Journal of Bone and Mineral Metabolism (2019) 37:243–255
https://doi.org/10.1007/s00774-018-0929-9

ORIGINAL ARTICLE

Evaluation of ostarine as a selective androgen receptor modulator in a rat model of postmenopausal osteoporosis

D. B. Hoffmann[1] · M. Komrakova[1] · S. Pflug[1] · M. von Oertzen[1] · D. Saul[1] · L. Weiser[1] · T. A. Walde[1] · M. Wassmann[2] · A. F. Schilling[1] · W. Lehmann[1] · S. Sehmisch[1]

Received: 2 August 2017 / Accepted: 17 April 2018 / Published online: 21 May 2018

Abstract

Selective androgen receptor modulators (SARMs) have shown beneficial effects on muscle wasting, general physical function and bone properties in male mammals. However, data on the effects of SARMs in postmenopausal osteoporotic bone are scarce. We evaluated the effects of the SARM drug ostarine on postmenopausal osteoporotic bone in a rat osteoporosis model. Ovariectomy was performed on 46 of 56 3-month-old female Sprague–Dawley rats. Eight weeks after ovariectomy, ostarine was orally administered daily for 5 weeks in dosages of 0.04 (low, OVX + Ost. 0.04), 0.4 (intermediate, OVX + Ost. 0.4), and 4 mg/kg (high, OVX + Ost. 4) body weight. Another ovariectomized group received no ostarine. Lumbar vertebrae and femora were removed for biomechanical, gene expression, ashing, and computer tomography analyses. Low dose showed no effects. The effects of intermediate and high doses were comparable overall. Improvements were mainly seen in structural properties such as bone mineral density and bone volume density. However, the effects in femora were superior to effects in vertebrae. Ostarine treatment for 5 weeks did not improve significantly biomechanical properties. mRNA expression of the receptor activator of NF-κB ligand decreased after treatment, and uterine weight increased. Serum levels of phosphorus increased following ostarine treatment in intermediate and high-dose groups. Short-term treatment of osteoporotic bone with ostarine leads to improvement of several microstructural bone indices. While we did not observe changes in biomechanics, it is conceivable that longer treatment may also improve biomechanical properties. Further studies are needed to characterize longer time effects and side effects of ostarine in osteoporosis.

Keywords Osteoporosis · SARM · Ostarine · Rat model of osteoporosis

Abbreviations

ALP	Alkaline phosphatase
BMD	Bone mineral density
BV/TV	Bone volume density
BW	Body weight
DHT	Dihydrotestosterone
ERα	Estrogen receptor alpha
F_{max}	Maximal load
N.Nd.	Trabecular nodes
OC	Osteocalcin
OPG	Osteoprotegerin
OVX	Ovariectomy
RANKL	Receptor activator of NF-κB ligand
S-4	S-3-(4-acetylamino-phenoxy)-2-hydroxy-2-methyl-*N*-(4-nitro-3-trifluoromethyl-phenyl)-propionamide
SARM	Selective androgen receptor modulator
SERM	Selective estrogen receptor modulator
TRAP	Tartrate-resistant acid phosphatase
Tb.Sp	Trabecular spacing
Tb.th	Trabecular thickness
Tb.Wi	Trabecular thickness (2-Dscan)

✉ D. B. Hoffmann
daniel.hoffmann@med.uni-goettingen.de

[1] Department of Trauma-, Orthopaedic- and Plastic Surgery, University Medical Center Goettingen, Robert Koch St. No. 40, 37075 Goettingen, Germany

[2] Department of Medical Microbiology, Subdivision of General Hygiene and Environmental Health, University of Goettingen, Humboldallee 34a, 37073 Goettingen, Germany

Introduction

Postmenopausal osteoporosis is a widespread but underestimated and under-served disease [1]. Several studies predict a notable rise in the prevalence of typical osteoporotic fractures over the next decades as the population ages [2]. In spite of improved treatment options currently available to patients, e.g., bisphosphonates as first-line therapy, treatment for postmenopausal osteoporosis is still a challenge [3].

Because of severe side effects, including an increased risk of cancer, stroke, and arteriosclerosis, the use of estrogen in postmenopausal osteoporosis has been considerably restricted [4, 5]. An alternative treatment option is the use of selective estrogen receptor modulators (SERMs) such as raloxifene. The drug exerts estrogen-like effects on bone and anti-estrogenic effects on the uterus and breast [6]. Raloxifene prevents vertebral fractures to a considerable extent, but there is little evidence for its role in preventing non-vertebral fractures [7, 8]. Furthermore, raloxifene increases the risk of deep vein thrombosis and pulmonary embolism, and can cause unpleasant side effects such as cramps, hot flashes, sinusitis, and edema [9]. A drug exerting osteoanabolic effects without affecting estrogen-dependent tissue would be preferable. Therefore, new therapy options are needed.

The beneficial effects of androgens on the skeletal system and bones were demonstrated in various studies over the past years [10–14], although the mechanisms of action of androgens and androgen receptors on bones are still not fully understood [15]. Their use has been limited because of their side effects, such as on the skin and prostate. Selective androgen receptor modulators (SARMs) have no testosterone steroid ring and were developed to exert anabolic effects on bone and muscle in males with minimal effects on other testosterone-dependent tissue. One of the SARM compounds is ostarine, also known as GTx-024, S-22, or enobosarm. In several studies, a positive effect of ostarine was shown for total lean body mass and physical function [16–18]. These studies focused on patients with cancer-induced muscle wasting and elderly men. Also in postmenopausal women, ostarine had a significant effect on physical function [17]. However, published data on ostarine's effects in bone are scarce and only available on male patients [17, 19]. For andarine (S-4) and LGD-3303, two other SARMs, bone-protective effects have already been shown in female rats [20–22]. These findings imply that SARMs appear to be a promising treatment option for postmenopausal osteoporosis.

The aim of the present study was to evaluate ostarine's effects on postmenopausal osteoporotic bone. We used the ovariectomized rat, which is the standard animal model in basic research of osteoporosis, to investigate ostarine in postmenopausal osteoporosis [23].

Materials and methods

General procedures

All procedures were approved by the local Institutional Animal Care and Use Committee (district authorities of Oldenburg, Oldenburg, Germany). We used the ovariectomized rat, which is the standard animal model for osteoporosis studies. Experiments were performed with 56 3-month-old female Sprague–Dawley rats (Fa. Winkelmann, Borchen, Germany). All rats were maintained according to German animal protection laws and fed a soy-free diet throughout the experiment (ssniff Special Diet, Soest, Germany).

Ten rats received no therapy and represent the healthy control group (intact group, $n = 10$). They did not develop any osteoporotic changes in bone. The remaining 46 rats were ovariectomized at 3 months of age (OVX).

Eight weeks after ovariectomy, the ovariectomized rats developed osteoporosis. At that time, ostarine treatment commenced (MK-2866, ShangHaiBiochempartner Co., Ltd., Shanghai, China) (Fig. 1). Ostarine was added to the chow (OVX) in three different concentrations. One group received a low dose of 0.04 mg/kg body weight (BW)/day (OVX + Ost. 0.04, $n = 12$), a second group received an intermediate dose of 0.4 mg/kg BW/day (OVX + Ost. 0.4, $n = 12$), and a third group received a high dose of 4 mg/kg BW/day (OVX + Ost. 4, $n = 12$) (Fig. 1). A fourth group of ovariectomized rats received no treatment (OVX, $n = 10$). Ostarine was administered for 5 weeks. We selected a dose of 0.04 mg/kg BW as low dose due to evidence for improvements of total lean body mass and physical function in this human-equivalent dose [17]. To investigate a dose-dependent effect we increased the dose 10-fold and 100-fold.

Thirteen weeks after ovariectomy, all animals were killed under CO_2 anesthesia. Femora, vertebrae and serum samples were collected for analysis. Gastrocnemius and soleus muscles were weighed. We used the first lumbar vertebra and right femur for micro-computed tomography, the second vertebra and left femur for ashing and mineral content analysis, the third vertebra and left femur for biomechanical testing, and the sixth vertebra for gene expression analysis. Vertebrae and femora were stored at − 20 °C until analysis. For gene expression analysis, the samples were stored at − 80 °C.

Biomechanical assessment

The biomechanical test was designed according to the procedure described by Sehmisch et al. and Tezval et al. [24, 25]. We utilized a Zwick mechanical testing instrument (type 145 660 Z020/TND, Zwick, Ulm, Germany) to perform a resistance test of the third lumbar vertebrae. The defrosted

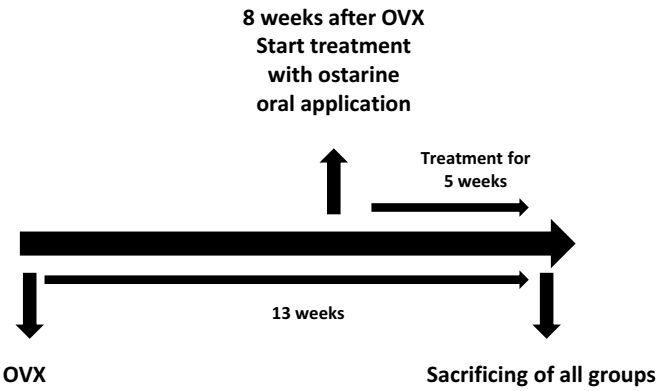

Fig. 1 Study was performed with 3-month-old Sprague–Dawley rats. Intact rats received no ovariectomy and no treatment. Eight weeks after ovariectomy ostarine was administered orally for 5 weeks in low, intermediate and high dose. The control group (OVX) received no therapy. 13 weeks after ovariectomy all rats were killed

Group	Treatment
Intact	No ovariectomy, no treatment
OVX	Ovariectomy, no treatment
OVX + Ost. 0.04	Low dose, 0.04mg/kg bw/day
OVX + Ost. 0.4	Intermediate dose, 0.4mg/kg bw/day
OVX + Ost. 4	High dose, 4mg/kg bw/day

bones were fixed to the lower plate of the testing machine and a stamp was lowered. The stamp had a driving force of 1 N with a speed of 50 mm/min. The test results were obtained with a relative accuracy of 0.2–0.4% over the range of 2–500 N. The test machine stopped automatically when the line of the curve declined more than 10 N. Throughout the test process, the compressive force was measured every 0.1 mm by the testXpert software. Femora were tested similarly using a three-point-bending mechanical test as described by Tezval et al. [25].

Following the biomechanical test described previously [25, 26], we analyzed the maximum load (F_{max}) and stiffness (S). For vertebrae, we also measured the yield load (y_L). Maximum load is the maximum force that the bone can withstand. Stiffness represents the elasticity of the bone, and the yield load represents the inflection point demarcating elastic and plastic deformation. In femora, the yield point at the femoral neck is usually very close to the maximum load; therefore, we did not analyze the yield point for our specimens.

Micro-computed tomography (CT)

To measure bone mineral density (BMD), bone volume density (BV/TV), and other morphological bone properties, we used the Quantum FX micro-CT (Caliper Life Sciences, Hopkinton, MA, USA) at 70 kVp and 200 µA, resulting in a $40 \times 40 \times 40$ µm^3 voxel size. In every scan, a phantom block with several known mineral densities was included. We used the 3DOsteo Analyze software (developed in our laboratory) to calculate bone parameters according to the American Society for Bone and Mineral Research (ASBMR) [27]. Furthermore, the cortical area (mm^2) was measured at the dorsal and ventral side of the vertebral body, cut sagittally on 3D images.

To obtain additional morphological data, we performed structural analyses on 2D images of micro-CT scans (Fig. 1). Four images of sagittally cut vertebrae and femora were analyzed with the MetaMorph Basic Acquisition Software (Leica MikrosystemeVertrieb GmbH, Wetzlar, Germany). Collected data were trabecular nodes (N.Nd), trabecular connectivity (N.Nd/mm^2), cortical and trabecular density, trabecular bone area, and trabecular thickness (Tb.Wi).

Ashing

We used ashing to measure the mass of mineralized bone. The left femora and the second lumbar vertebrae were heated in an oven at 750 °C for 30 min. Before and after ashing, the bones were weighed to the nearest 0.00001 g. The mineral content (ash weight) was expressed relative to the wet weight of each bone (%).

The calcium content was assessed using an atomic absorption spectrometer (4100, PerkinElmer, Waltham, MA, USA) [28]. Orthophosphate content was measured using a colorimetric method (spectral photometer DM4, Zeiss, Oberkochen Germany) [29, 30].

Gene expression analysis

The sixth lumbar vertebrae were used for gene expression analysis. The bones were homogenized with a micro-dismembrator (Sartorius, Goettingen, Germany). The RNeasy Mini Kit (Qiagen, Hilden, Germany) was utilized for RNA extraction. Afterwards, the RNA was reverse-transcribed with Superscript RNase H-reverse transcriptase (Promega, Fitchburg, USA). The expression levels of alkaline phosphatase (ALP) (Qiagen, Hilden, Germany, Cat. no: QT00190680), androgen receptor (Qiagen, Hilden, Germany, Cat. no: QT 00184394) and estrogen receptor α (Qiagen, Hilden, Germany, Cat. no: QT01595013), receptor activator of nuclear factor κB ligand (RANKL) (Qiagen, Hilden, Germany Cat. no: QT00195125), osteocalcin (Qiagen, Hilden, Germany Cat. no: QT01084573), and osteoprotegerin (OPG) (Qiagen, Hilden, Germany, Cat. no: QT00177170) were measured using quantitative real-time polymerase chain reaction (qRT-PCR) based on SYBR green detection (QuantiTect SYBR Green PCR Kit, Qiagen,Venlo, Netherlands) in an iCycler (CFX96, Bio-Rad Laboratories, Hercules, USA). Ready-to-use primers from Qiagen were used and qRT-PCR was performed according to the manufacturer's instructions. Gene expression was calculated using the $2^{\Delta\Delta C_T}$ method, and the results were normalized to gene expression levels in untreated female rats (Intact). The reference gene was β_2 microglobulin (Qiagen, Hilden, Germany, Cat. no: QT00176295) [31].

Muscle analyses

After killing the rats, gastrocnemius and soleus muscles were removed. Both muscles were weighed directly after removal. Weight of muscles is presented proportional to body weight to avoid false-positive differences due to weight gain.

Serum analysis

ALP, estradiol, calcium and phosphorus were measured using an Architect c16000 analyzer (Abbott, Chicago, USA) at the Department of Clinical Chemistry, University of Goettingen. ALP activity was measured by the para-nitrophenyl phosphate method at 404 nm, and calcium was quantified by arsenazo III dye (Abbott) at 660 nm using commercially available reagents according to the manufacturer's instructions [32]. For measurement of serum estradiol levels we used a commercial analysis kit (Abbot, Prod.-No: B7K721 Chicago, USA).

Statistical analysis

Differences between groups were analyzed by one-way ANOVA with a Tukey–Kramer post hoc test (GraphPad Prism, La Jolla, CA, USA). p values < 0.05 were considered significant. Data are presented as the mean and standard deviation (SD).

Results

Ostarine has no influence on bodyweight

Rats across the different treatment groups had similar BW at the beginning of the study (248 ± 8 g). After the ovariectomies were performed, typical metabolic changes occurred that induced increases in bodyweight. In the context of normal growth, intact rats gained weight as well, but significantly less than that gained by ovariectomized rats. Treatment with ostarine in ovariectomized rats did not significantly affect BW (Fig. 2a, b).

Uterine wet weight is increased after ostarine treatment in ovariectomized rats

Uterine wet weight decreased significantly after ovariectomy, compared with wet weight in intact animals (intact group: 522.2 ± 99.4 mg; OVX group: 104.4 ± 39.7 mg) (Fig. 2c). The groups which received intermediate and high-ostarine doses displayed significantly increased uterine wet weight compared with untreated ovariectomized rats (OVX: 104.4 ± 39.7 mg; OVX + Ost. 0.4: 327 ± 59.5 mg; OVX + Ost. 4: 394.5 ± 60.6 mg). Treatment with a low dose of ostarine did not result in a significant increase in uterine wet weight (Fig. 2c). To test, if the increased uterine wet weight can be explained by changes in estrogen levels in the rats, we measured E2 in the serum. We found a significantly decreased level of E2 in all ovariectomized rats in comparison with intact rats, but no significant change due to ostarine treatment irrespective of the doses (Fig. 2d).

Morphological bone properties in micro-CT are improved after ostarine treatment in intermediate and high dose

BMD and BV/TV increased significantly in femora after ostarine treatment in the intermediate and high-dose groups compared with values in untreated ovariectomized rats (Fig. 3a). Indeed, ostarine treatment in intermediate- and high-dose groups showed similar results as intact rats. Untreated ovariectomized rats had the lowest BMD and BV/TV (Fig. 3a). In vertebrae, both parameters increased significantly in the intermediate-dose group (Fig. 3b).

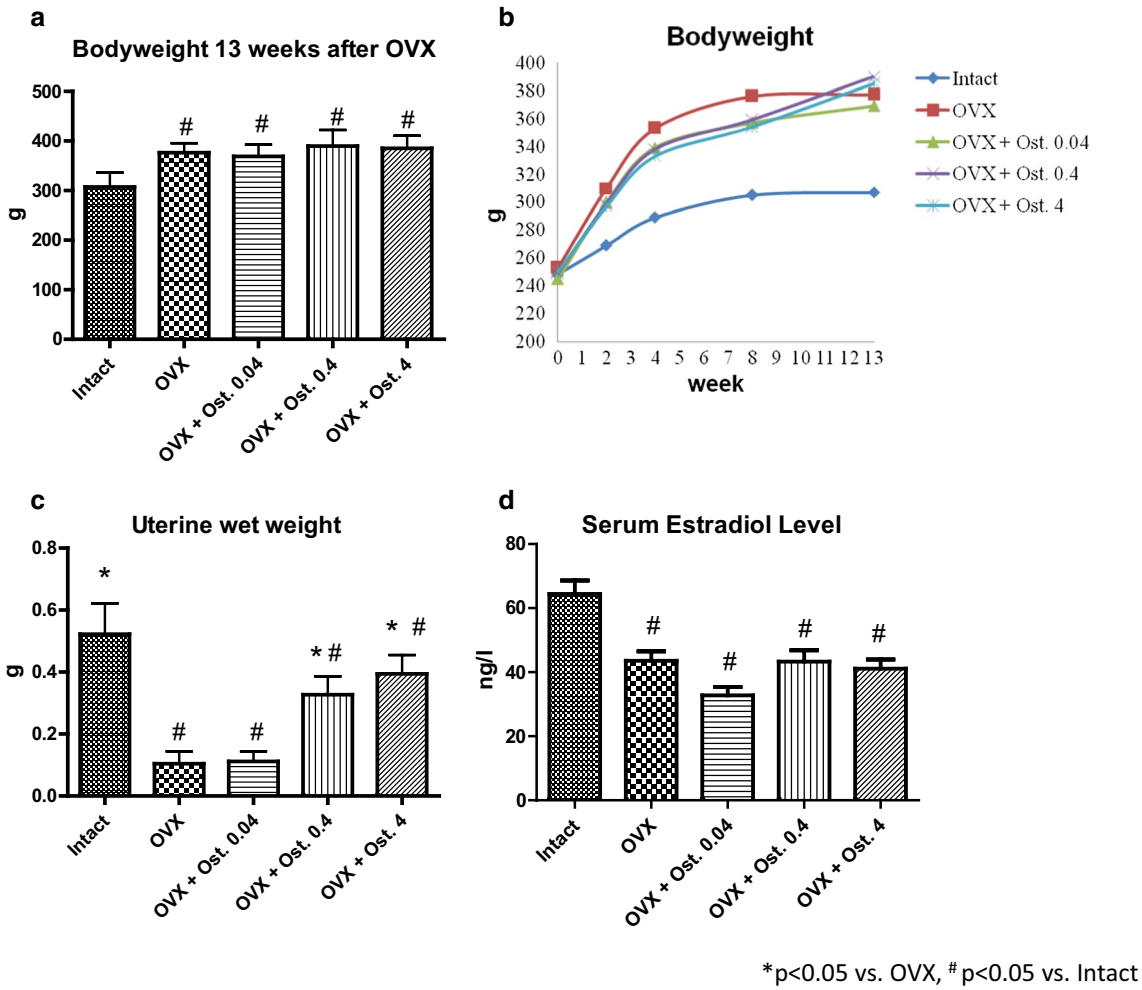

Fig. 2 **a** The figure shows the BW after killing 13 weeks after ovariectomy. Due to ovariectomy the rats gained weight because of typical metabolic changes. Application of ostarine in ovariectomized rats showed no difference in body weight compared with untreated rats. **b** Changes in bodyweight over time. The BW increased in all groups. Intact rats also increased in BW in context of normal growth, but significantly lower than ovariectomized rats. Application of ostarine showed no influence on body weight. **c** Treatment with ostarine in intermediate (0.4 mg/kg BW/day) and high (4 mg/kg BW/day) dose resulted in a significantly increased uterine wet weight compared with untreated ovariectomized rats. The increase was independent of serum estradiol level (**d**). **d** All ovariectomized rats showed significantly decreased levels of estradiol in serum 13 weeks after ovariectomy compared with intact rats. Ostarine had no influence in estradiol level in ovariectomized rats. $^{*}p < 0.05$ versus OVX, $^{\#}p < 0.05$ versus intact

Treatment with ostarine in intermediate dose showed comparable results with intact rats. Untreated ovariectomized rats and ovariectomized rats treated with ostarine in high dose showed lowest levels of BMD and BV/TV in vertebrae (Fig. 3b). Other morphological bone parameters such as trabecular spacing (Tb.Sp) and thickness (Tb.th) did not differ significantly between ostarine-treated groups and untreated ovariectomized rats in femora and vertebrae as shown in Table 1. Intact rats had the highest values.

Analysis of 2D images in femora showed a significant improvement following intermediate- and high-dose ostarine treatment in trabecular nodes (N.Nd.), trabecular density, trabecular bone area, and cortical density compared with untreated ovariectomized rats (Figs. 4, 5). The trabecular connectivity increased significantly in femora after high-dose ostarine treatment. Similar results were observed for trabecular thickness (Tb.Wi) between intact rats without osteoporosis and after rats were treated with intermediate and high doses of ostarine (Fig. 4). Intact rats showed highest values in all trabecular properties and were significantly superior to any ostarine treatment in N.Nd., trabecular density and trabecular connectivity. Values of intact rats and ovariectomized rats treated with ostarine were similar in trabecular bone area after treatment with the intermediate dose and in trabecular thickness after intermediate and high dose. Cortical density in femora was increased after ostarine treatment in low and intermediate dose compared with intact rats and untreated ovariectomized rats (Fig. 4).

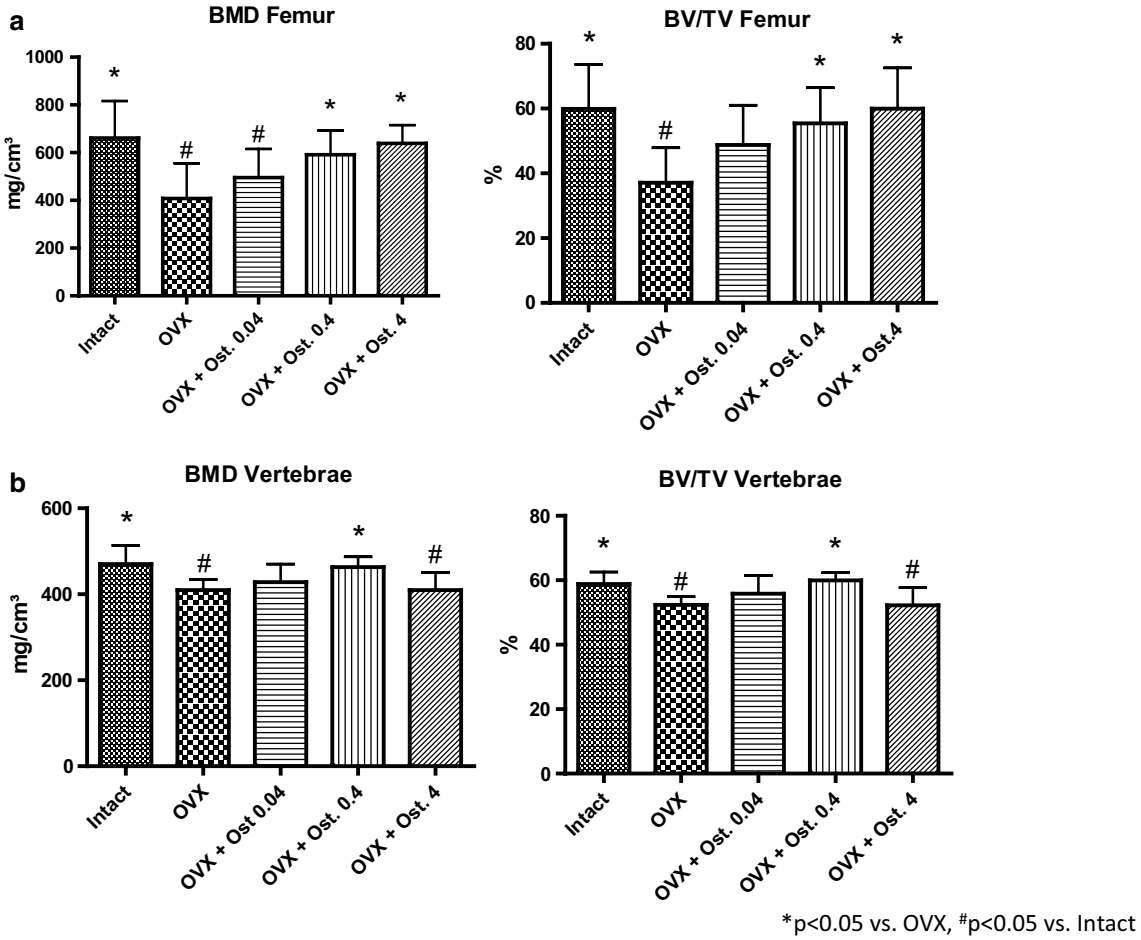

Fig. 3 **a** The BMD and BV/TV was significantly increased in femora after treatment with ostarine in intermediate and high dose compared with untreated ovariectomized rats and similar to values in intact rats. Application of ostarine in low dose showed no significant effect on femora in ovariectomized rats. **b** In vertebrae BMD and BV/TV parameters were significantly increased after application of ostarine in intermediate dose and were comparable with intact rats. Application of ostarine in low and high dose did not show a significant improvement in vertebrae. $^{*}p < 0.05$ versus OVX, $^{\#}p < 0.05$ versus intact

In vertebrae, similar trends after ostarine treatment were observed for trabecular and cortical properties, but without statistical significance (Table 1). In most trabecular properties intact rats showed significant superior values compared with all ovariectomized rats (Table 1). In cortical bone area no significant differences between intact and ovariectomized rats were found in vertebrae (Table 1).

Ostarine has influence on mineral content in femora and vertebrae

Mineral content in vertebrae increased significantly after ostarine treatment in the intermediate- and high-dose groups, and was greater than mineral content in vertebrae of intact animals (Fig. 6). In femora, higher mineral content was recorded in the high-dose group than in untreated ovariectomized rats. Untreated ovariectomized rats and rats treated with ostarine in low and intermediate dose showed significant lower mineral content compared with intact rats (Fig. 6).

Biomechanical properties were not improved by ostarine

In vertebrae significant lower maximum load and yield load was observed following high-dose ostarine treatment, whereas stiffness was significantly diminished in the low-dose group compared with intact rats (Fig. 7). Intact rats showed significant higher levels in all biomechanical properties in vertebrae compared with untreated ovariectomized rats (Fig. 7).

In femora, no changes were observed between untreated ovariectomized rats and ovariectomized rats treated with ostarine (Fig. 7). Intact rats showed superior results in maximum load compared with all other groups. Stiffness

Table 1 µCT analyses in vertebrae and femora after treatment with Ostarine in three different doses: trabecular spacing (Tb.Sp) and thickness (Tb.th) did not differ significantly between ostarine-treated groups and untreated ovariectomized rats in femora and vertebrae in contrast to differences in BMD and BV/TV (Fig. 3)

	Intact		OVX		OVX + Ost. 0.04		OVX + Ost. 0.4		OVX + Ost. 4	
Sample size	10		9		11		10		11	
	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD
µCT vertebrae										
Trabecular Thickness (Tb.th) (mm)	0.1681[a]	0.0461	0.0618[b]	0.0145	0.0780[b]	0.0147	0.0824[b]	0.0254	0.0917[b]	0.0295
Trabecular spacing (Tb.Sp) (mm)	0.3864[a]	0.0843	0.3247[b]	0.0275	0.3267[b]	0.0354	0.3137[b]	0.0329	0.3004[b]	0.0172
µCT analysis femora										
Trabecular Thickness (Tb.th) (mm)	0.0875[a]	0.0296	0.0283[b]	0.0091	0.0490[b]	0.022	0.0431[b]	0.015	0.0498[b]	0.0211
Trabecular spacing (Tb.Sp) (mm)	0.2816[a]	0.0258	0.2158[b]	0.0154	0.2541	0.0346	0.2458	0.0325	0.2293	0.0710
2D analysis of vertebrae										
Trabecular nodes (N. Nd.) (n)	27.4[a]	9.0	16.4[b]	10.3	16.4[b]	8.0	17.0[b]	7.0	14.6[b]	7.4
Trabecular connectivity (N/mm^2)	8.6	1.7	7.8	2.0	7.8	1.8	7.3	2.0	7.1[b]	2.2
Corticl density (%)	86.9	6.0	85.4	6.2	86.8	7.0	88.4	3.6	88.1	5.6
Trabecular density (%)	22.7[a]	9.1	12.2[b]	6.3	12.3[b]	4.5	14.0[b]	4.5	13.6[b]	5.1
Trabecular thickness (Tb Wi) (µm)	123[a]	62	73[b]	16	75[b]	23	71[b]	12	71[b]	17
Trabecular bone area (mm^2)	3.275[a]	1.354	2.005[b]	0.988	2.042[b]	0.795	2.283[b]	0.671	1.955[b]	0.680
Cortical bone area (mm^2)	2.209	0.471	2.016	0.532	1.947	0.373	2.305[c]	0.446	2.084	0.411
2D analysis of femora										
Trabecular bone area (mm^2)	4.08[a]	0.75	2.57[b]	0.72	2.89[b]	0.79	3.68[a,c]	1.35	3.53[a]	1.13

Analysis of 2D images of vertebrae showed only non-significant improvements after ostarine therapy

[a] $p < 0.05$ versus OVX

[b] $p < 0.05$ versus inact

[c] $p < 0.05$ versus OVX + Ost. 0.04

of femora did not differ significantly between the treatment groups (Fig. 7).

Slight, non-significant influence of ostarine on muscle weight

The mean muscle weights corrected by body weight showed lower values in untreated ovariectomized rats compared with intact rats, but these differences were not significant. Ovariectomized rats treated with ostarine showed same muscle weight like intact rats (Fig. 8a, b).

RANKL expression is suppressed after ostarine treatment in intermediate and high dose

RANKL mRNA expression decreased significantly after treatment with intermediate and high doses, compared with expression in untreated ovariectomized rats (Table 2). The differences in OPG mRNA expression were non-significant between the groups (Table 2).

ALP mRNA levels increased significantly in the intermediate-dose group. However, neither low-dose nor high-dose treatment led to a difference in ALP mRNA levels compared with levels in the control groups (Table 2).

No significant changes were observed in osteocalcin mRNA expression following ostarine treatment.

Expression of androgen and estrogen receptor α (ERα) did not differ between the groups (Table 2).

Ostarine influences serum phosphorus and ALP levels

Ovariectomized rats treated with intermediate and high doses of ostarine displayed significantly higher levels of ALP than intact rats (Table 2). Levels were also higher than in untreated ovariectomized rats, but with no statistical significance. The serum phosphorus levels increased significantly in the intermediate- and high-dose groups compared with intact rats, untreated ovariectomized rats and ovariectomized rats treated with ostarine in low dose. Changes in serum calcium levels in serum were not observed (Table 2).

Discussion

Studies of the effects of SARMs on bone are few, particularly in the case of postmenopausal osteoporosis. Previous studies on ostarine investigated either effects on

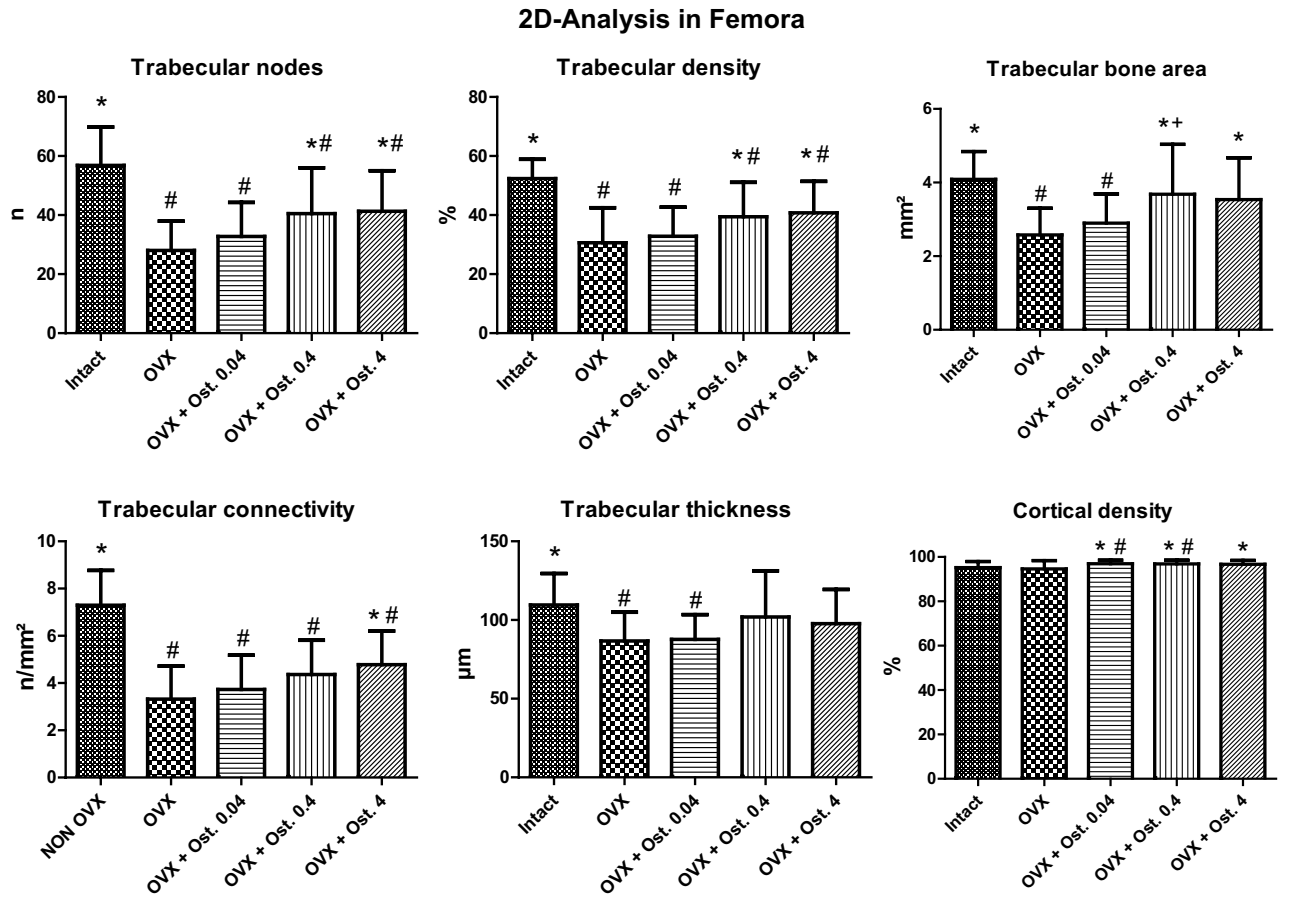

Fig. 4 Analysis of 2D images in femora: significant improvement in intermediate and high dosage in trabecular nodes (N.Nd.), trabecular density, trabecular bone area and cortical density. Trabecular connectivity was increased at high dose. The trabecular thickness (Tb.Wi) showed similar results between intact rats without osteoporosis and after treatment with ostarine in intermediate and high dose. Intact rats showed highest values in all trabecular properties. Results for cortical density after ostarine treatment in low and intermediate dose were superior to intact rats. $^{*}p < 0.05$ versus OVX, $^{\#}p < 0.05$ versus intact, + versus OVX + Ost. 0.04

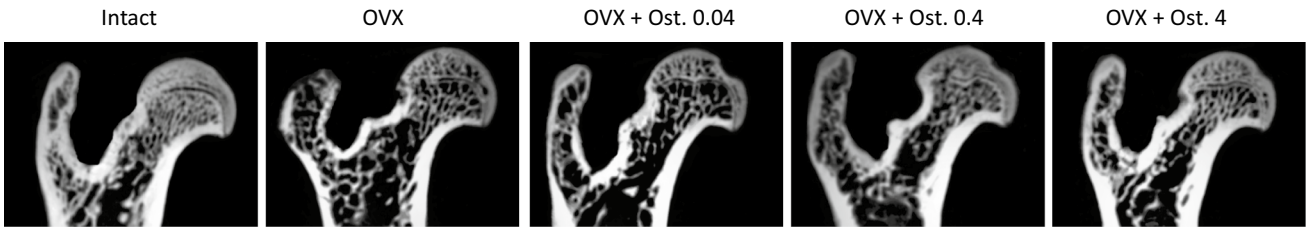

Fig. 5 2D images of micro-CT scans of femora. The images show the structural changes in trabecular and cortical bone 13 weeks after ovariectomy. Treatment with ostarine in a low dose showed no improvements. Groups which received intermediate- and high-ostarine doses showed improvements in trabecular and cortical properties

muscle wasting and physical function or effects on bone in male mammals [16–19]. Conclusive data on the effects of SARMs in general and ostarine in particular on postmenopausal metabolism and osteoporotic bones are still lacking.

In our experiments, BW increased in all groups. Intact rats gained weight as part of normal growth; the gain was less pronounced than in ovariectomized rats. In this study, ostarine had no apparent effect on BW in ovariectomized

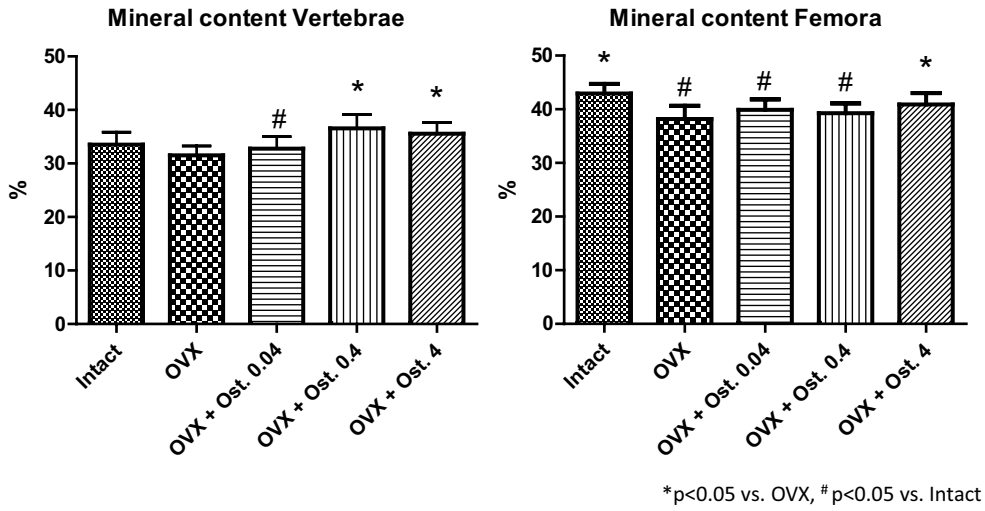

Fig. 6 The mineral content in vertebrae was significantly increased after treatment with ostarine at intermediate and high dose, and was even superior to intact animal. In femora the mineral content was improved after high dosage. $^{*}p < 0.05$ versus OVX, $^{\#}p < 0.05$ versus intact

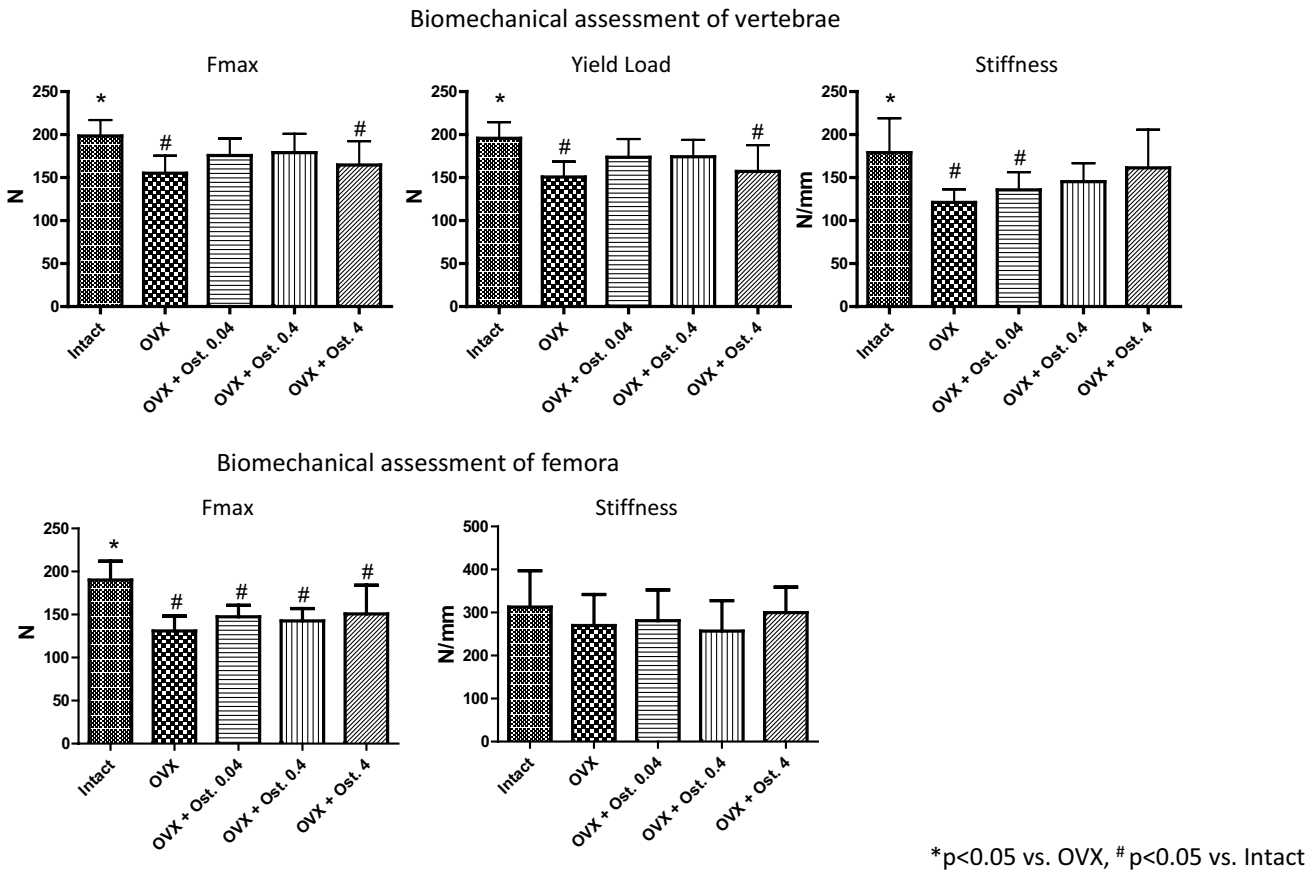

Fig. 7 In vertebrae only slight but non-significant improvement of maximum load and yield load were seen after low and intermediate ostarine dose compared with untreated ovariectomized rats. There was no difference to intact animals detectable at these doses. In contrast to OVX alone and ostarine at low-dose treatment, stiffness was not significantly distinct from intact after intermediate- and high-dose ostarine treatment. Compared with intact rats, maximum load and yield load were significant lower following ostarine treatment in high dose, whereas stiffness was significantly diminished in low-dose group. In femora no changes were observed after treatment with ostarine after 5 weeks. Intact rats showed superior results in maximum load compared with all other groups. Stiffness did not differ significantly between intact and ovariectomized rats. $^{*}p < 0.05$ versus OVX, $^{\#}p < 0.05$ versus intact

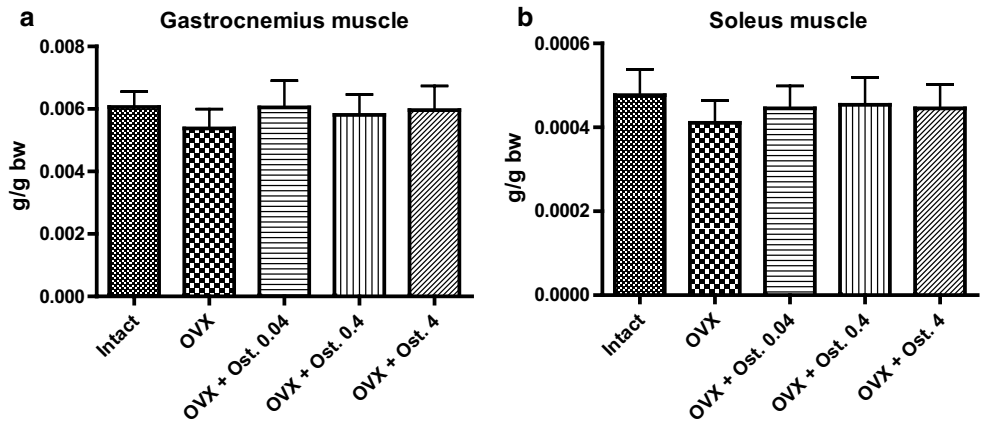

Fig. 8 Weight of gastrocnemius (**a**) and soleus (**b**) muscles. Muscle weight is presented proportional to bodyweight to avoid false-positive differences due to gain weight after ovariectomy. Untreated ovariectomized rats showed the lowest muscle weight values but with no statistically significant difference to the other groups

Table 2 Gene expression analysis in vertebrae: RANKL mRNA decreased significantly after treatment with intermediate and high doses

	Intact		OVX		OVX + Ost. 0.04		OVX + Ost. 0.4		OVX + Ost. 4	
Sample size	10		9		11		10		11	
	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD
Gene expression										
ALP	1.159	0.569	1.096	0.633	1.571	0.601	2.152[a,b,d]	0.825	1.189	0.754
Osteocalcin	1.313	0.893	4.042	4.242	3.647	3.194	3.294	3.281	4.126	3.757
RANKL	1.045	0.370	1.616	0.638	1.439	0.431	1.010[a]	0.477	0.758[a,c]	0.270
OPG	1.111	0.538	0.868	0.246	1.108	0.373	0.849	0.233	0.746	0.441
TRAP	1.221	0.864	0.913	0.364	1.023	0.611	0.916	0.616	0.934	0.553
Androgen receptor	0.940	0.359	1.094	0.353	1.134	0.461	0.893	0.217	1.330	0.393
Estrogen receptor alpha	1.148	0.514	1.201	0.705	1.670	0.679	1.221	0.722	1.340	0.393
Serum analysis										
Phosphorus mmol/l	1.60	0.33	1.73	0.28	1.68	0.21	2.12[a,b,c]	0.26	2.12[a,b,c]	0.32
Calcium mmol/l	2.09	0.16	2.00	0.13	1.92	0.16	2.10	0.14	2.08	0.22
ALP (U/I)	87.6	16.8	135.0	38.4	104.7	25.9	141.4[b]	54.9	168.3[b,c]	49.3

The differences in OPG mRNA expression were non-significant between the groups. ALP mRNA increased significantly in the intermediate-dose group. Serum analysis: rats treated with intermediate and high doses of ostarine displayed significantly higher levels of ALP than intact rats. Serum phosphorus levels increased significantly in the intermediate- and high-dose groups compared with all other groups

ALP alkaline phosphatase, *OPG* osteoprotegerin, *RANKL* receptor activator of NF-κB ligand, *TRAP* tartrate-resistant acid phosphatase

[a] $p < 0.05$ versus OVX

[b] $p < 0.05$ versus intact

[c] $p < 0.05$ versus OVX + Ost. 0.04

[d] $p < 0.05$ versus OVX + Ost. 4

rats. Vajda et al. showed increasing effects on BW by the SARM LGD-3303, whereas Kearbey et al. could not demonstrate weight gain following treatment with S-4 [20, 22]. Thus, an influence on BW would appear to depend on the individual SARM drug. Furthermore, the influence of SARMs on muscles can affect BW. In our study we could show slight, non-significant effects on muscle growth by ostarine. Stronger effects on muscle growth by other SARMs can have more influence on bodyweight.

In this study, we demonstrated considerable and statistically significant improvements in cortical and trabecular bone by ostarine treatment in primary affected osteoporotic bones.

The positive effects of ostarine were mainly shown in the structural properties of vertebrae and femora. However,

the effects in femora were superior to those in vertebrae, particularly in the analysis of 2D images. This is probably due to the heterogeneous changes of different skeletal parts in case of osteoporosis [33–35]. Not all bones are affected with the same strength by osteoporosis. Trabecular bone loss differs between different bones [33]. Previous studies also demonstrated different effects between femora and vertebrae following anti-osteoporotic treatment [36]. Mineral content increased after treatment with ostarine in both femora and vertebrae. mRNA expression of the osteoclast activator RANKL decreased following treatment. However, mRNA expression of ALP, a marker for increased bone turnover, was only significantly elevated after treatment with an intermediate-range dose of ostarine. The increase in serum ALP levels in the intermediate- and high-dose groups was non-significant. Biomechanical properties did not show statistically significant improvements in comparison with ovariectomized rats without ostarine treatment; however, in some of the treatment groups there was no significant difference to intact bones detectable.

Overall, the low dose of 0.04 mg/kg BW/day showed no effects. The effects of the intermediate (0.4 mg/kg BW/day) and high (4 mg/kg BW/day) doses were comparable. Parameters such as BV/TV and BMD in femora improved following high-dose ostarine treatment, but the intermediate dose often yielded equal or superior results. A tenfold higher dose must show distinctly improved results to be acceptable; therefore, treatment with the intermediate dose of ostarine would appear to be more reasonable.

Our results on the osteoprotective effects of ostarine in cortical and trabecular osteoporotic bone are in general consistent with published data on other SARM drugs (S-4, LGD-3303) in postmenopausal osteoporotic bones [20–22]. However, in contrast with our study, those studies also demonstrated a substantial improvement in biomechanical properties after SARM treatment. It may be that biomechanical properties would have improved in our study had we administered ostarine longer than 5 weeks. In the aforementioned studies, SARMs were administered longer than 10 weeks [20–22]. Furthermore, the observed effects may have been the result of varying drug properties. In contrast to the present study, a previous study by Dalton et al. in elderly men showed no changes in BMD after treatment with ostarine. This difference is probably due to a considerably variation of ostarine dosage [17]. The highest dosage for elderly men was 0.04 mg/kg BW. This represents the low-dose group in our study. However, we have to consider different equivalent doses for rats due to different body surfaces and specific physiology [37]. But even with respect to the equivalent dose (0.04 mg/kg BW in human represents around 0.25 mg/kg BW in rats [38]), the dose in the study of Dalton et al. is between the low and intermediate dose in our study. From the authors point of view, an improvement in BMD in humans would be, therefore, also possible when ostarine was given in a higher dose. On the other hand it is conceivable that a decrease in RANKL leads to decreased osteoclastic resorption which is necessary for bone remodeling. If bone remodeling is suppressed, the non-remodeled bone accumulates mineral and microcracks. This then leads to increased stiffness and decreased fracture toughness, respectively, which would be consistent with our observations. Thus, it may be possible that suppressed osteoclastic resorption plays a role for the phenotype of ostarine-treated bone. Unfortunately, due to the preprocessing of our bones we were not able to perform cellular histomorphometry to support this hypothesis.

Uterine tissue is an estrogen- and androgen-responsive tissue; therefore, the effects of ostarine on the uterus were investigated. We found an increase in uterine wet weight in ovariectomized rats following ostarine treatment which was independent of serum estradiol levels. This can be an important safety limitation in the drug's use in postmenopausal osteoporosis, even if ostarine treatment did not reach the uterine wet weight of intact rats. Ostarine and SARMs in general can only offer a promising new treatment option for postmenopausal osteoporosis if they do not cause cancer. An increase in uterine weight has already been shown in other SARMs [39–41]. However, in studies which found beneficial effects of the SARMs S-4 and LGD-3303 in postmenopausal bone, uterine weight was not considered [20, 22]. Thus, the risks of short-term and long-term ostarine use and development of endometrial cancer should be investigated in future studies. Perhaps only a short-term ostarine regimen is reasonable in cases of severe osteoporosis or fracture healing; this question was not settled in the present study.

We demonstrated a significant increase in serum levels of phosphorus after treatment with ostarine in intermediate- and high-dose groups. To our knowledge, there are no former studies investigating phosphorus level after ostarine treatment or SARM treatment in general. High levels of serum phosphorus are associated with vascular calcification and endothelial dysfunction [42]. This can be another safety limitation of ostarine in treatment of postmenopausal osteoporosis. However, this was not under investigation in the present study and further studies are needed to analyze potential cardiovascular side effects following ostarine treatment.

In conclusion, ostarine treatment in a rat model of postmenopausal osteoporosis showed a significant improvement in cortical and trabecular bone after 5 weeks. Only minor differences were observed in effects of intermediate and high doses. Further improvements in biomechanical properties are conceivable, if the ostarine treatment duration is extended. A limitation of ostarine use in postmenopausal osteoporosis is the significant increase in uterine weight we observed. Further studies are needed to characterize

the effects of ostarine on fracture healing in osteoporosis-affected bones, as well as the risk of endometrial cancer and cardiovascular events.

Acknowledgements The present study was funded by the German Research Foundation (DFG SE 1966/6-1, KO 4646/3-1). The authors are grateful to R. Castro-Machguth and A. Witt for technical support.

Compliance with ethical standards

Conflict of interest All authors have no conflicts of interest.

References

1. Duquet N (2014) Osteoporosis: treatment and pharmaceutical care. J Pharm Belg 2:14–24
2. Osterkamp R (2005) Population developments in Germany until 2050. Chirurg 76:10–18
3. Papaioannou A, Morin S, Cheung AM, Atkinson S, Brown JP, Feldman S, Hanley DA, Hodsman A, Jamal SA, Kaiser SM, Kvern B (2010) 2010 clinical practice guidelines for the diagnosis and management of osteoporosis in Canada: summary. CMAJ 182:1864–1873
4. Rossouw JE, Anderson GL, Prentice RL, LaCroix AZ, Kooperberg C, Stefanick ML, Jackson RD, Beresford SA, Howard BV, Johnson KC, Kotchen JM, Ockene J (2002) Risks and benefits of estrogen plus progestin in healthy postmenopausal women: principal results. From the Women's Health Initiative randomized controlled trial. JAMA 288:321–333
5. Chlebowski RT, Kuller LH, Prentice RL, Stefanick ML, Manson JE, Gass M, Aragaki AK, Ockene JK, Lane DS, Sarto GE, Rajkovic A (2009) Breast cancer after use of estrogen plus progestin in postmenopausal women. N Engl J Med 360:573–587
6. Muchmore DB (2000) Raloxifene: a selective estrogen receptor modulator (SERM) with multiple target system effects. Oncologist 5:388–392
7. Ettinger B, Black DM, Mitlak BH, Knickerbocker RK, Nickelsen T, Genant HK, Christiansen C, Delmas PD, Zanchetta JR, Stakkestad J, Glüer CC (1999) Reduction of vertebral fracture risk in postmenopausal women with osteoporosis treated with raloxifene: results from a 3-year randomized clinical trial. Multiple Outcomes of Raloxifene Evaluation (MORE) Investigators. JAMA 282:637–645
8. Kanis JA, Johansson H, Oden A, McCloskey EV (2010) A meta-analysis of the efficacy of raloxifene on all clinical and vertebral fractures and its dependency on FRAX. Bone 47:729–735
9. An KC (2016) Selective estrogen receptor modulators. Asian Spine J. 10:787–791
10. Anderson FH, Francis RM, Faulkner K (1996) Androgen supplementation in eugonadal men with osteoporosis-effects of 6 months of treatment on bone mineral density and cardiovascular risk factors. Bone 18:171–177
11. Tobias JH, Gallagher A, Chambers TJ (1994) 5 alpha-dihydrotestosterone partially restores cancellous bone volume in osteopenic ovariectomized rats. Am J Physiol 267:E853–E859
12. Katznelson L, Finkelstein JS, Schoenfeld DA, Rosenthal DI, Anderson EJ, Klibanski A (1996) Increase in bone density and lean body mass during testosterone administration in men with acquired hypogonadism. J Clin Endocrinol Metab 81:4358–4365
13. Lea CK, Flanagan AM (1998) Physiological plasma levels of androgens reduce bone loss in the ovariectomized rat. Am J Physiol 274:E328–E335
14. Amory JK, Watts NB, Easley KA, Sutton PR, Anawalt BD, Matsumoto AM, Bremner WJ, Tenover JL (2004) Exogenous testosterone or testosterone with finasteride increases bone mineral density in older men with low serum testosterone. J Clin Endocrinol Metab 89:503–510
15. Otto-Duessel M, He M, Jones JO (2012) Tissue-selective regulation of androgen-responsive genes. Endocr Res 37:203–215
16. Dobs AS, Boccia RV, Croot CC, Gabrail NY, Dalton JT, Hancock ML, Johnston MA, Steiner MS (2013) Effects of enobosarm on muscle wasting and physical function in patients with cancer: a double-blind, randomised controlled phase 2 trial. Lancet Oncol 14:335–345
17. Dalton JT, Barnette KG, Bohl CE, Hancock ML, Rodriguez D, Dodson ST, Morton RA, Steiner MS (2011) The selective androgen receptor modulator GTx-024 (enobosarm) improves lean body mass and physical function in healthy elderly men and postmenopausal women: results of a double-blind, placebo-controlled phase II trial. J Cachexia Sarcopenia Muscle 2:153–161
18. Girgis CM, Mokbel N, Digirolamo DJ (2014) Therapies for musculoskeletal disease: can we treat two birds with one stone? Curr Osteoporos Rep 12:142–153
19. Zhang X, Lanter JC, Sui Z (2009) Recent advances in the development of selective androgen receptor modulators. Expert Opin Ther Pat 19:1239–1258
20. Kearbey JD, Gao W, Narayanan R, Fisher SJ, Wu D, Miller DD, Dalton JT (2007) Selective androgen receptor modulator (SARM) treatment prevents bone loss and reduces body fat in ovariectomized rats. Pharm Res 24:328–335
21. Kearbey JD, Gao W, Fisher SJ, Wu D, Miller DD, Dalton JT (2009) Effects of selective androgen receptor modulator (SARM) treatment in osteopenic female rats. Pharm Res 26:2471–2477
22. Vajda EG, Hogue A, Griffiths KN, Chang WY, Burnett K, Chen Y, Marschke K, Mais DE, Pedram B, Shen Y, van Oeveren A (2009) Combination treatment with a selective androgen receptor modulator q(SARM) and a bisphosphonate has additive effects in osteopenic female rats. J Bone Miner Res 24:231–240
23. Kalu DN (1991) The ovariectomized rat model of postmenopausal bone loss. Bone Miner 15:175–191
24. Sehmisch S, Erren M, Rack T, Tezval M, Seidlova-Wuttke D, Richter J, Wuttke W, Stuermer KM, Stuermer EK (2009) Short-term effects of parathyroid hormone on rat lumbar vertebrae. Spine (Phila Pa 1976) 34:2014–2021
25. Tezval M, Stuermer EK, Sehmisch S, Rack T, Stary A, Stebener M, Konietschke F, Stuermer KM (2010) Improvement of trochanteric bone quality in an osteoporosis model after short-term treatment with parathyroid hormone: a new mechanical test for trochanteric region of rat femur. Osteoporos Int 21:251–261
26. Sehmisch S, Erren M, Kolios L, Tezval M, Seidlova-Wuttke D, Wuttke W, Stuermer KM, Stuermer EK (2010) Effects of isoflavones equol and genistein on bone quality in a rat osteopenia model. Phytother Res 24:S168–S174
27. Dempster DW, Compston JE, Drezner MK, Glorieux FH, Kanis JA, Malluche H, Meunier PJ, Ott SM, Recker RR, Parfitt AM (2013) Standardized nomenclature, symbols, and units for bone histomorphometry: a 2012 update of the report of the ASBMR Histomorphometry Nomenclature Committee. J Bone Miner Res 28:2–17
28. CEN (2002) European committee for standardization. Determination of calcium and magnesium. EN ISO 7980
29. CEN (2004) European committee for standardization. Determination of orthophosphate. EN ISO 6878
30. Hoffmann DB, Griesel MH, Brockhusen B, Tezval M, Komrakova M, Menger B, Wassmann M, Stuermer KM, Sehmisch S (2016) Effects of 8-prenylnaringenin and whole-body vibration therapy on a rat model of osteopenia. J Nutr Metab 2016:6893137

31. Komrakova M, Hoffmann DB, Nuehnen V, Stueber H, Wassmann M, Wicke M, Tezval M, Stuermer KM, Sehmisch S (2016) The effect of vibration treatments combined with teriparatide or strontium ranelate on bone healing and muscle in ovariectomized rats. Calcif Tissue Int 99:408–422
32. Tezval M, Hansen S, Schmelz U, Komrakova M, Stuermer KM, Sehmisch S (2014) Effect of urocortin on strength and microarchitecture of osteopenic rat femur. J Bone Miner Metab 33:154–160
33. Chen H, Zhou X, Fujita H, Onozuka M, Kubo KY (2013) Age-related changes in trabecular and cortical bone microstructure. Int J Endocrinol 2013:213234
34. Ritzel H, Amling M, Posl M, Hahn M, Delling G (1997) The thickness of human vertebral cortical bone and its changes in aging and osteoporosis: a histomorphometric analysis of the complete spinal column from thirty-seven autopsy specimens. J Bone Miner Res 12:89–95
35. Amling M, Posl M, Ritzel H, Hahn M, Vogel M, Wening VJ, Delling G (1996) Architecture and distribution of cancellous bone yield vertebral fracture clues. A histomorphometric analysis of the complete spinal column from 40 autopsy specimens. Arch Orthop Trauma Surg 115:262–269
36. Kavuncu V, Sahin S, Baydas G, Ilhan N, Ozercan I, Yasar A, Pekkutucu I, Ilhan N, Ozercan R (2003) A comparison of estrogen and two different doses of calcitonin in ovariectomized rats. Yonsei Med J 44:508–516
37. Nair AB, Jacob S (2016) A simple practice guide for dose conversion between animals and human. J Basic Clin Pharm 7:27–31
38. USFDA (2005) Guidance for industry: estimating the maximum safe starting dose in adult healthy volunteer. US Food and Drug Administration
39. Jones A, Hwang DJ, Duke CB 3rd, He Y, Siddam A, Miller DD, Dalton JT (2010) Nonsteroidal selective androgen receptor modulators enhance female sexual motivation. J Pharmacol Exp Ther 334:439–448
40. Furuya K, Yamamoto N, Ohyabu Y, Morikyu T, Ishige H, Albers M, Endo Y (2013) Mechanism of the tissue-specific action of the selective androgen receptor modulator S-101479. Biol Pharm Bull 36:442–451
41. Nagata N, Furuya K, Oguro N, Nishiyama D, Kawai K, Yamamoto N, Ohyabu Y, Satsukawa M, Miyakawa M (2014) Lead evaluation of tetrahydroquinolines as nonsteroidal selective androgen receptor modulators for the treatment of osteoporosis. ChemMedChem 9:197–206
42. Scialla JJ, Wolf M (2014) Roles of phosphate and fibroblast growth factor 23 in cardiovascular disease. Nat Rev Nephrol 10:268–278