. Selected unconjugated EDs of the bisphenol group – BPA, BPS, BPF, BPAF, and the paraben family – methyl-, ethyl-, propyl-, butyl-, and benzyl-parabens – were measured by high performance liquid chromatography-tandem mass spectrometry in the plasma of 24 postmenopausal women. Parameters of calcium-phosphate metabolism and bone mineral density were assessed. Osteoporosis was classified in 14 women, and 10 women were put into the control group. The impact of EDs on calcium-phosphate metabolism was evaluated by multiple linear regressions. In women with osteoporosis, concentrations of BPA ranged from the lower limit of quantification (LLOQ) – 104 pg/ml and methyl paraben (MP) from LLOQ – 1120 pg/ml. The alternative bisphenols BPS, BPF and BPAF were all under the LLOQ. Except for MP, no further parabens were detected in the majority of samples. The multiple linear regression model found a positive association of BPA (β=0.07, $p<0.05$) on calcium (Ca) concentrations. Furthermore, MP (β=-0.232, $p<0.05$) was negatively associated with C-terminal telopeptide. These preliminary results suggest that these EDs may have effects on calcium-phosphate metabolism.

Key words

Osteoporosis • Endocrine disruptor • Bisphenol • Paraben

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Introduction

Age-associated osteoporosis is a significant public health problem because it is related to increased bone fragility and associated morbidities. In the Czech Republic, there are around 500,000 women and 200,000 men diagnosed as having osteoporosis (Zofkova and Blahos 2017). The risk of hip fractures increases approximately 13-fold from age 60-80 (De Laet *et al.* 1997). Recent evidence suggests that exposure to environmental toxicants such as lead, cadmium, and mercury is associated with higher risks for osteoporosis and fractures (Pollack *et al.* 2013, Zofkova *et al.* 2017).

Many chemicals occurring in the environment have the ability to interfere with the endocrine system, and these substances have been termed endocrine disruptors (EDs). There is growing evidence of their negative impacts on living organisms (reviewed in Diamanti-Kandarakis *et al.* 2009), which are exposed to EDs mainly by the intake of contaminated food and fluids, breathing contaminated air or transdermally (Darbre 2015). EDs may alter the hormonal and homeostatic system and thus affect the metabolism, sexual development, growth, stress response, insulin production, gender behavior, reproduction and even fetal development of the living body (Kabir *et al.* 2015).

The term endocrine disruptor was initially defined as any substance affecting the endocrine system. A more precise definition according to the Environmental Protection Agency defines an ED as an exogenous substance interfering with the synthesis, secretion, transport, binding, action or elimination of natural

hormones responsible for the maintenance of homeostasis, reproduction, development and/or behavior. This term currently includes thousands of substances, and their number is still increasing. The most widely discussed are bisphenol A, phthalates, dioxins, perfluoroalkyl substances and parabens. EDs are found in everyday products (e.g. food and drink packaging, cosmetic products, thermal receipts) and throughout the environment (e.g. water, soil and air). A substantial number of EDs are considered to act on the basis of their ability to bind to estrogen receptors (ER) and thus mimic the endogenous estrogens. In this role they can either act as estrogens or block the binding of estrogenic hormones and act as anti-estrogens. Estrogenic activity has been confirmed in more than 8 thousands chemicals, as can be found in the Estrogenic Activity Database EDAB (Shen *et al.* 2013).

One system in which estrogens play an important role is bone turnover. The decline of estrogen production after menopause increases the risk of osteoporosis (Compston 2001, Stransky and Rysava 2009). Environmental pollutants that disrupt endocrine system by their estrogen-like activity might also affect the modeling and remodeling of bone. There are only a few reports on the effect of EDs on bone mineral density or bone turnover in humans, including on phthalate metabolites (DeFlorio-Barker and Turyk 2016, Min and Min 2014), dioxin levels (Eskenazi *et al.* 2014), organochlorine compounds (Glynn *et al.* 2000, Hodgson *et al.* 2008, Wallin *et al.* 2005), polyaromatic hydrocarbons (Guo *et al.* 2018), perfluoroalkyl substances (Khalil *et al.* 2016) and bisphenol A (BPA) (Kim *et al.* 2012, Zhao *et al.* 2012). Therefore, we conducted a preliminary study to evaluate the associations between unconjugated EDs of a group of bisphenols (BPA plus the alternative bisphenols BPS, BPF, and BPAF) and the paraben family (methyl paraben – MP, ethyl paraben – EP, propyl paraben – PP, butyl paraben – BP, benzyl paraben – BenzylP) on the one hand and plasma parameters of calcium-phosphate metabolism (calcium – Ca, ionized calcium – ionCa, phosphate – P, parathormone – TH, osteocalcin, C-terminal telopeptide-crosslaps – CTx, vitamin D – vitamin D) and bone mineral density (BMD) on the other.

Materials and Methods

Chemicals and reagents

The steroids estrone (E1), 17β-estradiol (E2) and estriol (E3) and deuterated standards of estrone (d4E1) and estriol (d2E3) were from Steraloids (Newport, RI, USA). Standards of MP, EP, PP, BP, BenzylP, BPA, BPS, BPF, BPAF and deuterated standards of BPA (d16BPA), 17β-estradiol (d3E2) were obtained from Sigma-Aldrich (St. Louis, MO, USA), as were 99.9 % tert-butyl methyl ether (MTBE), acetone, sodium bicarbonate, sodium hydroxide and dansyl chloride. The deuterated parabens – d4-MP and d4-PP – were purchased from Chiron (Trondheim, Norway). Deuterated standards of EP (d4EP) and BP (d4BP) were from EQ Laboratories GmbH (Augsburg, Germany). D4-BPS was synthesized as described previously (Kolatorova Sosvorova *et al.* 2017). Methanol and water for chromatography were purchased from Merck (Darmstadt, Germany). All solvents and reagents were of HPLC grade.

Study population and sample collection

This preliminary study consisted of 14 post-menopausal women with classified osteoporosis and 10 age-matched healthy women. Women with osteoporosis were 63.21±4.93 (age ± SD) years old and healthy women were 63.30±4.80 years old. All women underwent examination in the Osteocentrum of the Military University Hospital, Prague. Osteoporosis was classified when decreased bone density (BMD, measured using densitometry by DEXA – dual-energy x-ray absorptiometry) was observed in at least one measured area (lumbar spine, left proximal femur, left femoral neck and/or in the distal third of radius). BMD was expressed by a T-score, which is the number of standard deviations above or below the mean for healthy 30-year-old adult women.

The patients enrolled in the study had a body mass index (BMI)≤30, did not suffer any endocrinopathy, nephropathy, hepatopathy, celiac disease, idiopathic bowel diseases, rheumatoid arthritis, or systemic connective tissue disease, and were not undergoing any treatment that might interfere with calcium-phosphate metabolism (glucocorticoids, diuretics, warfarin, hormone replacement therapy). They had no nutritional disorders, lactose intolerance, or excessive alcohol consumption and did not use dietary supplements containing calcium or vitamin D.

All women underwent blood sampling, with plasma used for an initial routine osteological examination related to calcium-phosphate metabolism (levels of Ca, ionCa, P, vitamin D, PTH, osteocalcin and CTx), for assessment of biochemical parameters of liver and kidney

function (AST, ALT, ALP, GGT, albumin, creatinine, urea), and further for bisphenol and paraben analyses. The latter were performed using all glass equipment, e.g. Pasteur pipettes, glass syringes and glass tubes, and controlled for contamination by bisphenols. The only plastic the blood was in contact with was in plasma collection tubes with K2EDTA. However, these tubes were tested for possible BPA contamination with satisfactory results (Vitku *et al.* 2015). To avoid paraben contamination, all reusable laboratory glassware was washed in ultrapure water, acetonitrile, methanol p.a. and heated for 8 h at 400 °C. After heating, glassware was sequentially rinsed with acetonitrile, methanol and water of HPLC grade. Furthermore, paraben-free gloves were worn during sample preparation. All laboratory surfaces were washed with methanol p.a. before sample processing, the laboratory floor was washed with methanol p.a. as well, and only paraben free soap was used in the laboratory. This protocol was summarized and discussed in a recently published paper (Kolatorova Sosvorova *et al.* 2017). Samples were stored in glass tubes -20 °C until analysis.

The protocol was approved by the Ethical Committee of the Military University Hospital Prague. All patients signed an informed consent form before participating in the project. The study was performed in accordance with the Declaration of Helsinki (2000) of the World Medical Association.

Analyses of biochemical markers of bone metabolism

The biochemical markers albumin, alkaline phosphatase, and gamma-glutamyltransferase were measured by colorimetric analysis (Roche Diagnostics, Mannheim, Germany). A colorimetric assay with bromcresol green was used for albumin and an enzymatic colorimetric assay for gamma-glutamyltransferase. A photometric assay with pyridoxal phosphate activation was used to measure alanine and aspartate aminotransferase (Roche Diagnostics). Creatinine was analyzed by a kinetic colorimetric assay based on the Jaffé method, and urea by a kinetic UV test with urease and glutamate dehydrogenase (Roche Diagnostics).

Total Ca was analyzed using a photometric assay with 5-nitro-5-methyl BAPTA (1,2-bis(o-aminophenoxy)ethane-N,N,N′,N′-tetraacetic acid) (Roche Diagnostics). IonCa was measured by potenciometry with an ion-selective electrode (Radiometer medical Aps, Bronshoj, Denmark). P was analyzed by a photometric UV assay with ammonium molybdate (Roche Diagnostics). Thyroid stimulating hormone (TSH), PTH, osteocalcin, CTx and vitamin D were analyzed by an electrochemiluminiscence immunoassay (ECLIA) kit (Roche Diagnostics). A CTx immunoassay specifically recognizes C-terminal fragments of type I collagen containing the β-isomerized octapeptide EKAHD-β-GGR. Analysis of vitamin D was based on measurements of levels two metabolites: 25-hydroxycholecalciferol and 25-hydroxyergocalciferol.

Sample preparation for liquid chromatography – mass spectrometry analyses

Estrogens (E1, E2, E3), bisphenols (BPA, BPS, BPF, BPAF) and parabens (MP, EP, PP, BP, BenzylP) in plasma samples were measured by an already published method (Kolatorova Sosvorova *et al.* 2017, Vitku *et al.* 2015). Briefly, 500 µl of plasma was spiked with a mixture of deuterated standards (d16BPA, d4BPS, d4MP, d4EP, d4PP, d4BP, d4E1, d3E2, d2E3) and diluted with 500 µl of physiological solution. Samples were shaken, and a liquid-liquid extraction using MTBE (2 ml, 1 min) was performed. The organic layer was transferred to a clean glass tube and evaporated until dryness on a vacuum concentrator. Then a derivatization step was performed as follows; a volume of 50 µl of bicarbonate buffer (100 mM, pH 10.5) and 50 µl of dansyl chloride in methanol (1 mg/ml) were added to the dry residue and shortly mixed. The mixture was incubated at 60 °C for 5 min in a thermoblock and let to cool down to laboratory temperature before evaporation in the vacuum concentrator. The dry residues were reconstituted with 300 µl of methanol. 50 µl of the sample was transferred to a vial with a glass insert where 50 µl of 10 mM ammonium formate in ultrapure water was pre-pipetted. 50 µl of the sample was injected into the ultra-high performance liquid chromatograph-tandem mass spectrometer (LC-MS/MS). Specifically, analysis was performed on an Eksigent ultraLC 110 system (Redwood City, CA, USA) equipped with a Kinetex C18 1.7 µm (150x3 mm) column together with an API 3200 (Sciex, Concord, Canada) triple quadrupole mass spectrometer with electrospray ionization (ESI) in positive mode. More detailed information about the analytical method can be found in (Kolatorova Sosvorova *et al.* 2017).

Statistical analysis

The Mann-Whitney test was used to compare groups of patients with classified osteoporosis and the control group, since a majority of the data had

a non-Gaussian distribution. Where there was normal data distribution, the Student´s t-test was used to compare the groups. ED levels below the lower limit of quantification (LLOQ) were replaced by LLOQ/√2, similarly as in Hornung and Reed (1990). Subsequently, data were transformed by Box-Cox transformation to achieve a normal distribution and multiple linear regression analysis was used to explore associations between measured BPA and MP on one hand and each parameter of calcium-phosphate metabolism on the other. Each model was adjusted for BMI and age as potential confounders. Both covariates were modeled as continuous independent variables. Furthermore, osteoporosis was added to the model as a possible confounding factor in a categorical scale. A backward stepwise selection procedure was used to construct the linear regression model – variables with the highest p-value were gradually removed from the model until only variables with p-value less than $p<0.05$ remained. Data analysis was performed using the statistical software Statgraphics Centurion XVI from Statpoint Inc. (Warrenton, VA, USA).

Results

Table 1 shows a comparison between plasma estrogen levels in women with osteoporosis and the healthy control group. Estrogen levels were always higher in the control group; however, p-values did not reach statistical significance.

Table 1. Comparison of plasma estrogen levels in women with osteoporosis and the control group.

Estrogen	Women with osteoporosis (n=14)	Control group (n=10)	p-value
E1 (pg/ml)	25 (11; 39)	38 (21; 54)	0.6605
E2 (pg/ml)	5 (2; 7)	8 (5; 11)	0.1567
E3 (pg/ml)	15 (7; 22)	24 (15; 34)	0.1688

Data are shown as means and 95 % confidence intervals (in parentheses) for each group; the level of significance is provided in the last column.

All women underwent bone densitometry and a biochemical examination in relation to calcium-phosphate metabolism. Differences or similarities in biochemical markers can be seen in Table 2.

Furthermore, a comparison of ED levels between the group of women with osteoporosis and the control group was performed (Table 3). The most abundant bisphenol in plasma was BPA, which could be measured in 87.5 % of samples. Concentrations varied from LLOQ to 162 pg/ml in the control group and LLOQ to 104 pg/ml in the group of women with classified osteoporosis. The alternative bisphenols (BPS, BPF, BPAF) were under LLOQ in all of the samples. From the paraben family, the highest concentrations were found for MP, which was detected in 50 % of samples. Its concentrations varied greatly; however, from concentrations below LLOQ to 8.27 ng/ml in the healthy group and to 1.31 ng/ml in the osteoporosis group. EP was detected in 3 of 24 samples, PP and BP each in 1 of 24 samples. Based on these results, the alternative bisphenols and parabens other than MP were excluded from statistical data analysis. Regarding BPA and MP, no statistically significant differences were found between groups of women with osteoporosis and healthy women (Table 3).

Finally, multiple linear regressions were used to evaluate associations between each parameter of calcium-phosphate metabolism and the EDs MP and BPA (Table 4). BPA ($\beta=0.07$, $p<0.05$) was positively associated with Ca levels in plasma. There was also a negative association between MP ($\beta=-0.232$, $p<0.05$) and CTx along with a positive association between osteoporosis ($\beta=0.375$, $p<0.001$) and CTx. The model explained 60 % of variability.

Discussion

This preliminary study aimed to investigate the effects of several bisphenol and paraben EDs on the parameters of bone turnover and density, and to compare all these parameters in a group of postmenopausal women with osteoporosis and age-matched women with normal bone density.

Table 2. Comparison of parameters of calcium-phosphate metabolism in women with osteoporosis and the control group.

Parameter	Women with osteoporosis (n=14)	Control group (n=10)	p-value
Age (years)	63.21 (60.39; 66.04)	63.30 (59.96; 66.64)	0.9719
BMI (kg/m^2)	23.81 (22.41; 25.21)	27.09 (25.43; 28.75)	**0.0048**
Ca (mmol/l)	2.44 (2.39; 2.48)	2.42 (2.36; 2.47)	0.5128
IonCa (mmol/l)	1.235 (1.214; 1.256)	1.239 (1.214; 1.264)	0.8019
P (mmol/l)	1.206 (1.134; 1.277)	1.193 (1.108; 1.278)	0.8139
PTH (pmol/l)	6.01 (4.63; 7.39)	5.28 (3.56; 7.00)	0.4579
Osteocalcin (µg/l)	27.427 (23.909; 30.945)	20.198 (16.036; 24.361)	**0.0117**
CTx (µg/l)	0.599 (0.531; 0.667)	0.349 (0.268; 0.429)	**0.0001**
Vitamin D (nmol/l)	55.11 (37.45; 72.49)	63.49 (44.29; 82.70)	0.5344
T score – lumbar spine	-2.44 (-3.03; -1.85)	0.26 (-0.44; 0.96)	**0.0001**
T score – proximal femur	-1.16 (-1.56; -0.75)	0.37 (-0.11; 0.85)	**0.0000**
T score – femoral neck	-1.5 (-1.98; -1.02)	-0.12 (-0.68; 0.44)	**0.0008**

Data are shown as means and 95 % confidence intervals (in parentheses) for each group; the level of significance is provided in the last column.

Table 3. Comparison of plasma levels of EDs in women with osteoporosis and the control group.

Endocrine disruptor	Women with osteoporosis (n=14)	Control group (n=10)	p-value
BPA (ng/ml)	0.060 (0.043; 0.076)	0.062 (0.043; 0.082)	0.4117
MP (ng/ml)	0.195 (0.167; 0.237)	0.231 (0.185; 0.325)	0.3321

Data are shown as means and 95 % confidence intervals (in parentheses) for each group; the level of significance is provided in the last column.

By examining biochemical markers of bone remodeling, we found significantly higher osteocalcin and CTx concentrations in women with osteoporosis. Other studies (but not all) have also reported higher levels of plasma osteocalcin (Atalay *et al.* 2012, Singh *et al.* 2015, Tian *et al.* 2017, Kalaiselvi *et al.* 2013) as well as CTx (Biver *et al.* 2012, Kawana *et al.* 2002, Tian *et al.* 2017) in women with osteoporosis. These results are in agreement with the general concept of high bone turnover in postmenopausal osteoporosis. However, an imbalance between bone resorption and formation occurs, which is attributed to the prolonged lifespan of osteoclasts (so more bone is resorbed) and decreased lifespan of osteoblasts (so less bone is formed) (Hendrickx *et al.* 2015, Seeman 2002).

As expected from the clinical practice, the group of healthy women had a higher BMI in comparison with osteoporosis women, as women with a lower BMI are at increased risk of osteoporosis (Asomaning *et al.* 2006). Similarly, there was also a positive correlation between BMI and T-scores from all measured regions (T-score lumbar spine – r=0.55, p=0.005; T-score proximal femur – r=0.46, p=0.023; T-score femoral neck – r=0.49, p=0.016). A correlation was also found between BMI and estrone (r=0.48, p=0.017). The mechanisms of these associations *in vivo* may include increased loading, adipokines such as leptin, and higher aromatase activity (reviewed in Walsh and Vilaca 2017).

Considering the relationships of menopause and estrogen deficiency, some effects of EDs with estrogenic properties on bone turnover can be expected. Results of *in vitro* studies suggest adverse effects of BPA on bone turnover by inhibiting both osteoblastic and osteoclastic activities (Hwang *et al.* 2013, Suzuki and Hattori 2003).

Table 4. Multiple regression models showing associations between each parameter of calcium-phosphate metabolism and endocrine disruptors.

Dependent variable	Predictors - EDs				Additional confounders						R^2 adjusted for d.f.
	BPA		MP		BMI		Age		Osteoporosis		
	β (95 % CI)	p-value	β (95 % CI)	p-value	β (95 % CI)	p-value	β (95 % CI)	p-value	β (95 % CI)	p-value	
Vitamin D	x	x	x	x	x	x	x	x	x	x	0
Ca	0.077 (0.007; 0.147)	0.033	x	x	x	x	x	x	x	x	15
IonCa	x	x	x	x	x	x	x	x	x	x	0
P	x	x	x	x	x	x	x	x	x	x	0
PTH	x	x	x	x	x	x	x	x	x	x	0
Osteocalcin	x	x	x	x	x	x	x	x	0.191 (0.048; 0.335)	0.0114	22
CTx	x	x	-0.232 (-0.436; -0.028)	0.0279	x	x	x	x	0.375 (0.208; 0.542)	0.001	59
T score – lumbar spine	x	x	x	x	x	x	x	x	-0.263 (-0.353; -0.172)	0.0000	60
T score – proximal femur	x	x	x	x	x	x	x	x	-0.505 (-0.724; -0.286)	0.0001	49
T score – femoral neck	x	x	x	x	x	x	x	x	-0.515 (-0.798; -0.232)	0.0010	37

Variables that where during elimination process subtracted from the model based on p-value>0.05 were marked as x. CI – confidence interval, d.f. – degrees of freedom.

There have been a few studies on the associations of EDs and bone mineral density or parameters of calcium-phosphate metabolism in humans. BPA was reported to not be associated with BMD in women with osteoporosis (Kim *et al.* 2012) or in premenopausal women (Zhao *et al.* 2012). These results are in accordance with our preliminary results. Serum BPA concentrations in postmenopausal women measured by an enzyme linked immunosorbent assay (ELISA) in the study of Kim *et al.* (2012) were 1.44±0.52 ng/ml, which were higher than in our osteoporotic women. Nonetheless, a substantial number of studies report differences in analyte concentrations measured by immunoassays and mass spectrometric techniques (Fanelli *et al.* 2011, Krasowski *et al.* 2014, Wood *et al.* 2008). The results from immunoassays can sometimes lead to false positive results (Sun F. *et al.* 2016), while antibodies sometimes recognize not only the target analyte but also structurally related molecules (in this case possibly 4,4-ethylidenebisphenol or 4-cumylphenol) (Maiolini *et al.* 2014). No studies have measured alternative bisphenols in relation to bone metabolism. Alternative bisphenols are often substituted for BPA in materials when BPA usage is restricted (Kolatorova *et al.* 2017). In our study, BPA was still the most abundant bisphenol in women with osteoporosis as well as in the control group, suggesting that alternative bisphenols are not yet being widely used as BPA replacements.

Multiple linear regression showed a positive association between BPA levels and Ca levels in plasma. Significantly higher plasma Ca levels have also been found in teleosts after BPA treatment at 4 days; however, at 8 days plasma Ca had decreased (Suzuki *et al.* 2003). We are aware that we cannot draw conclusions on the molecular mechanisms, and we can only suggest a hypothesis. Whereas no decrease in bone density in association with BPA increase has been found, the Ca levels in plasma are likely to be from sources other than bone, e.g. there could be higher intestinal absorption of Ca or/and reabsorption of Ca in the kidneys by multiple mechanisms. However, studies on mice have not supported this hypothesis; a decrease in Ca plasma levels through a reduction of intestine Ca absorption and reabsorption in the kidneys was observed in pregnant mice treated with BPA (Kim *et al.* 2013, Otsuka *et al.* 2012).

Surprisingly the other group of EDs – parabens – have not yet been investigated in relation to bone density and calcium-phosphate metabolism in humans, although its estrogenic activities have already been reported (Kolatorova Sosvorova *et al.* 2017, Okubo *et al.* 2001, Routledge *et al.* 1998, Sun L. *et al.* 2016, Watanabe *et al.* 2013). Concentrations of unconjugated MP in plasma in our group of women were within the same order of magnitude as measured in another study using the same analytical method (Kolatorova Sosvorova *et al.* 2017) as well as by methods developed in other laboratories (Frederiksen *et al.* 2011, Sandanger *et al.* 2011, Tahan *et al.* 2016, Ye *et al.* 2008). Concentrations of MP have been reported to be higher where paraben-containing products are used (Tahan *et al.* 2016). In accordance with our results, few parabens other than MP have been detected (above the limit of detection) (Frederiksen *et al.* 2011, Sandanger *et al.* 2011, Ye *et al.* 2008).

The results of our study suggest that MP has a "positive" effect on bone, while it is negatively associated with CTx, a marker of bone resorption. This effect could be attributed to its estrogenic properties. However, all these results need to be interpreted with caution, mainly because of our small sample size. The only study that we can compare our results with is the study of Hu *et al.* (2016) on a mice experimental model. Contrary to our results, they found no effects on CTx levels but observed significantly decreased serum procollagen type 1 N-terminal propeptide (P1NP), which is a marker of bone formation.

Conclusion

The results of this preliminary study contribute further knowledge on the role of endocrine disruptors, suggesting that bisphenol and paraben EDs can influence bone metabolism. The effects of different EDs were not uniform, pointing to the fact that EDs can target multiple pathways in the organism. Future studies are needed to confirm these findings and to discover the molecular mechanisms of the action of EDs in relation to bone metabolism.

Conflict of Interest

There is no conflict of interest.

Acknowledgements

This work was supported by the project MH CZ - DRO (Institute of Endocrinology - EÚ, 00023761).

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