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Implications of combined Ovariectomy/Multi-Deficiency Diet on rat bone with age-related variation in Bone Parameters and Bone Loss at Multiple Skeletal Sites by DEXA

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Background: Osteoporosis is a multi-factorial, chronic, skeletal disease highly prevalent in post-menopausal women and is influenced by hormonal and dietary factors. Because animal models are imperative for disease diagnostics, the present study establishes and evaluates enhanced osteoporosis obtained through combined ovariectomy and deficient diet by DEXA (dual-energy X-ray absorptiometry) for a prolonged time period.

Material/Methods: Sprague-Dawley rats were randomly divided into sham (laparotomized) and OVX-diet (ovariectomized and fed with deficient diet) groups. Different skeletal sites were scanned by DEXA at the following time points: M0 (baseline), M12 (12 months post-surgery), and M14 (14 months post-surgery). Parameters analyzed included BMD (bone mineral density), BMC (bone mineral content), bone area, and fat (%). Regression analysis was performed to determine the interrelationships between BMC, BMD, and bone area from M0 to M14.

Results: BMD and BMC were significantly lower in OVX-diet rats at M12 and M14 compared to sham rats. The Z-scores were below –5 in OVX-diet rats at M12, but still decreased at M14 in OVX-diet rats. Bone area and percent fat were significantly lower in OVX-diet rats at M14 compared to sham rats. The regression coefficients for BMD *vs.* bone area, BMC *vs.* bone area, and BMC *vs.* BMD of OVX-diet rats increased with time. This is explained by differential percent change in BMD, BMC, and bone area with respect to time and disease progression.

Conclusions: Combined ovariectomy and deficient diet in rats caused significant reduction of BMD, BMC, and bone area, with nearly 40% bone loss after 14 months, indicating the development of severe osteoporosis. An increasing regression coefficient of BMD *vs.* bone area with disease progression emphasizes bone area as an important parameter, along with BMD and BMC, for prediction of fracture risk.

Key words: OVX-diet | skeletal site | animal model | DEXA | bone mineral density

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Background

Osteoporosis is a common, chronic, and multi-factorial skeletal disease causing reduced bone mass and bone micro-architecture deterioration [1,2]. Its pathophysiology is complex, involving a broad spectrum of endogenous (genetic and hormonal) and environmental factors [3]. It is an important socio-medical problem because of high morbidity, mortality, and medical costs [4,5]. The estimated direct costs are currently $31.7 billion (£21.165 billion) and expected to increase to $76.7 billion (£51.1 billion) in 2050 based on the expected European demographic changes [6]. It is highly prevalent, especially in post-menopausal women [7–9], due to estrogenic deficiency [10], and the lifetime risk for women to have an osteoporotic fracture is 30–40% worldwide [11].

The progression of the disease is slow, which necessitates long-term studies capable of providing a clear and detailed understanding of the disease. This in turn causes difficulties regarding cost, management, and ethics in human subjects. Animal models are required for preclinical assessment of potential therapies for osteoporosis [12,13]. Therefore, animal models simulating humans, but with faster biology and aging, such as rat models [14], are of major interest in osteoporotic research. Thus, to understand disease pathogenesis and develop new therapies, prosthetics, and biomaterials [15], further characterization of post-menopausal osteoporosis in animal models is needed.

Ovariectomized mature rats are recognized as the best animal model of post-menopausal osteoporosis [16]. Thereby, many studies have focused on the effect of ovariectomy on osteoporosis [7,17–22]. On the other hand, the multi-factorial nature of the disease demands more focus on a diet-combined ovariectomy model [23]. Calcium and vitamin D are important nutrients known to maintain bone mass in healthy bone, and their deficiency enhances calcium mobilization from bone [24]. Nonetheless, few studies have addressed ovariectomy combined with either low calcium diet [25–27] or vitamin D deficiency [28]. Moreover, scant data have been reported on combined ovariectomy with deficient calcium, vitamin D, vitamin C, phytoestrogen, and scarce phosphorous supply involving long-term analysis at different skeletal sites.

Thus, in an attempt to establish severe osteoporotic status, we performed ovariectomies on rats and used multiple deficient diet treatment to address the multifactorial nature of the disease, along with postmenopausal osteoporosis on bone parameters. Further, the long-term observations of this study also focused on senile osteoporosis apart from post-menopausal osteoporosis. Although DEXA is crucial in diagnosing osteoporosis, its intrinsic limitation (e.g., areal BMD = Bone Mineral Density by DEXA) is a 2-dimensional measurement of a 3-dimensional structure, liable to be confounded by bone size [29,30]. Therefore, we also performed a regression analysis to determine the relationship between the parameters measured at different ages of rats. We also investigated the importance of the bone area as a parameter that influences bone mineral density in DEXA measurements.

Material and Methods

Maintenance of animals

Ten-week-old female Sprague-Dawley rats were purchased from Charles River (Sulzfeld, Germany). The range of average initial body weights of the rats was 250–290 g. Rats were maintained under standard laboratory conditions and underwent an acclimatization period of 4 weeks before implementation of the experimental procedures. The treatment of animals and all the experimental procedures complied with German animal protection laws of the district government "RP" Giessen (89/2009).

Grouping of animals

Rats were assigned to either the control group (sham operated) or the experimental group (ovariectomy and diet). The animals were monitored at baseline (M0), M12 (12 months post-surgery, and M14 (14 months post-surgery, and each group contained 10 animals at each time point. At the age of 14 weeks the control group animals underwent laparotomy; a large incision was made in the abdominal wall to avoid discrepancies due to surgery between groups, after being anaesthetized with intra-peritoneal injection of 62.5 mg/kg body weight ketamine (Hostaket®, Hoechst) and 7.5 mg/kg body weight xylazine (Rompun®, Bayer), and were fed with normal feed throughout the experimental procedure. Experimental rats were ovariectomized bilaterally with a dorsal approach, using anesthesia as mentioned above. The OVX-diet rats were fed with normal feed up to 2 weeks post-surgery, after which they were fed a diet deficient in vitamin D2/D3, vitamin C, calcium, and phosphorus, and which was free of soy and phytoestrogen, purchased from Altromin (Altromin-C1034, Altromin Spezialfutter GmbH, Lage, Germany), throughout the experimental time period.

Determination of body weight

Body weights (g) of rats of both groups (sham and OVX-diet) were measured immediately after laparotomy and ovariectomy to obtain the baseline measurements (M0), and at M12 and M14 post-surgery.

Dual-Energy X-ray Absorptiometry (DEXA)

Rats were scanned by DEXA (Lunar Prodigy, GE Healthcare, Germany), as a non-invasive, rapid, and precise imaging method

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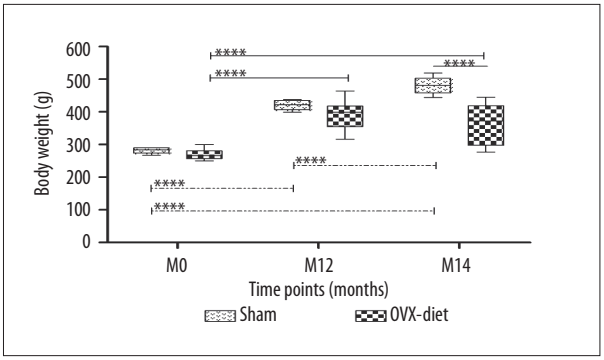

Figure 1. Changes in body weight of sham and OVX-diet rats at M0 (baseline), M12 and M14 post treatment. Though body weight increased with time (dotted lines – sham, straight line – OVX-diet), significant reduction was observed in OVX-diet rats at M14. Asterisks indicate the significance level (**** as p<0.0001) for two way ANOVA (between groups) and one way ANOVA (within group across time points).

[31,32]. The rats were anaesthetized as described previously, and then ventrally positioned on a DEXA table with legs separated from the trunk to scan the whole body. After the scan, regions of interest (ROI) were marked respective to the spine, pelvis, left femur, right femur, left tibia, and right tibia. The measured parameters included bone mineral density = BMD (g/cm^2), bone mineral content = BMC (g), bone area (cm^2), and fat (%). Rats were scanned immediately after ovariectomy and laparotomy to obtain the baseline measurements (M0). A follow-up scan was done at M12 and M14 post-surgery. Qualitative analysis was performed using the small-animal mode of the enCORE software (GE Healthcare, v. 13.40) according to the developer's procedure.

Statistical analysis

Body weight (g), BMD (g/cm^2), BMC (g), fat (%), area, and Z score were examined for statistical significance. Column statistics were performed to determine skewness and kurtosis of the data to check for normal distribution. Two-way ANOVA and Bonferroni multiple comparison test were done to determine the variation between groups at each particular time point. Further, 1-way ANOVA followed by Bonferroni multiple comparison test were done to determine variation across time points in a particular group.

Z-scores were calculated with the formula Z-score = [measured BMD – age matched BMD]/age-matched population SD [9]. Regression analysis to determine the interrelationships between BMD, BMC, and area of 2 skeletal sites (spine and pelvis) was done at M0, M1, M3, M12, and M14 time points, as these sites are highly prone to osteoporotic fractures [6]. Percent bone loss was calculated by the formula [mean BMD – mean peak BMD] / mean peak BMD*100 [33]. Percent change calculated by the formula for M0-M1 period = BMD (M1) – BMD (M0) / BMD (M0) * 100, and calculated for each time period. Our previously published DEXA scan raw data of M1 and M3 are used for regression, percent bone loss, and percent change determination to observe progressive change due to age and disease (supplementary file). All the above analyses were done using GraphPad prism version 5 statistical software. Unless otherwise indicated, asterisks indicate the significance levels (* p<0.05, ** p<0.01, *** p<0.001, and **** p<0.0001).

Results

Body weight

Sham rats and OVX-diet rats showed an increase in body weight over time, showing significance at M12 and M14 compared to baseline. Significant increase in body weight was observed in sham rats at M14 compared to M12; however, between groups OVX-diet rats showed significant differences in their body weight at M14 when compared to sham rats (Figure 1).

Bone mineral density (BMD), bone mineral content (BMC) and bone area

Comparison of BMD between groups at M12 and M14 showed significant differences at all skeletal sites except at baseline measurements (M0). Sham rats showed gradual, significant increase in BMD in the spine and tibia at M12 compared to baseline. In the pelvis and femur of sham rats, significant increase was observed both at M12 and M14 compared to baseline. Sham rats showed a slight decrease in BMD at M14 compared to M12 in all skeletal sites. OVX-diet rats showed a gradual, significant decrease in BMD at M12 and M14 compared to baseline in all skeletal sites. BMD values at M14 were significantly lower than M12, especially in the tibia (Figure 2).

BMC values of the OVX-diet group were significantly decreased when compared to sham rats at all examined skeletal sites at M12 and M14, but not at baseline. Across time points, the sham group showed significant increase in BMC in all skeletal sites at both time points. In contrast, OVX-diet rats showed a significant decrease in BMC at M12 and M14 compared to baseline in the pelvis, femur, and tibia (Figure 3).

Areal comparison between groups showed a significant decrease in bone area of OVX-diet rats in spine and femur at M14, whereas the pelvis showed significant decrease at both M12 and M14. There were no significant differences observed between groups in the tibia. The bone area of the sham group, across time points, was skeletal site-dependent, with the spine

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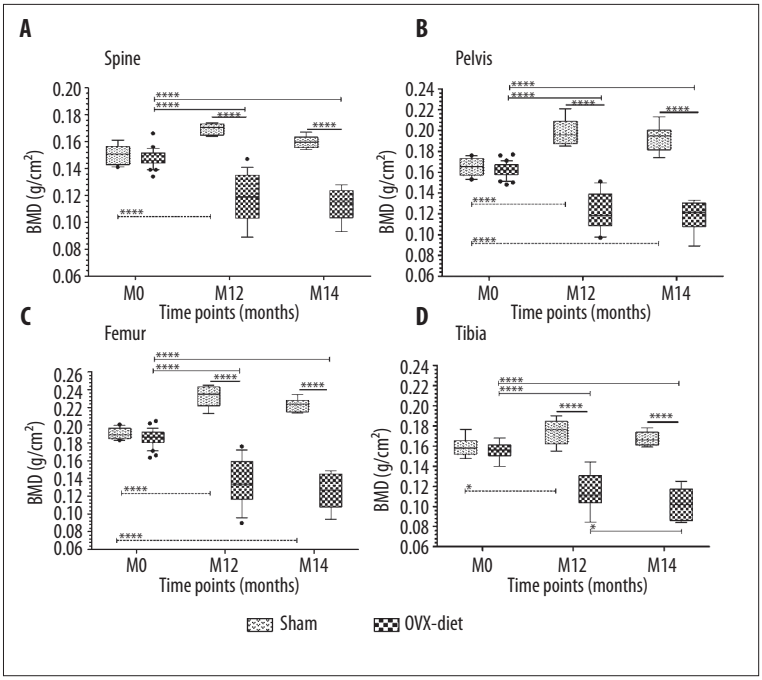

Figure 2. Variation in BMD in different skeletal sites, (**A**) Spine, (**B**) Pelvis, (**C**) Femur, and (**D**) Tibia across time points in a group and between groups at each time points. In all skeletal sites, BMD increased in sham at M12 with slight decrease at M14 unlike OVX-diet which showed a reduction in BMD with time. Asterisks indicate the significance level.

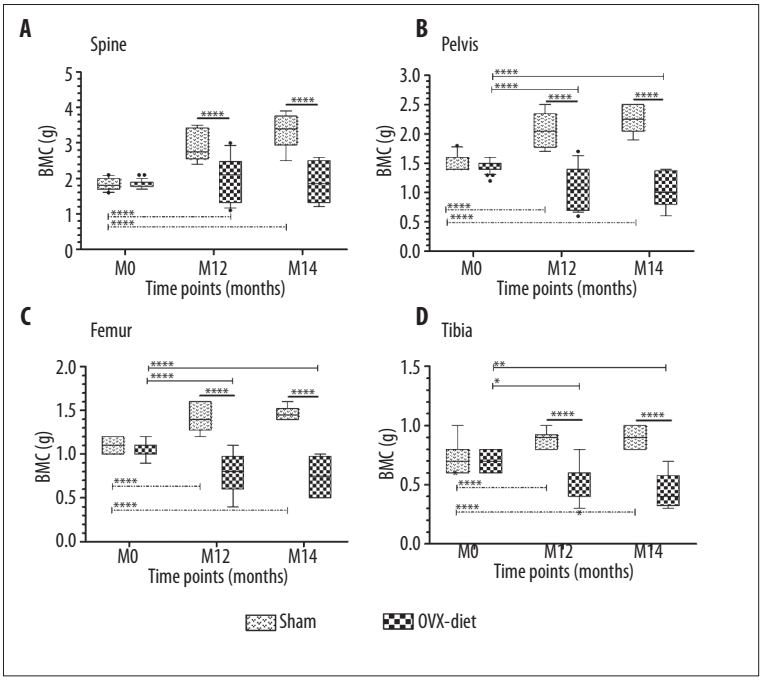

Figure 3. Variation in BMC in different skeletal sites, (**A**) Spine, (**B**) Pelvis, (**C**) Femur, and (**D**) Tibia across time points in a group and between groups at each time points. Significant differences between sham and OVX-diet group both at M12 and M14 indicated that BMC increased with time in sham whereas it decreased with time in OVX-diet rats.

and pelvis showing significant difference at M12 and M14 compared to baseline. Significant areal increase was observed at M14 compared to M12. No change in area was observed for the femur across time points; unlike the tibia, which exhibited an areal increase only at M14. In contrast, the spine of the OVX-diet group showed significant increases at M12 and M14, but were lower than in sham rats. No time-dependent changes were observed for the pelvis, femur, or tibia of OVX-diet rats (Figure 4).

Z-score and percent bone loss

Considering the Z-score, in which the BMD was compared to age-matched rats, significant decreases were observed between groups at M12 and M14 in all skeletal sites. The Z-scores for OVX-diet rats further decreased over time, showing significance at M12 and M14 compared to baseline. A significant decrease was observed at M14 compared to M12 in the tibia of OVX-diet rats (Figure 5).

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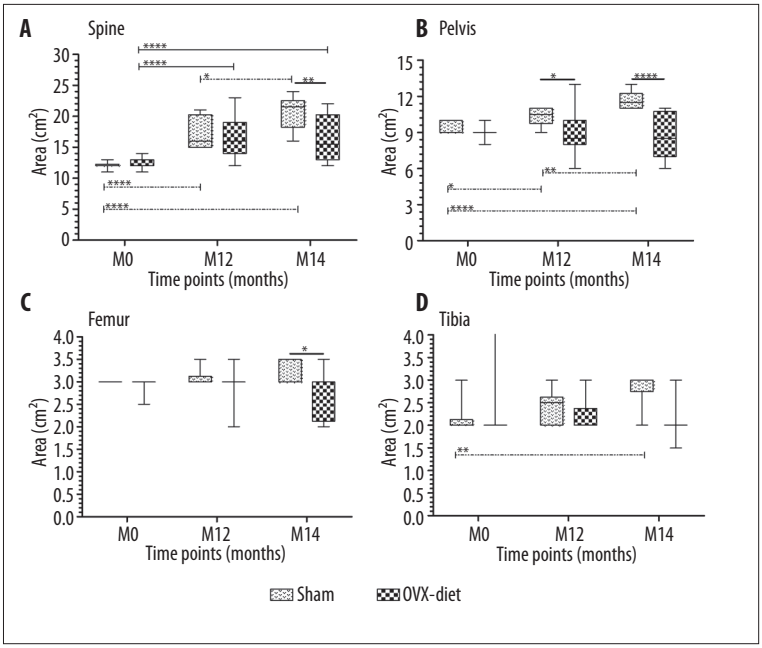

Figure 4. Variation in bone area with time in different skeletal sites. Area in OVX-diet rats significantly decreased at M14 in (**A**) Spine, (**B**) Pelvis and (**C**) Femur unlike tibia when compared to sham. While sham showed time dependent increase in spine, pelvis and tibia (dotted line), only spine of OVX-diet rats (straight lines) showed increase with time unlike other skeletal sites where no change was observed. Asterisks indicate the significance level.

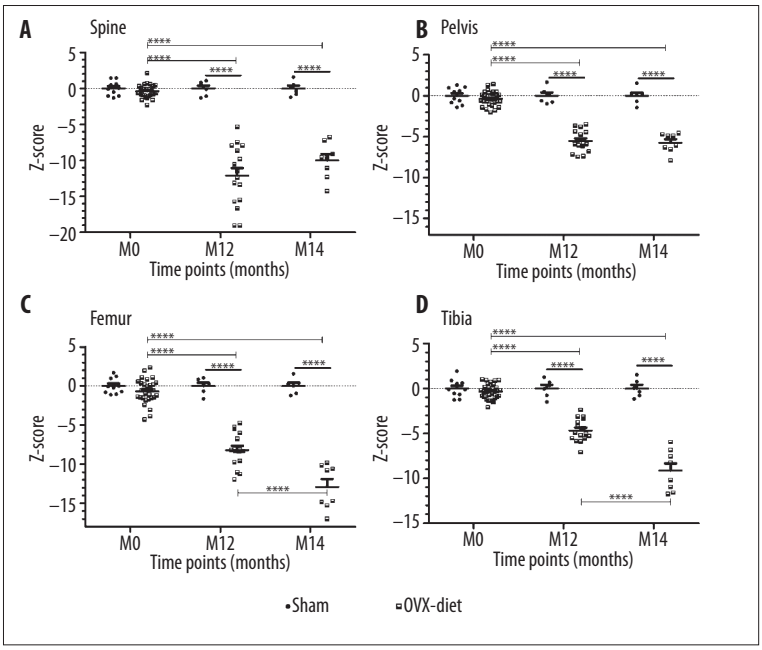

Figure 5. Shows the Z scores determination where BMD was compared to age matched rats to nullify the age related variation between rat groups. Significant differences between groups were observed at M12 and M14 along with time dependent decrease in OVX-diet group and with no change in sham rats.

Percent bone loss increased with time from M0 up to M14 in OVX-diet rats, and was skeletal site-dependent (Table 1). In the spine, the bone loss increased from –1.8% at M0 to –25.52% at M3 and –30.23% at M12. The highest bone losses were observed in the pelvis, femur, and tibia at M14: –39.00%, –43.82%, and –38.75%, respectively.

Tissue fat (%)

Comparison between groups showed significant decrease in percent fat of OVX-diet rats only at M14 in the spine, pelvis, and femur. No significant differences were seen in the percent fat in the tibia when comparing the sham and OVX-diet groups. Across time points, the sham group showed significant increases in the spine, pelvis, and femur at M12 and M14 compared to baseline, whereas the tibia showed significant increase only at M14. Additionally, a significant increase was observed at M14 in the spine compared to M12. In the case of the OVX-diet group, there was significant increase in percent fat in all skeletal sites at M12 and M14. Although no significance was observed between M12 and M14 in OVX-diet rats, the percent fat was slightly lower at M14 compared to M12 in all skeletal sites (Figure 6).

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Table 1. Bone loss (%) variation with time and skeletal site.

Time points (month)	Skeletal site (percent bone loss)			
	Spine	Pelvis	Femur	Tibia
M0	–1.8814	–1.8788	–2.2643	–2.0853
M1	–18.3077	–19.9154	–17.0587	–16.8300
M3	–25.5263	–26.5806	–22.6510	–22.9795
M12	–30.2360	–37.9723	–42.1304	–34.0158
M14	–29.2276	–39.0017	–43.8272	–38.7587

Bone loss was calculated using the formula, [mean BMD – mean peak BMD]/mean peak BMD*100, where mean BMD (Bone Mineral Density) indicates the mean BMD of OVX-diet rats at each time point and mean peak BMD is the mean BMD of the sham rats at each time point. Bone loss increased with time indicating an elevation in bone osteoporosis with disease progression.

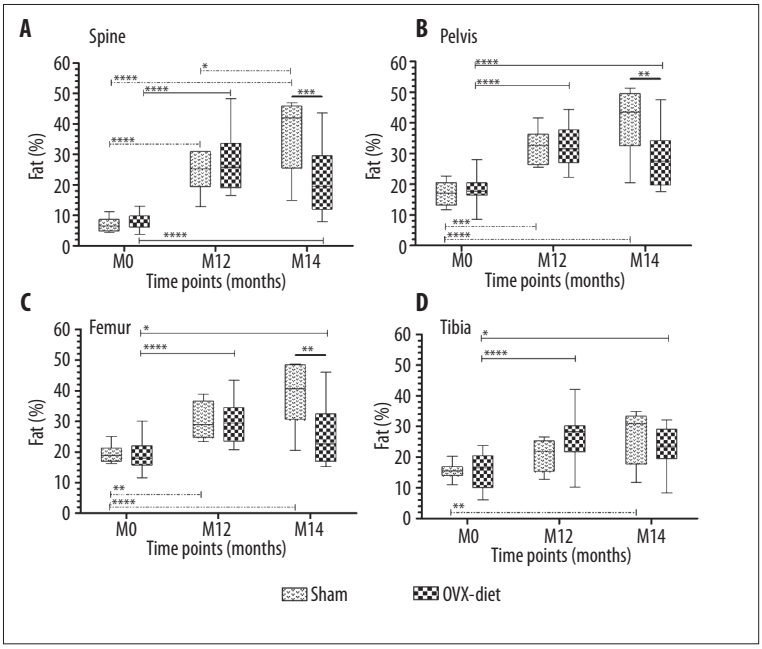

Figure 6. Shows the variation in tissue fat (%) between groups (short line) and across time points in each group. Tissue fat increased with time irrespective of groups upto M12, but significant reduction was observed in OVX-diet rats at M14 in skeletal sites, (**A**) Spine, (**B**) Pelvis, and (**C**) Femur. Asteriscks indicate the significance level (* p<0.05, ** p<0.01, *** p<0.001 and **** p<0.0001).

Dependency and relationship between BMC, BMD, and bone area with time

Linear regression analysis was performed between BMD, BMC, and bone area to evaluate the influence of change in one variable on the other in the spine and pelvis of OVX-diet rats. In the spine there was a linear, gradually increasing relationship between BMC and BMD, with R^2 increasing from 0.2739 at M0 and R^2 of 0.7904 at M12, after which it decreased to R^2 of 0.6630 at M14. In the case of BMC *vs.* bone area, the relationship increased, with a small value of R^2 of 0.3086 at M3, with high value at M14 of about 0. 9536. In the case of BMD with bone area, a negative relationship, which was not significant, was observed up to M3, after which there was an increasing, positive relationship at M12 and M14, with R^2 of 0.4529 and 0.4633, respectively (Figure 7).

In the pelvis, the relationship between BMC and BMD was higher than that of the spine at M0, with R^2 being 0.7537. The R^2 value gradually increased, reaching R^2 of 0.8885 at M14, after a slight decrease at M3, with R^2 of 0.8192. Regression values for BMC *vs.* bone area showed a pattern similar to that in the spine, starting with R^2 of 0.3032 at M0, and reaching 0.9542 at M14. In the case of BMD *vs.* bone area, negative and low correlations were observed at M0 and M1, after which it increased gradually, reaching R^2 of 0.8178 at M14 (Figure 8).

Percent change in BMD, BMC, and bone area with respect to time was evaluated in the skeletal sites of the spine and pelvis of sham and OVX-diet groups, based on their respective average values (Table 2). In the spine in sham rats during the M0-M1 period, there was a 3.5% increase in BMD, with a 16% increase in BMC and a 13% increase in area, in contrast to the

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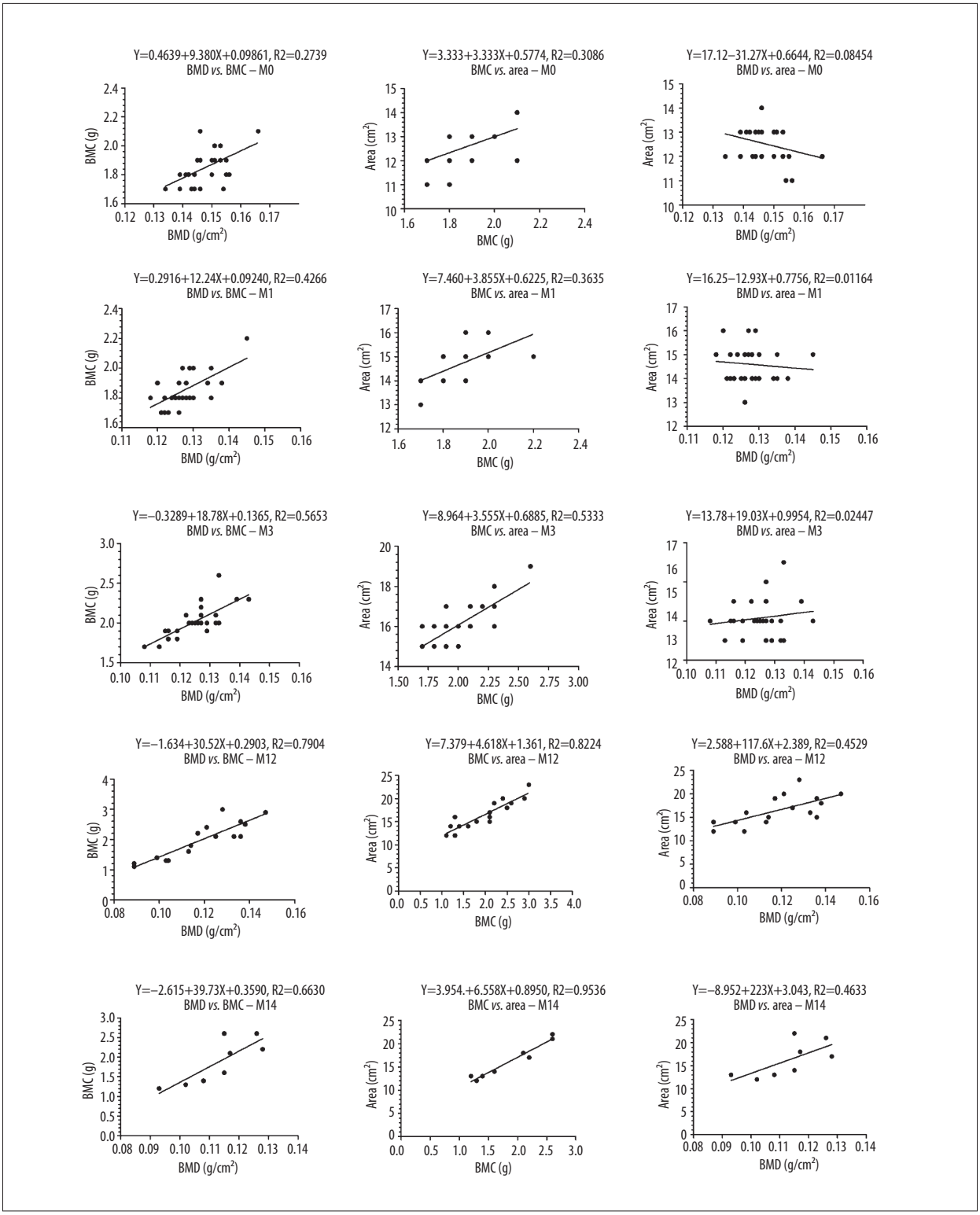

Figure 7. Shows the interrelationships between BMD *vs.* BMC, BMC *vs.* Area and BMD *vs.* area at different time points of M0, M1, M3, M12 and M14 in spine of OVX-diet rats. Increasing regression values was observed for BMD *vs.* BMC from M0 upto M12 with decrease at M14, whereas BMC *vs.* area showed increasing values upto M14 unlike BMD *vs.* area where regression remained low upto 3 months with increase in further time points. Values were always less for BMD *vs.* area, when compared to BMD *vs.* BMC, BMC *vs.* area relationships. Our published M3 figure [42] is used here for time dependent observation.

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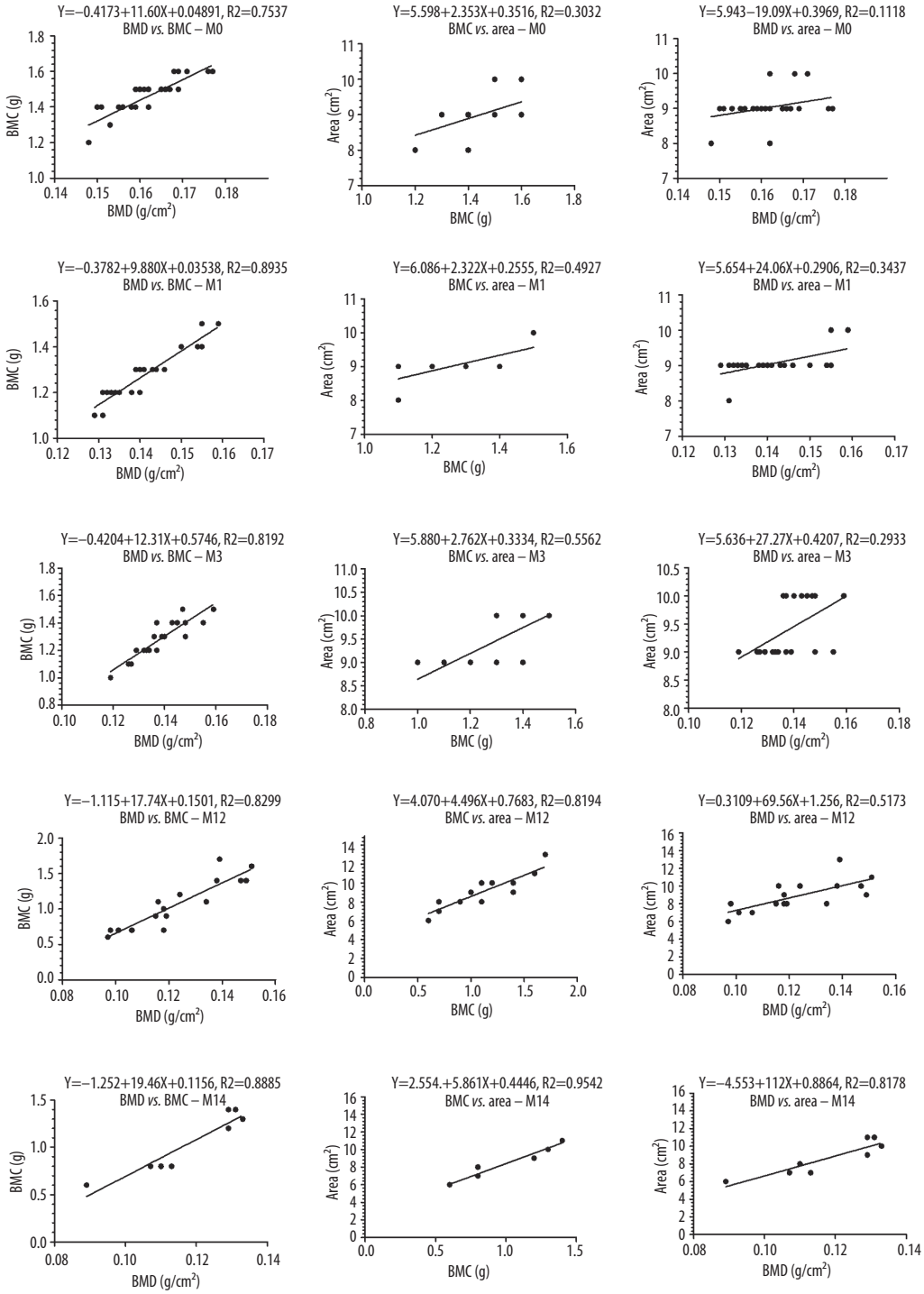

Figure 8. Shows the interrelationships between BMD *vs.* BMC, BMC *vs.* area and BMD *vs.* area at different time points of M0, M1, M3, M12 and M14 in pelvis of OVX-diet rats. BMD *vs.* BMC reached a high value at M1 with no change or increase upto M14 whereas relationships for BMC *vs.* area and BMD *vs.* area increased with time. Our published M3 figure [42] is used here for time dependent observation.

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Table 2. Proportion change (%) in osteoporosis determinants (BMD, BMC and area) related to time in spine and pelvis.

Skeletal site	Time period (month)	Sham (% change)			OVX-diet (% change)		
		BMD	BMC	Area	BMD	BMC	Area
Spine	M0–M1	3.5857	16.3154	13.3412	–13.7559	0.1081	16.8000
	M1–M3	7.7991	14.3177	5.7292	–1.7263	9.2873	10.6849
	M3–M12	0.7929	19.1781	18.3908	–5.5813	–2.4209	2.1040
	M12–M14	–5.8014	14.9425	20.3883	–4.4397	–5.0633	–1.5152
Pelvis	M0–M1	6.4935	5.9908	2.9186	–13.0821	–12.8767	0.0738
	M1–M3	5.8537	8.5507	–0.7463	–2.9562	–0.9434	3.5398
	M3–M12	6.7204	15.8879	8.7719	–9.8382	–15.1786	–5.1816
	M12–M14	–2.8547	8.8710	12.9032	–4.4670	–2.9240	–2.8169

Proportion change (%) was calculated by the formula, for 0M–1M period = [BMD (1M) – BMD (0M)]/BMD (0M)*100. Likewise, calculated for each time period for both sham and OVX-diet rats to indicate how the variation in each parameter influences the relationship between the BMC *vs.* BMD or BMD *vs.* area or BMC *vs.* area. BMD and BMC indicates Bone Mineral Density and Bone Mineral Content respectively.

OVX-diet group, in which there was a 13% decrease in BMD, with a 0.10% increase in BMC and a 16% increase in area. Percent change varied with time, slowly progressing to a negative percent change in BMD at M14 in sham group, whereas in the OVX-diet group there was a decrease in BMD, with varying negative percent changes with time influenced by BMC and area. The pelvis also showed variation in percent change of BMC, BMD and bone area with time.

Discussion

A severe osteoporotic status as determined by Z-Score (reaching below –5 at M14) was observed in this study in OVX-diet rats. This was achieved through ovariectomy plus nutrient depletion (calcium, vitamin, phosphorous, phytoestrogen). This supports the concept that estrogen depletion and calcium and vitamin deficient diets cause bone loss, leading to a progression of the osteoporotic bone status by 14 months after ovariectomy. Parameters of bone quality (BMD, BMC, and bone area, Z-score), as well as other parameters such as percent fat, were examined at the skeletal sites of the spine, pelvis, femur, and tibia.

This study showed that BMD and BMC exhibited a significant decrease in all examined skeletal sites when compared with sham rats at all time points except baseline. Body weight of the OVX-diet group increased with time, but was significantly lower than the sham group at M14. Furthermore, percent fat was also significantly lower at M14 in the OVX-diet group compared to sham rats. Previous epidemiological data have shown that high body weight or body mass index (BMI) is correlated with high bone mass, and that a reduction in body weight may cause bone loss, acting as a catalyst for future osteoporotic disease. Furthermore, positive relationships between calcium intake, body weight, and bone mass were reported [34,35]. An increase in adipose tissue leads to enhanced estrogen production and osteoclast suppression, resulting in increased bone mass [36]. Our study also corroborates that lower body weight is associated with low bone mass.

Additionally, vitamin D acts as a key regulator of intestinal calcium absorption and bone resorption to ensure constant serum calcium levels. Vitamin D deficiency and serum low free calcium leads to enhanced secretion of parathyroid hormone, leading to osteoporosis [24,37]. A previous pilot study in a sheep model has indicated that malnutrition with calcium- and vitamin D-deficient diet after ovariectomy supported induction of severe osteoporosis [7]. Thus, a combined multi-deficiency diet along with ovariectomy was used to obtain the cumulative effect on bone loss.

The Z-score, which compares the bone mineral density to age-matched sham rats, was below –2.0. In humans this value is considered as the threshold of osteoporotic fracture status [38]. Despite reaching 17.5 months of age, Z-score results confirmed osteoporotic status in OVX-diet rats compared to the sham group, thus precluding the age factor and supporting the validity of our established osteoporotic rat model. Bone mass is influenced by age, body size, and behavioural factors. Analysis of the extent to which all of these factors contribute to BMD and age-dependent bone loss is important in fracture risk prediction [39].

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The measured bone areas at most skeletal sites were decreased in the OVX-diet group at M14 compared to the sham rats. But previous studies have also shown a greater increase in bone area of OVX-diet rats relative to a decrease in BMC, which yielded a BMD significantly less than that of sham rats [40]. It very important to know if BMD is influenced only by bone area. As BMD is the ratio of BMC to bone area, controversy arises in defining statements of bone strength with respect to bone size [41], in addition to the fact that DEXA is a 2-dimensional assay. In this study, we used regression analysis to investigate the relationship between bone parameters with disease progression. BMD is directly proportional to BMC, and thus is indirectly correlated with the bone area. On the other hand, the BMC parameter has direct relationships to both BMD and bone area. Theoretically, BMC/bone area and BMD/BMC relationship is expected to be direct and steady (with high regression values) at all time points, but it tends to increase as osteoporosis progresses. This emphasizes the importance of proportion change in each parameter with time and how this impacts BMD and how it tends to change with age and disease progression.

As discussed earlier, R^2 between BMD and bone area of OVX-diet rats increased with time because BMD initially decreased by about –13.75% at M0–M1 in OVX-diet rats, whereas the bone area had a 16% change, showing a negative relationship. However, with time, there was a –5.58% change in BMD at M3–M12, with a 2% change in bone area reaching a high regression value due to a –4.43% change in BMD at 14M with a –1.5% change in area. Although BMC and area were directly proportional, they also showed increasing relationships with time, indicating that percent change in bone area was not the same as that of BMC initially. This correlated with time and disease at M14, when both BMC and area changed in a negative manner. In the case of BMD and BMC, the highest correlation was observed in the spine at M14, when percent change in BMD and BMC were equal. The same percent variation was determined in sham skeletal sites of the spine and pelvis to determine the influence of age on BMD of normal rats. Percent change in BMD gradually decreased with time, reaching negative values at M12–M14 of –5.80 and –2.85% in the spine and pelvis, respectively, indicating the point of BMD decline in sham rats. Thus, bone size affects the apparent density due to the non-linear relationship between area and volume, and could be improved by considering area as a determinant of skeletal strength [39].

Conclusions

The study showed significantly lower BMD, BMC, percent fat, and body weight in OVX-diet rats compared to sham rats. The low Z-score, with percent bone loss of nearly 40% at M14, indicates that severe osteoporosis was established in rats by combined diet accompanied by ovariectomy. The proportional change of BMD with respect to time point shows that BMD increases in sham rats up to M12, but decreases at M14, while bone area tends to increase gradually. This indicates senile osteoporosis in sham rats at M14. In OVX-diet rats, BMD decreased. A decrease in bone area were also observed, thus causing the regression co-efficiencies to increase with time, indicating lower area along with lower BMD, which leads to enhanced fracture risk in OVX-diet rats. Finally, our results suggest that BMD, BMC, and bone area are important parameters in analyzing fracture risk. These parameters should be individually analyzed in osteoporosis with aging to avoid biased BMD due to the variation of bone area with age. This would lead to a better understanding of various aspects of the disease.

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