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Sclerostin and Pref-1 have differential effects on bone mineral density and strength parameters in adolescent athletes compared with non-athletes

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Abstract

Purpose—Exercise activity is common in female adolescents, however, excessive exercise can have detrimental effects on bone mineral density (BMD). Mechanisms underlying this decrease in bone mass are not well understood. We investigated the effects of sclerostin, a potent inhibitor of bone formation via WNT signaling inhibition, and pre-adipocyte factor (Pref)-1, a suppressor of osteoblast differentiation, on BMD, bone turnover markers and bone strength in adolescent athletes.

Methods—We studied 50 adolescents between 15-21 years of age: 17 amenorrheic athletes (AA), 17 eumenorrheic athletes (EA) and 16 nonathletic controls (NA). We measured spine and hip BMD by dual energy x-ray absorptiometry and estimated failure load and stiffness at the distal radius and tibia using micro-finite element analysis. We also measured fasting sclerostin, Pref-1, N-terminal propeptide of type 1 procollagen (P1NP) and C-terminal collagen crosslinks (CTX) levels.

Results—Sclerostin levels were higher in AA and EA compared with NA (AA: 0.42 ± 0.15 ng/mL, EA: 0.44 ± 0.09 ng/mL, NA: 0.33 ± 0.14 ng/mL; p=0.047). In EA, sclerostin was positively associated with lumbar spine (LS) BMD and its Z-score (R=0.52, p=0.03 and R=0.55, p=0.02, respectively) whereas in NA, sclerostin was inversely associated with LS BMD (R=−0.61, p=0.01). Pref-1 levels were similar in all three groups and there were significant inverse associations between Pref-1, BMD and estimated bone strength in NA.

Conclusions—Sclerostin and Pref-1 may have differential effects on bone in adolescent athletes compared to non-athletes.

Keywords

Sclerostin; Pref-1; Bone strength; Adolescent athletes

Introduction

Sports activities are common in female adolescents and weight-bearing activities are known to have beneficial effects on bone [1]. However, excessive athletic activity, which results in amenorrhea, has negative effects on bone mineral density (BMD). Amenorrheic athletes

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(AA) have lower BMD compared to eumenorrheic athletes (EA) [2] and we have previously shown that excessive athletic activity in adolescent AA is associated with lower BMD and impaired parameters of bone microarchitecture compared with EA and non-athletes (NA) [3]. Hormonal alterations such as hypogonadism contribute partially to these changes; however other determinants of impaired bone health in excessive exercisers are not well understood. Two hormones, sclerostin and preadipocyte factor (Pref-1), have negative effects on bone formation and therefore may be potential mediators of the bone phenotype of adolescent athletes.

Sclerostin is a glycoprotein, secreted primarily by the osteocyte and a potent inhibitor of bone formation through inhibition of the WNT signaling pathway. Effects of sclerostin on bone accrual are best exemplified in human disease models of sclerostin deficiency, sclerosteosis and van Buchem's disease, both of which are characterized by increased bone mass [4-6]. Individuals with sclerosteosis have loss-of-function mutations in the sclerostin-encoding SOST gene and undetectable levels of sclerostin [7]; N-terminal propeptide of type 1 procollagen (P1NP), a marker of bone formation, and BMD are increased in individuals with sclerosteosis as compared to healthy controls [4, 7].

In adults, sclerostin levels are influenced by gonadal status and age. Sclerostin increases with increasing age and is inversely associated with estradiol levels in women [8-10]. Treatment with estradiol in postmenopausal women causes a significant decrease in sclerostin [11, 12]. In adults and children, sclerostin is higher in males than females [8, 13], and in children, sclerostin declines after pubertal onset in both sexes [13].

Sclerostin levels are also affected by mechanical loading. In murine models, loading of the ulna and tibia results in a decrease in sclerostin-positive osteocytes in cortical and trabecular bone [14, 15]. In obese adults, sclerostin increases with diet-induced weight loss, yet this increase is not observed when weight loss is due to both diet and exercise [16]. In a study of healthy men and premenopausal women, individuals in the most physically active quartile had lower sclerostin compared to individuals in the least active quartile [17]. Yet in postmenopausal women, a year of weight bearing exercise did not result in changes in sclerostin [18]. Therefore, beneficial effects of exercise on BMD may partly be mediated by changes in sclerostin in some populations.

Preadipocyte factor (Pref-1) is a member of the epidermal growth factor-like family of proteins, which is expressed in progenitor cell types and is also a known suppressor of osteoblast differentiation. Pref-1 has been reported to be high in hypothalamic amenorrhea and anorexia nervosa [19, 20], and is inversely associated with BMD.

Although both sclerostin and Pref-1 have a negative effect on bone formation, they have not been investigated in adolescent athletes, and their relationship to bone parameters, amenorrhea and exercise in this group is unknown. We investigated the effects of sclerostin and Pref-1 on BMD and estimated bone strength in adolescent AA, EA and NA in order to compare a state of intense physical activity versus a sedentary state (athletes vs. non-athletes) and effects of a hypogonadal versus eugonadal state (AA vs. EA). We hypothesized that AA would have higher sclerostin and Pref-1 than EA and NA and that higher sclerostin and Pref-1 would be associated with lower BMD and bone strength.

Methods

Subjects

We studied 50 adolescent females between 15-21 years of age: 17 AA (mean age ± SD: 19.8 ± 1.7 years), 17 EA (18.7 ± 1.7 years) and 16 NA (19.4 ± 1.2 years). Subjects were recruited

through medical clinics and advertising in local newspapers and colleges. A bone age of at least 15 years and a body mass index (BMI) between the 10^{th} and 90^{th} percentiles were required for inclusion in the study. Athletes participating in the study participated in at least 4 hours of aerobic, weight-bearing exercise of the legs or at least 20 miles of running/week for the preceding 6 months, at a minimum. These criteria were established in consultation with exercise physiologists. Non-weight bearing athletes, including cyclists and swimmers were excluded from participation. Gymnasts and rowers were also excluded due to differences in the nature of the impact and weight-bearing in these activities as compared to runners [21-23]. Nonathletic controls were included if they did not participate in more than 2 hrs of weight-bearing exercise per week and did not participate in organized team sports. Participants were excluded if they used medications known to affect bone metabolism, including oral contraceptives, in the three months prior to enrollment or if conditions other than exercise were a possible cause of amenorrhea.

Amenorrhea was defined as the absence of menses for ≥ 3 months in a period of oligomenorrhea (cycle length > 6 weeks) lasting at least 6 months or the absence of menarche by the age of ≥ 16 years. Eumenorrhea was defined as at least nine menstrual periods in the preceding year with a cycle length of 21-35 days. The Partners HealthCare institutional review board approved this study. Written informed consent was obtained from all participants ≥ 18 years of age or parents of those participants < 18 years old and assent was obtained from all participants < 18 years of age. Clinical characteristics, bone mineral density, bone microarchitecture and finite element analysis data have previously been reported for this cohort [3, 24].

Experimental Protocol

All participants had a history (including a detailed assessment of exercise activity) and physical exam performed and blood was drawn for laboratory studies. Height was measured as the average of 3 readings on a single, wall-mounted stadiometer and subjects were weighed on an electronic scale. BMI was calculated using the formula [weight (kg)/height (meter)^2]. A bone age x-ray of the left hand and wrist was obtained and assessed using the standards of Greulich and Pyle [25]. Participants underwent DXA to measure BMD of the lumbar spine and hip and lumbar spine bone mineral apparent density (BMAD) was calculated using published methods [26]. All participants underwent high-resolution peripheral quantitative CT (HR-pQCT) of the non-dominant distal radius and tibia as previously described [3].

Microfinite Element Analysis

We used linear micro-finite element analysis (FEA) of HR-pQCT images to estimate biomechanical properties of the distal radius and distal tibia under uniaxial compression loading as previously described [27-29]. Outcomes from micro-FEA included stiffness (kN/m) and failure load (kN). A prior study has reported strong associations between the biomechanical properties derived from microFEA-estimated and those measured directly via ex vivo testing of elderly human cadaveric radii [30].

Biochemical Assays

Sclerostin was measured using an enzyme immunoassay (TECOmedical Group, Quidel Corporation, San Diego, CA) with a minimum detectable level of detection of 0.13 ng/mL and an intra-assay coefficient of variation (CV) of 5.48% and inter-assay CV of 5.78%. Pref-1 was measured with the Quantikine Human Pref-1 immunoassay (ELISA) (R&D Systems, Minneapolis, MN) with a mean minimum detectable level of 0.012 ng/mL, intra-assay CV of 3.7% and inter-assay CV of 6.2%. An ultrasensitive ELISA was used to measure estradiol (ALPCO Diagnostics, Salem, NH) with a minimum level of detection of

1.399 pg/mL, intra-assay CV of 6.36% and inter-assay CV of 7.60%. P1NP was measured by radioimmunoassay (Orion Diagnostics, Espoo, Finland) with a minimum reportable value of 0.7 μg/L and an intra-assay CV of 3.5-5.3% and an inter-assay CV of 3.6-5.4%. C-terminal collagen cross-links (CTX) levels were measured by immunoradiometric assay (Immunodiagnostic Systems, Fountain Hills, AZ) with a minimum reportable value of 0.02 ng/mL and intra-assay CV of 5.2-6.8% and inter-assay CV of 5.6-7.4%.

Statistical Methods

Statistical analysis was performed using JMP (version 9; SAS Institute, Inc., Cary, NC) software. Means and standard deviations are reported. The means were compared using an ANOVA and the Tukey-Kramer adjustment was used to account for multiple comparisons. A two-tailed p-value of < 0.05 was used to indicate significance. For non-normal distributions, the Wilcoxon test was used to make comparisons between the groups and the Bonferroni correction was used to account for multiple comparisons, in which case a p-value of < 0.017 was used to indicate significance. Pearson correlation coefficients, or Spearman's coefficients, if the data were not normally distributed, were calculated to assess univariate relationships. Multivariable analyses were performed using least-squares linear regression to control for potential confounders. Outlier analysis was performed and one outlier was excluded in reporting associations between sclerostin and P1NP.

Results

Clinical characteristics

Table 1 lists the clinical characteristics of subjects in the AA, EA and NA groups. The three groups did not differ in chronologic age, bone age, BMI or height (Table 1). The AA group had a significantly older menarchal age as compared to the NA group (AA: 14.2 ± 2.4 years versus HC: 12.1 ± 1.7 years; $p < 0.01$) but there were no significant differences in menarchal age between AA and EA or between EA and NA. Hip BMD, Hip BMD Z-score and femoral neck (FN) BMD Z-score were significantly higher in EA as compared to both AA and NA (Table 1). Lumbar spine (LS) BMD, LS BMD Z-score and LS BMAD were all significantly lower in AA as compared to both EA and NA (Table 1). CTX levels were significantly higher in EA as compared to NA whereas P1NP levels were not significantly different in the three groups.

Stiffness and failure load in the radius were significantly lower in AA as compared to NA (Table 1). In the tibia, stiffness and failure load were significantly higher in EA as compared to NA (Table 1).

Sclerostin and Pref-1 Levels

Sclerostin was higher in AA and EA as compared to NA (AA: 0.42 ± 0.15 ng/mL; EA: 0.44 ± 0.09 ng/mL; NA: 0.33 ± 0.14 ng/mL; p=0.047) (Figure 1). This relationship remained significant after controlling for total body bone mineral content ($p < 0.04$), or for menarchal age (p=0.03). Pref-1 levels were not significantly different in the three groups (AA: 0.35 ± 0.16 ng/mL; EA: 0.38 ± 0.25 ng/mL; NA: 0.38 ± 0.28 ng/mL; p=0.71).

Associations of Sclerostin and Pref-1 with Bone Mineral Density, Bone Turnover Markers and Estimated Bone Strength in Non-Athletes and Athletes

a. Non-athletes

BMD: In NA, there were inverse associations between **sclerostin** and LS BMD (R=−0.61; p= 0.01), LS BMAD (R=−0.60; p=0.01) (Figure 2), and total hip BMD (R=−0.55; p<0.03). These relationships remained significant after controlling for estradiol levels or for

menarchal age. There was also a trend towards an inverse association between sclerostin and FN BMD (R= −0.49; p=0.05) and LS BMD Z-scores (R=−0.48; p=0.06).

There were significant inverse associations of **Pref-1** with LS BMD (Spearman's rho= −0.85; p<0.0001) and its Z-score (Spearman's rho=−0.68; p< 0.01), LS BMAD (Spearman's rho=−0.86; p<0.0001), hip BMD (Spearman's rho=−0.82; p=0.0001) and its Z-score (Spearman's rho=−0.86; p< 0.0001), and FN BMD (Spearman's rho =−0.69; p<0.01) and its Z-score (Spearman's rho =−0.73; p=0.002). These relationships remained significant after controlling for estradiol or for menarchal age.

Bone Turnover Markers: In NA, there were significant inverse associations between **sclerostin** and P1NP (R=−0.59; p<0.02) and CTX (R=−0.57; p=0.02), which remained significant after controlling for estradiol (p=0.02 and p=0.03, respectively) or for menarchal age (p=0.002 and p=0.02, respectively). There were no significant associations of **Pref-1** with P1NP or CTX.

Estimated Bone Strength: In NA, **sclerostin** was inversely associated with stiffness and failure load at the radius (stiffness: R=−0.65; p=0.006; failure load: R=−0.67; p=0.005), even after controlling for estradiol or for menarchal age. There were trends towards an inverse association of sclerostin with estimated stiffness and failure load at the tibia (stiffness: R=−0.44; p<0.09; failure load: R=−0.47; p<0.07).

Pref-1 was also inversely associated with stiffness and failure load at the radius (stiffness: Spearman's rho= −0.82; p<0.0001; failure load: Spearman's rho= −0.80; p=0.0002) and tibia (stiffness: Spearman's rho= −0.73; p=0.001; failure load: Spearman's rho= −0.73; p=0.001). All relationships remained significant after controlling for estradiol or for menarchal age.

b. Eumenorrheic Athletes

BMD: In EA, there were significant positive correlations between **sclerostin** and LS BMD (R=0.52; p=0.03) and its Z-score (R=0.55; p=0.02), and LS BMAD (R=0.71; p=0.001) (Figure 2b). These relationships remained significant after controlling for estradiol, and associations with LS BMAD remained significant after controlling for menarchal age. We did not find an association between **Pref-1** and BMD in EA.

Bone Turnover Markers: In EA, there was a positive association between **sclerostin** and P1NP (R=0.56; p=0.03). There were no significant associations between **Pref-1** and P1NP or CTX in EA.

Estimated Bone Strength: In EA, there was a trend towards a positive association between **Pref-1** and failure load in the radius (Spearman's rho= 0.44; p<0.08). There were no significant associations between **sclerostin** and estimates of bone strength in EA.

c. Amenorrheic Athletes

BMD: We found no associations between sclerostin or Pref-1 and BMD in AA. **Bone Turnover Markers:** In AA, **Pref-1** was positively associated with P1NP (R=0.68; p=0.004) and this relationship remained significant after controlling for estradiol. There was also a trend towards a positive association between Pref-1 and CTX (R=0.47; p=0.06). There were no associations between **sclerostin** and bone turnover markers in AA.

Estimated Bone Strength: We found no associations between sclerostin or Pref-1 and estimated bone strength in AA.

Discussion

We have shown that whereas sclerostin and Pref-1 have the predicted inverse association with bone parameters in non-athletes, sclerostin is positively associated with BMD in eumenorrheic adolescent athletes and Pref-1 is positively associated with bone turnover markers in amenorrheic adolescent athletes. Therefore sclerostin and Pref-1 appear to have differential effects on bone parameters in adolescent athletes as compared to non-athletes.

Sclerostin is a potent inhibitor of WNT signaling and therefore of bone formation. Both estrogen [11, 12] and physical activity [16, 17] are important potential regulators of sclerostin levels. This is the first study that uses the model of amenorrheic and eumenorrheic adolescent athletes to potentially delineate the important effects of estrogen status and physical activity on sclerostin levels. When estrogen status was held constant (by comparing menstruating athletes to menstruating non-athletes), we observed that those with higher levels of physical activity had higher sclerostin levels, in contrast to animal models showing decreased SOST expression with increased mechanical loading [14] and cross-sectional studies in adults in which increased physical was associated with lower sclerostin levels [17]. Of interest, one prior study investigating sclerostin levels in elite male and female athletes found higher sclerostin levels in males performing weight-bearing activities as compared to those performing non-weight-bearing activities [31]. All athletes in our study were weight-bearing athletes; therefore, it is possible that different types of physical activity result in differences in sclerostin secretion.

It is not clear why sclerostin levels are higher in weight-bearing adolescent athletes compared to non-athletes. One possibility is that sclerostin levels are higher in individuals with greater absolute bone mineral content, as sclerostin is secreted by osteocytes. In our study, EA had significantly higher total bone mineral content as compared to AA but similar levels compared to NA. However, even after controlling for total bone mineral content, athletes had higher sclerostin compared with NA. Of interest, in obese adults, weight loss mediated by dietary changes leads to increased sclerostin levels [16], suggesting that nutritional deficiency is associated with increased sclerostin. Therefore, it is possible that higher sclerostin in adolescent athletes is consequent to a state of relatively lower energy availability from excessive exercise compared to NA. It is also possible that effects of nutritional deprivation exceed effects of amenorrhea on sclerostin, and therefore levels do not differ between AA and EA (although we expect energy availability to be even lower in AA compared with EA). Finally, we speculate that higher sclerostin in EA (who have higher BMD at cortical weight-bearing sites than healthy NA) may act as a physiological ‘brake’ to prevent continued increases in bone formation and bone mass from repetitive mechanical loading.

Pref-1, a trans-membrane protein highly expressed in both pre-adipocytes and osteoblastic cell lines [32], is a member of the EGF-like family of proteins. Pref-1 is an important negative regular of both adipocyte and osteoblast differentiation. In murine models, over-expression of osteoblast-specific Pref-1 results in mice with significantly reduced BMD [33]. We have previously shown Pref-1 to be inversely associated with PA and lateral spine BMD in women with anorexia nervosa and healthy controls [20]. In this study, we also demonstrated significant inverse associations between Pref-1 and BMD in NA. However, in AA, we demonstrated significant positive associations between Pref-1 and P1NP, a marker of bone formation. We have previously shown that bone marrow fat, which is inversely associated with BMD in multiple populations including healthy Caucasian women [34], women with anorexia nervosa [35] and obese women [36], is positively associated with Pref-1 in women with anorexia nervosa, but inversely associated with Pref-1 in normal-weight controls [37]. Therefore, Pref-1 may act differentially in states of nutritional

sufficiency compared with states of nutritional deficiency [37]. It is possible that Pref-1 also has differential effects on bone formation in states of intense physical activity compared to a sedentary state.

Although menarchal age was significantly greater in AA as compared to NA, there was no significant difference between menarchal age in EA as compared to NA and therefore this does not explain the contrasting relationships found in the EA and NA groups with respect to sclerostin and BMD and Pref-1 and bone strength. Additionally, even after controlling for menarchal age, reported associations of sclerostin and Pref-1 with bone density, bone turnover markers and estimated bone strength persisted.

Therefore, we have shown that both sclerostin and Pref-1 may have contrasting effects on BMD, bone microarchitecture and bone strength in athletes as compared to non-athletes. Further studies are needed to further delineate the role of sclerostin and Pref-1 in mediating BMD, bone microarchitecture and bone strength, and the association of sclerostin and Pref-1 with exercise activity, and nutritional and estrogen status.

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Non-Standard Abbreviations

AA	Amenorrheic athletes
EA	Eumenorrheic athletes
NA	Non-athletes
LS	Lumbar spine

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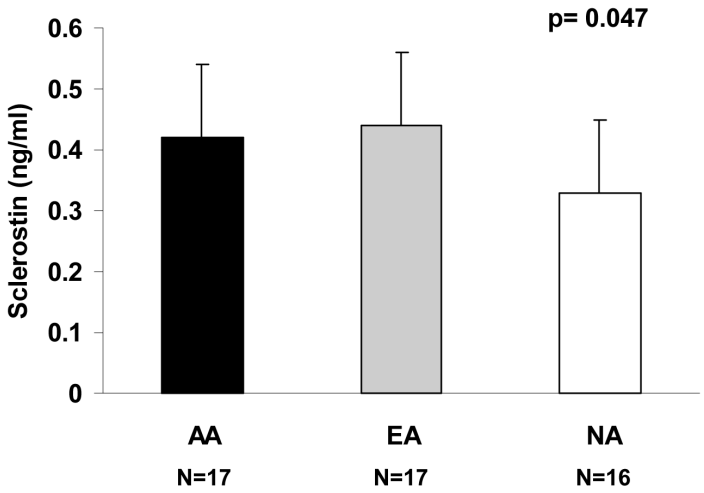

Figure 1.
Sclerostin levels were higher in athletes as compared to non-athletes (p=0.047). AA: amenorrheic athletes; EA: eumenorrheic athletes; NA: non-athletes.

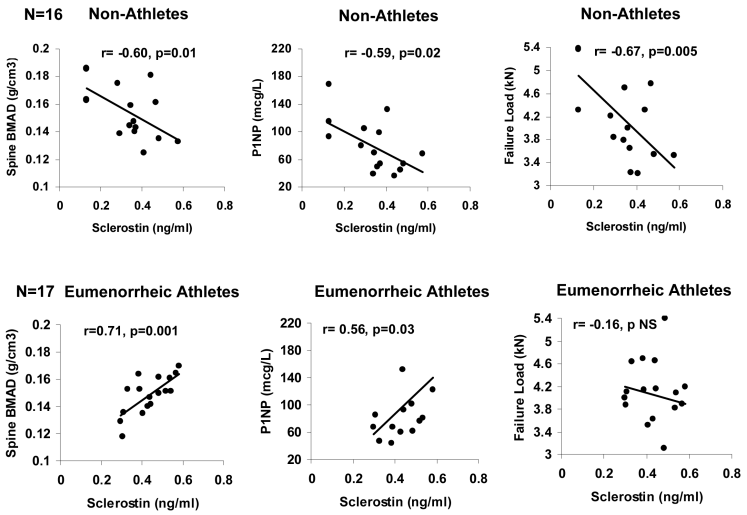

Figure 2.

In non-athletes, sclerostin was inversely associated with lumbar spine bone mineral apparent density (R=−0.60; p=0.01), P1NP (R=−0.59; p=0.02) and failure load at the radius (R=−0.67; p=0.005) (upper panel). In **eumenorrheic athletes**, sclerostin was positively associated with lumbar spine bone mineral apparent density (R=0.71; p=0.001) and P1NP (R=0.56, p=0.03) (lower panel).

Table 1

Clinical Characteristics of Study Subjects

	Amenorrheic Athletes (n=17)	Eumenorrheic Athletes (n=17)	Non-athletes (n=16)	*p*-value for overall group comparisons
Age (years)	19.8 ± 1.7	18.7 ± 1.7	19.4 ± 1.2	0.11
Bone Age (years) *	17.6 ± 0.7	17.5 ± 0.9	17.7 ± 0.9	0.25
BMI (kg/ m^2) *	20.8 ± 2.2	22.1 ± 2.2	21.5 ± 2.4	0.16
Height (cm)	166.3 ± 5.6	164.8 ± 6.7	163.2 ± 6.2	0.34
Menarchal Age (years)	14.2 ± 2.4	12.9 ± 1.2	12.1 ± 1.7	**<0.01**
LS Spine BMD (g/cm^2) *	0.89 ± 0.13[a,c]	1.01 ± 0.08	1.02 ± 0.13	**0.004**
LS Spine BMD Z-score *	−1.22 ± 1.25[a,c]	0.02 ± 0.86	−0.11 ± 1.02	**0.004**
LS Spine BMAD(g/cm^3) *	0.13 ± 0.02[a,c]	0.15 ± 0.01	0.16 ± 0.02	**0.002**
Total Hip BMD (g/cm^2)	0.94 ± 0.10[a]	1.05 ± 0.11[d]	0.96 ± 0.08	**0.003**
Total Hip BMD Z-score	−0.18 ± 0.91[a]	0.85 ± 0.90[g]	−0.33 ± 0.59	**0.0002**
Femoral Neck BMD (g/cm^2)	0.82 ± 0.09	0.90 ± 0.11	0.84 ± 0.10	0.06
Femoral Neck BMD Z-score	−0.49 ± 0.83[b]	0.26 ± 0.95[d]	−0.61 ± 0.71	**<0.01**
RADIUS				
Stiffness (kN/m)	70.5 ± 14.8[e]	79.5 ± 12.1	83.0 ± 15.6	**0.04**
Failure Load (kN)	3.6 ± 0.8[f]	4.0 ± 0.6	4.2 ± 0.7	**0.048**
TIBIA				
Stiffness (kN/m)	223.2 ± 25.8	242.9 ± 30.8[c]	211.5 ± 30.1	**0.01**
Failure Load (kN)	11.2 ± 1.3	12.1 ± 1.5[c]	10.6 ± 1.4	**0.01**
P1NP (μg/L) *	88.9 ± 40.1	99.4 ± 59.8	81.1 ± 37.2	0.79
CTX (ng/mL)	1.10 ± 0.34	1.19 ± 0.39[e]	0.87 ± 0.26	**<0.03**
Estradiol (pg/mL) *	27.6 ± 25.3	20.5 ± 11.6	43 ± 50.1	0.54

* Wilcoxon test used to analyze differences between groups and if p-value was significant, Bonferroni adjustment used to account for multiple comparisons

[a] p≤0.01 as compared to EA

[b] p=0.03 as compared to EA

[c] p<0.01 as compared to NA

[d] p<0.02 as compared to NA

[e] p<0.04 as compared to NA

[f] p<0.05 as compared to NA

[g] p<0.001 as compared to NA.