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Title: Impaired Vascular Function in Exercising Anovulatory Premenopausal Women is Associated with Low Bone Mineral Density

Short title: Vascular and bone associations in young women

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Abstract

In estrogen deficient postmenopausal women, osteoporosis shares a common link with cardiovascular disease risk, including endothelial dysfunction. The current study sought to examine associations between bone mineral density (BMD) and endothelial function in estrogen deficient premenopausal women with exercise-associated menstrual disturbances. Recreationally trained women (24.3 ± 0.8 years; overall mean $\pm$ SEM) who were estrogen deficient (amenorrheic or eumenorrheic anovulatory cycles; E2Def; $n=13$) or estrogen replete (eumenorrheic ovulatory cycles; E2Rep; $n=14$) were studied. Total body and lumbar BMD (L1-L4) were determined using dual energy X-ray absorptiometry. Serum markers of oxidative stress (oxidised low-density lipoprotein; OxLDL), energy deficiency (triiodothyronine), and bone turnover (osteocalcin, c-telopeptide X, P1NP) were assessed. Estrogen exposure was determined by assessing daily urinary estrone-3-glucuronide (E1G) across a monitoring period. Calf blood flow (CBF), an index of endothelial function, was measured using strain-gauge plethysmography. CBF, total body and L1-L4 BMD, triiodothyronine and E1G were lower ($p<0.05$), and c-telopeptide crosslinks higher ($p<0.05$) in E2Def. Osteocalcin and OxLDL did not differ ($p>0.05$) between groups. L1-L4 BMD, osteocalcin and E1G were the strongest predictors of CBF ($R^2=0.615$, $p<0.001$). CBF was the strongest predictor of L1-L4 BMD ($R^2=0.478$, $p<0.001$). L1-L4

($r=0.558$, $p=0.008$) and CBF ($r=0.534$, $p=0.004$) were independently correlated with E1G. In young recreationally trained premenopausal women with anovulatory menstrual disturbances, low CBF predicts decreased lumbar BMD, suggesting impaired peripheral endothelial function may predict early unfavorable changes in bone metabolism. This finding may be of relevance in the early detection of cardiovascular and bone health decrements in otherwise healthy estrogen deficient premenopausal women.

Introduction

Cardiovascular disease (CVD) and osteoporosis are recognized major public health problems^{1,2}, with growing evidence indicating that these diseases are linked. Low bone mineral density (BMD) is associated with endothelial dysfunction³, coronary artery disease^{1,2}, peripheral vascular disease⁴, stroke⁵ and cardiovascular mortality^{2,6}. Sex differences in this relationship are also reported, with some studies demonstrating a stronger link between bone health and CVD in women compared with age-matched men^{5,7}, suggesting a possible role for estrogen^{5,7}. Estrogen is known to upregulate the production and bioavailability of endothelium-derived nitric oxide (NO), a key molecule that modulates both vascular tone and bone resorption^{8,9}. Conversely, estrogen deficiency is associated with decreased circulating levels of NO⁹ and increased risk of osteoporosis and cardiovascular disease^{8,9}.

Estrogen deficiency is not exclusive to the postmenopausal period. During the premenopausal years, exercise-associated menstrual disturbances such as hypothalamic anovulation, either with eumenorrhea or amenorrhea, are characterized by marked hypoestrogenemia^{10,11}. The prevalence of eumenorrheic anovulation (16-25%) and amenorrhea (1-60%) is markedly higher in recreationally and competitively active women compared with sedentary women (5-8% and 2-5%, respectively)¹²⁻¹⁵. Chronic energy deficiency due to high energy expenditure that is often combined with restrictive eating patterns that can occur with or without obvious caloric deficits has been identified as the primary cause of these menstrual disturbances¹⁶.

The current study sought to examine the relationship between BMD and endothelial function, a surrogate marker of CVD risk, in estrogen deficient recreationally active women with exercise-associated menstrual disturbances. We also investigated whether estrogen exposure, as determined by daily urinary analysis of estrogenic metabolites across a monitoring period, and serum measures of triiodothyronine, a marker of energy status ¹⁷, commonly linked impaired bone and vascular health. Oxidative stress, a known contributing factor to both osteoporosis and CVD ^{18,19}, was also assessed for possible shared links due to the known anti-oxidant effects of estrogen ²⁰. In keeping with previous findings in amenorrheic athletes ^{10,21-23}, we anticipated that estrogen deficient women would demonstrate impaired endothelial function and lower BMD compared with their estrogen replete counterpart. We hypothesized that impaired endothelial function would be associated with lower BMD, and that these perturbations would be commonly linked with estrogen exposure, and markers of energy deficiency and oxidative stress.

Materials and Methods

Participants: Participants were recruited by posters. Physical activity status was required to have been consistent for the previous 6 months. Eligibility criteria for the study included: 1) age 18 to 35 years; 2) self-reported good health; 3) no known chronic illness, including hyperprolactinemia, metabolic syndrome, polycystic ovarian syndrome and thyroid disease; 4) stable menstrual status over preceding 3 months (menstruating or absence of menses); 5) non-smoking; 6) not currently dieting and weight stable for the preceding 3 months, as determined by self-report; 7) absence of hormonal therapy for at least 12 months; 8) no history or current clinical diagnosis of eating disorders and 9) no other contraindications that would preclude participation in the study. The study was approved by the institutional committee on human research by the Ethics Review Board, and confirmed to the standards set by the latest revision of the Declaration of Helsinki. All volunteers signed an approved informed consent document.

Experimental Design: The current investigation forms part of a larger prospective observational study examining relationships between physical activity, reproductive function, and bone and cardiovascular health. Fifty-two women completed 1-3 studies from the larger study over a 2-3 year window, with each woman being assessed across 2-3 consecutive menstrual cycles. From this cohort, we have previously published data on calf blood flow ^{10,24} and bone health ²¹. The current study presents novel analysis of the association between cardiovascular and bone health in two unique groups comprised of age-, body-composition-, and fitness-matched women who were either estrogen deficient (functional hypothalamic amenorrhea or eumenorrheic anovulatory cycles), or estrogen replete (eumenorrheic ovulatory cycles).

Experimental Protocol: All testing was completed in an ambient room (22-24°C) between the hours of 08:00 and 11:00. Except for maximal oxygen capacity testing, participants were 8 hours (overnight) fasted and had abstained from physical activity and caffeine for 24 hours and alcohol 12 hours prior to each study visit. All measures, except urine measures which were assessed daily, were recorded during the first 6-days of each menstrual cycle and during the first 6-days of each 30-day monitoring period for amenorrheic women. Subjects were monitored for 2 to 3 consecutive menstrual cycles or monitoring periods. Measures collected during 2 to 3 menstrual cycles were averaged for statistical analyses.

Exercise Status: Participants were required to be participating in purposeful exercise eliciting a heart rate (HR) greater than 55% of maximal HR (220 minus age) for more than 2 hours per week, and demonstrate a VO_2 peak $>40 \text{ ml/kg/min}$ ¹⁰. Exercise logs documented exercise type, duration, and HR.

Menstrual Status: Menstrual status was determined from daily first morning void urine samples. Urine samples were assayed for luteinizing hormone (LH), pregnanediol 3-glucuronide (PdG), and estrone 3-glucuronide (E1G) to assess ovulatory status and

estrogen exposure. We have previously described the criteria for detection of positive ovulation and menstrual cycle status ¹⁰. Amenorrhea was defined as cessation of menses for >90 days. Anovulatory menstrual status was identified as presentation of consistent anovulatory menstrual cycles for the study duration.

Study Groups: Two groupings of physically active women were retrospectively established: 1) estrogen replete (E2Rep; n=14) with eumenorrheic ovulatory menstrual cycles; and 2) estrogen deficient (E2Def; n=13) with eumenorrheic anovulatory menstrual cycles (n=4) and functional hypothalamic amenorrhea (n=9).

Estrogen Exposure: Using daily urinary estrogen levels, estrogen exposure (E1G) across each menstrual cycle or monitoring period was calculated by the trapezoidal area under the curve (AUC) method ¹⁰.

Anthropometric Measures: Average total body mass was determined from weekly measures on a physician's balance scale (Detecto, Webb City, MO). Height was measured at the beginning of the study period. Body mass index (BMI; kg/m²) was calculated (mean body weight (kg) divided by height²).

Body Composition and Bone Mineral Density: DXA was utilized to determine body composition and BMD (Prodigy, General Electric Lunar Corporation, Madison, WI, enCORE 2002 software, version 6.50.069). Total body and lumbar spine (L1-L4) BMD (absolute, g/cm² and Z-scores) were determined. A 28 subject precision study demonstrated 0.6 and 0.7 % precision for the total body and lumbar spine, respectively. The DXA scanner has a precision of < 1% coefficient of variation for body composition measurements.

Peak Aerobic Capacity: VO₂ peak was measured once by a progressive treadmill test to volitional exhaustion. Expired gases were collected continuously to measure inspired air volumes and to analyze breath-by-breath samples (Moxus Modular VO₂ System, Applied Electrochemistry Inc., Pittsburgh, PA).

Calf Blood Flow, Heart Rate and Blood Pressure: Calf blood flow (CBF), an index of endothelial function, was assessed using venous occlusion strain-gauge plethysmography as previously described by our group^{10,24}. In brief, with the subject in a supine position, a cuff was placed at the ankle (200mm Hg) and above the knee (55mmHg). An indium-gallium strain gauge (Vasculab SPG16 Medasonics, Newark, CA) was placed around the widest girth of the calf. Baseline resting and peak-ischemic measures were acquired. Ischemic plantar flexion exercise to fatigue was employed to elicit a maximal peak-response. Blood flow (mL/100mL/min), vascular conductance (blood flow/ mean arterial pressure (MAP); mL/100mL/min/mmHg), and vascular resistance (MAP/blood flow; U) were calculated. Measurement of HR and mean arterial pressure blood pressure (BP) were recorded continuously throughout each testing session on a beat-to-beat basis using the photoplethysmographic method (Finapres, Ohmeda, Madison, WI).

Serum Measures: Eight-hour fasted blood samples were analyzed for osteocalcin, c-telopeptide (CTX), procollagen type 1 N propeptide (P1NP), total triiodothyronine (TT3), and oxidized low-density lipoproteins (OxLDL). Serum samples were stored at -80°C until analyses were run. Osteocalcin was analyzed using an enzyme immunoassay technique (Quantikine Assay Kit, DRP300, R&D Systems Inc., Minneapolis, MN). Sensitivity was 0.246 ng/mL. The intraassay and interassay coefficients of variation (CV) were 4.7%, and 6.9% respectively. CTX was analyzed using a direct sandwich enzyme-linked immunosorbent assay (ELISA, Linco Research, Inc., St. Charles, MO). Analytical sensitivity was 0.5 ng/mL. The intra- and interassay CV were 4.6% and 6.2% respectively. P1NP was determined using a chemiluminescence-based immunoassay analyzer (Immulite, Diagnostic

Products Corp.). The analytical sensitivity of the assay was 2 $\mu\text{IU/ml}$ (14.4 pmol/L). The intra and interassay CV were 6.4% and 8.0%, respectively. TT_3 , an indirect marker of energy status¹⁷, was analyzed using a chemiluminescence-based immunoassay analyzer (Immulin, Diagnostics Products Corporation, Los Angeles, CA). Analytical sensitivity for insulin was 13.89 pmol L^{-1} , and for the TT_3 assay was 0.54 nmol L^{-1} . OxLDL, an indirect measure of systemic oxidative stress, specifically lipid oxidative damage, was determined with an ELISA plate assay (Alpco Diagnostics, Windham, NH, and R&D Systems, Minneapolis, MN). Analytical sensitivity was $\geq 0.03 \text{ U/I}$. Intra- and interassay coefficients of variation were both 5.75 U/I.

Urinary Measures: Measures of daily urinary metabolites were determined using microtiter plate competitive enzyme immunoassays to detect pregnanediol 3-glucuronide (PdG), and estrone 3-glucuronide (E1G). Detailed methods for these immunoassays have been described previously^{10,24}. The inter-assay coefficients of variation for high and low internal controls were 14.7% and 13.1% for E1G and 15.68% and 17.7% for PdG. To account for hydration status, urine samples were corrected for specific gravity using a hand refractometer (NSG Precision Cells, Inc., Farmingdale, NY).

Urinary LH, assessed in ovulatory women only, was determined by double antibody radioimmunoassay (Diagnostic Products Corp., Los Angeles, CA). The sensitivity of the LH assay was 0.6 mIU L^{-1} . The intra-assay and inter-assay coefficients of variation were 1.6% and 7.1%, respectively.

Statistics: Data screening for outliers and heteroscedasticity was conducted prior to statistical analysis. No outliers were detected and all data were normally distributed. Comparison of data between the groups was analyzed using independent samples *t*-test. Using pooled data, Pearson's bivariate correlational analyses were used to determine significant linear independent associations of interest. Running separate analyses, mixed

model linear regression using stepwise methods ($P=0.15$ for entry, and $P=0.20$ to leave the model) were used to explore predictors of calf blood flow and bone health. This method of inclusion was selected to account for potential confounding effects of various variables (e.g., inflation of the standard error due to collinearity), and to rule out the masking of associations of independent variables with the dependent variable because of confounders. Data were analyzed using SPSS version 23.0 (SPSS Inc., Chicago, IL). A significance level of $p<0.05$ was used to detect group differences. All data are presented as the mean $\pm$ SEM.

Results

Subject characteristics: Group characteristics are shown in Table 1. E2Def and E2Rep did not differ ($p>0.05$) in age, height, weight, body composition, or cardiorespiratory fitness. In contrast, MAP, HR and TT3 were lower ($p<0.05$) in E2Def. Estrogen deficient amenorrheic women and estrogen deficient women with eumenorrheic anovulatory menstrual cycles did not differ ($p>0.05$) in age, body composition, cardiorespiratory fitness level, BMD measures, serum bone turnover markers, resting calf blood flow, triiodothyronine, or estrogen exposure levels (data not shown).

Menstrual Cycle Characteristics and Urinary Ovarian Steroids: Average cycle length did not differ ($p>0.05$) between eumenorrheic ovulatory women (29 ± 1 day) and eumenorrheic anovulatory women (28 ± 1 day). Age of menarche was higher ($p<0.05$) in E2Def (13.2 ± 0.4 years) versus E2Rep (12.1 ± 0.3 years). Average duration of amenorrhea for the amenorrheic women was 176 ± 45 days at study entry. Urinary E1G AUC and E1G mean, and PdG AUC and PdG mean were significantly lower ($p<0.05$) in E2Def (Table 1).

Bone Mineral Density and Calf Blood Flow: Total body BMD and L1-L4 BMD were lower ($p<0.05$) in E2Def (Table 2). Serum P1NP and osteocalcin did not differ ($p>0.05$) between groups. In contrast, CTX was higher ($p<0.05$) in E2Def.

Resting CBF was lower ($p<0.05$) in E2Def, but peak CBF did not differ ($p>0.05$) between the groups (Table 2). In keeping with this, resting calf vascular conductance was lower ($p<0.05$) and vascular resistance higher ($p<0.05$) in E2Def, but peak vascular conductance and resistance did not differ ($p>0.05$) between groups.

Independent Correlations of Bone and Cardiovascular Health: Using pooled data for bivariate correlations (see Figure 1), L1-L4 BMD was independently associated with CBF ($r=0.701$, $p<0.001$), CTX ($r= -0.502$, $p=0.012$), and E1G AUC ($r=0.512$, $p=0.008$). CBF was independently associated with L1-L4 BMD ($r=0.701$, $p<0.001$), and E1G AUC ($r=0.541$, $p=0.004$). Triiodothyronine and OxLDL did not correlate ($p>0.05$) with either BMD or CBF.

Bone Indices as a Predictor of Endothelial Function: Using pooled data for stepwise linear regression, we explored whether estrogen and varied indices of bone health were able to predict endothelial function, as determined by resting CBF. Variables in the regression model included: L1-L4 BMD, E1G AUC, and osteocalcin. Osteocalcin levels are positively associated with cardiovascular disease risk in older estrogen deficient women²⁵. Using this model, CBF was predicted by L1-L4 BMD, osteocalcin and E1G AUC (see Table 3), explaining ~63% of the variance (adjusted $R^2=0.633$, $F=14.827$, $p<0.001$).

Cardiovascular Indices as a Predictor of Bone health: Using pooled data for stepwise regression, we examined whether estrogen and varied indices of cardiovascular health could predict L1-L4 BMD. Variables in the regression model included: resting CBF, E1G AUC and OxLDL. OxLDL is reported to modulate the crosstalk between osteoblasts/osteoclasts, promoting increased osteoclast differentiation and bone resorption¹⁸. We report that the strongest predictor of L1-L4 BMD was CBF (Table 3), explaining ~48% of the variance (adjusted $R^2=0.478$, $F=22.937$, $p<0.001$).

Discussion

Consistent with previous studies in ^{10,21,26}, we demonstrate that compared with their estrogen replete counterpart, estrogen deficient women with exercise associated menstrual disturbances demonstrate impaired endothelial function and lower total body and lumbar spine BMD. For the first time, we report endothelial function in the lower limb skeletal muscle vascular bed is predicted by L1-L4 BMD and osteocalcin. A reciprocal link was also observed, with L1-L4 BMD identified as the strongest predictor of endothelial function. Estrogen exposure, but not OxLDL or TT3, was positively associated with both L1-L4 BMD and endothelial function.

These observations suggest that in recreationally trained premenopausal women with menstrual disturbances: i) impaired vascular and bone health are linked; ii) peripheral vascular function may be a sentinel for early changes in bone metabolism, and vice versa; and iii) estrogen deficiency, but not oxidative stress or energy deficiency, may be a common mechanism in this bidirectional association.

Associations between vascular and bone health

In the current study, we report lower total body and L1-L4 BMD and lower CBF, an index of endothelial function, in our estrogen deplete versus replete women. These findings are consistent with previous studies in amenorrheic versus eumenorrheic recreational and competitive athletes ^{10,21,23,26}. Our novel findings identify that these perturbations are bidirectionally associated such that L1-L4 BMD predicts CBF, and vice versa. It is not yet known whether such associations can predict future bone or cardiovascular health risk. Notwithstanding, low bone mineral content in the forearm at menopause is associated with an increased future risk of cardiovascular mortality ²⁷. Endothelial dysfunction is also a recognised key permissive factor in the development and progression of atherosclerosis ²⁸ and is reported to be evident decades prior to overt coronary artery disease ²⁹. However, the long-term clinical consequence of impaired endothelial function in young otherwise healthy estrogen deficient physically active women are not yet known. Nevertheless, independent of other risk factors, premenopausal estrogen deficiency due to

functional hypothalamic amenorrhea is reported to be a risk factor for angiographically documented coronary artery disease during the late premenopausal years³⁰. Prospective studies in estrogen deficient physically active women to determine whether impaired endothelial function elicits premature development of atherosclerosis, or whether lumbar bone loss during the premenopausal years can predict future cardiovascular disease risk, will be of clinical interest.

Our observations are in keeping with studies in older estrogen deficient postmenopausal women^{3,31,32}. For example, impaired brachial artery endothelial function using ultrasonography is associated with lower L2-L4 BMD using DXA, with age-adjusted analysis revealing osteoporotic women demonstrate significantly greater impairments to endothelial function than those with normal BMD³. Decreased vascular blood flow in the lower extremities detected with Doppler flowmetry has also been reported to be associated with low BMD at the hip and calcaneus as assessed by single photon absorptiometry in postmenopausal women³¹. In contrast to the previous study³, adjustment for age removed these associations. Equivocal findings for the effects of age on these associations likely reflect in part the shared risk of ageing on both diseases¹.

Smoking, low physical activity, and elevated BMI are also recognized common risk factors for decreased BMD and increased cardiovascular disease risk¹. In the current study, participants were young, non-smoking, of normal body composition and body weight, and demonstrated good cardiorespiratory fitness levels. Thus, in the absence of shared risk factors, it is plausible that in our young otherwise healthy premenopausal women, estrogen deficiency *per se* may be a shared mechanism in the pathophysiology of these conditions. In support of this notion, and consistent the known biological effects of estrogen on cardiovascular and bone health, we observed strong independent and positive correlations between urinary E1G and CBF and BMD. In the vasculature, estrogen is a key regulator of vascular function in women. Estrogen elicits rapid vasodilation through upregulation of the production of nitric oxide (NO) via the endothelium nitric oxide synthase (eNOS) pathway⁹. NO derived from this pathway increases blood flow, both within the arterial tree⁹ and in the bone circulation³³. Bone is highly vascularized, and circulation is vital to bone

development, maintenance and repair^{1,33}. Thus, it is plausible that blood flow may be impaired in both the peripheral and bone circulation in estrogen deficient women and that, if evident, peripheral blood flow could be a surrogate marker of impaired bone circulation and/or bone health. In keeping with this notion, ovariectomy in animals induces decreased vertebral bone health in association with increased vascular dysfunction in the periphery, and decreased bone vessel motility and blood flow³⁴.

Exercise Training and Bone and Vascular Health

In the vasculature, dynamic exercise training elicits changes in functional (e.g., increased sensitivity to vasoactive substances) and structural components (e.g., increased vessel diameter) as a consequence of repeated hemodynamic forces on the vessel wall³⁵. In bone, weight-bearing exercise exerts mechanical forces on the skeleton that can stimulate osteogenic and bone anabolic effects, having beneficial effects on bone density, size, and shape³⁶. These exercise-associated benefits are associated with decreased risk of cardiovascular disease and osteoporosis^{35,36}. In contrast, we report that despite participation in regular load-bearing exercise training, our estrogen deplete women demonstrate lower lumbar and total BMD and lower CBF than their estrogen replete counterpart. Given that both groups report similar levels of exercise training and demonstrate similar cardiorespiratory fitness, these findings suggest the bone and vascular benefit of exercise may be obviated in the face of estrogen deficiency. This notion is consistent with findings in exercise trained estrogen deficient postmenopausal women. For example, negligible osteogenic and bone anabolic effects of dynamic weight-bearing exercise training³⁷ and lack of an attenuated age-associated increase in endothelial function in habitually endurance trained versus untrained postmenopausal women has been reported³⁸. While estrogen deficiency *per se* is implicated in these findings, estrogen deficiency-mediated increases in oxidative stress is also postulated to play a role^{19,38}. Estrogen exerts potent anti-oxidant effects by scavenging free radicals and preventing lipid peroxidation²⁰. In contrast, we report similar oxidative stress levels, as determined by lipid

peroxidation, despite marked differences in estrogen exposure between our estrogen deplete versus replete women. We also did not observe independent associations between OxLDL and BMD or endothelial function. This finding suggests that unlike older estrogen deficient women, oxidative stress may not be augmented, and/or antioxidant status is well preserved in otherwise healthy young estrogen deficient women.

Limitations

In the current study, we report associations only, not mechanisms of action, nor do we demonstrate causality. As such, our findings should be interpreted appropriately. The primary cause of menstrual disturbances in physically active women has been identified as energy deficiency¹⁶. Although we assessed circulating levels of triiodothyronine, a recognized marker of energy status, we were not able to directly assess the effects of energy deficiency *per se* on bone and cardiovascular health. Endothelial dysfunction is recognized as a key permissive factor in the development and progression of atherosclerosis²⁸. Regional blood flow, as assessed using plethysmographic techniques, is a validated surrogate measure of endothelial function³⁹. However, the long-term cardiovascular consequences of decreased calf blood flow in amenorrheic physically active young women has yet to be determined. Examination of premenopausal women with similar menstrual disturbances due to different etiology (e.g., non-exercise associated menstrual disturbances), or participating in high impact versus low impact exercise training, may result in different associations between estrogen status, and cardiovascular and bone health. Bone quantity (i.e., BMD by DXA) is only one component of bone health. Bone quality parameters, such as microarchitecture and strength, might also be important to assess. We also note that while the E2Def group had lower total body and L1-L4 BMD compared to the E2Rep group (~5% and ~13% lower, respectively), Z-scores (~-61% and ~-143% lower, respectively) were still within the expected range for age. Notwithstanding, these early

changes may influence bone health trajectory such that they may be, or become, clinically relevant over time.

Conclusion

We report for the first time that in otherwise healthy young estrogen deficient physically active premenopausal women with menstrual disturbances, impaired endothelial function in the lower limb vasculature is associated with decrements in lumbar BMD, and vice versa. These alterations were commonly linked with low estrogen exposure, but not markers of oxidative stress or energy status. These findings suggest that in physically active women with menstrual disturbances: 1) estrogen deficiency may be a shared mechanism of pathophysiology in cardiovascular and bone health; 2) impairments in peripheral endothelial function may predict early unfavorable changes in bone metabolism, or vice versa; and 3) the known favorable bone and vascular adaptations to exercise training appear to be obviated. Whether early changes in bone or vascular health can predict future risk of cardiovascular disease or osteoporosis, respectively, remains to be determined.

Notwithstanding, current health guidelines recommend regular exercise for the prevention of osteoporosis and cardiovascular disease⁴⁰. Thus, studies designed to investigate the independent and shared mechanisms of altered bone metabolism and vascular function in young women with exercise-associated menstrual disturbances is of clinical importance.

Such studies may help further our understanding of the early origins of, and possible connections between, cardiovascular disease and osteoporosis in women that are independent of the effects of ageing and other shared risk factors for these diseases.

Perspective

Menstrual disturbances are commonly reported in recreational and competitive athletes¹². Both the Female Athlete Triad and the Relative Energy Deficiency Syndrome identify that functionally altered menstrual status in physically active women is primarily attributed to

chronic energy deficiency in association with high energy expenditure combined with insufficient caloric intake^{15,41}. The known clinical consequences of altered menstrual status include decreased BMD^{15,41}. Impaired endothelial function, a permissive factor in the development of atherosclerosis, has also been reported in amenorrheic athletes^{10,23}. For the first time, we report a bidirectional association between lumbar BMD and peripheral endothelial function in recreationally active women with amenorrhea and anovulatory menstrual cycles. These associations were shown to be commonly linked with decreased estrogen exposure. Although it is not yet known whether subclinical changes in bone health can be used as a surrogate for impaired vascular health and/or increased future risk of cardiovascular disease, these findings may be of relevance in the early detection and prevention of cardiovascular and bone health decrements in otherwise healthy young women. Given that anovulatory menstrual cycles are asymptomatic, and are common among physically active women, these associations may be of particular importance to this group of women.

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Table 1. Subject characteristics.

	E2Rep (n=14)	E2Def (n=13)	P (main effect)
<i>Demographics</i>			
Age (years)	24.7 ± 0.9	23.9 ± 1.3	0.622
Age of menarche (years)	12.2 ± 0.3	13.2 ± 0.4	0.037
Weight (kg)	57.5 ± 1.3	57.7 ± 1.5	0.902
Height (cm)	164.8 ± 1.3	166.6 ± 1.3	0.445
BMI (kg/m ²)	21.3 ± 0.4	20.9 ± 0.5	0.618
Body fat (%)	24.3 ± 1.3	21.7 ± 1.9	0.281
FFM (kg)	40.9 ± 1.0	43.2 ± 0.9	0.112
MAP (mmHg)	70.1 ± 1.5	65.9 ± 0.9	0.022
HR (b/min)	56.1 ± 2.2	49.5 ± 2.	0.041
VO ₂ peak (ml/kg/min)	46.3 ± 1.3	44.7 ± 1.3	0.429
<i>Urinary Reproductive Hormones</i>			
E1G AUC (ng/ml)	2047.9 ± 256.4	834.3 ± 149.2	0.001
E1G mean (ng/ml)	70.8 ± 10.1	33.5 ± 3.9	0.005
PdG AUC (µg/ml)	65.3 ± 14.7	17.8 ± 3.2	0.004
PdG mean AUC (µg/ml)	2.7 ± 0.7	0.6 ± 0.2	0.008
<i>Serum Biomarkers</i>			
TT3 (nmol/L)	1.59 ± 0.05	1.36 ± 0.09	0.022
Prolactin (mIU/L)	380.8 ± 78.7	270.2 ± 72.3	0.334
OxLDL (U/l)	47.2 ± 2.3	42.5 ± 3.2	0.243
CRP (mg/dL)	11.3 ± 3.0	7.4 ± 2.2	0.306

Values are mean ± SEM.

AUC, area under the curve; BMI, body mass index; CRP, c-reactive protein; E1G, urinary oestrone 3-glucuronide; FFM, fat free mass; HR, resting heart rate; MAP, resting mean arterial pressure; OxLDL, oxidized low-density lipoprotein; PdG, pregnanediol 3-glucuronide; TT3, total triiodothyronine; VO₂ peak, peak oxygen uptake.

Table 2. Bone and calf blood flow measures for the groups.

	E2Rep (n=14)	E2Def (n=13)	P (main effect)
<i>Bone Mineral Density</i>			
L1-L4 BMD (g/cm ²)	1.28 ± 0.05	1.11 ± 0.03	0.006
L1-L4 BMD Z-score	1.05 ± 0.39	-0.46 ± 0.24	0.003
Total body BMD (g/cm ²)	1.20 ± 0.02	1.14 ± 0.02	0.036
Total body BMD Z-score	1.24 ± 0.22	0.48 ± 0.21	0.020
<i>Serum Bone Markers</i>			
Osteocalcin (ng/mL)	7.9 ± 0.9	10.3 ± 1.1	0.104
U-CTX (µg/L)	176.9 ± 18.2	256.7 ± 24.1	0.013
P1NP (µg/L)	109.8 ± 9.5	123.9 ± 15.4	0.426
<i>Calf Blood Flow</i>			
Resting CBF (ml/100ml/min)	4.52 ± 0.38	3.05 ± 0.34	0.008
Peak CBF (ml/100ml/min)	48.97 ± 1.94	46.21 ± 3.67	0.513

Values are mean±SEM.

CTX, c-telopeptide; P1NP, procollagen type 1 N propeptide.

Table 3. Regression analysis (stepwise regression) of predictors of calf blood flow, and total body and lumbar bone mineral density.

	<i>B</i>	<i>SE B</i>	<i>β</i>	<i>F ratio</i>	<i>R</i> ²	<i>R</i> ² adj	<i>P</i>
<i>L1-L4 BMD</i>							
Intercept	0.876	0.171					
CBFrest	0.087	0.018	0.707	22.937	0.499	0.478	<0.001
<i>Total Body BMD</i>							
Intercept	1.066	0.039					
CBFrest	0.029	0.01	0.523	8.656	0.273	0.242	0.007
<i>CBF Resting</i>							
Intercept	-2.957	1.500					
L1-L4 BMD	5.022	1.199	0.624	20.444	0.482	0.458	<0.001
Osteocalcin	0.127	0.045	0.371	17.083	0.519	0.583	<0.001
E1G AUC	0.004	0.000	0.245	14.827	0.679	0.633	<0.001

CBF, calf blood flow; CBFrest, resting calf blood flow; E1G AUC, urinary oestrone 3-glucuronide area under the curve.

A

B

Figure 1. Scatterplots showing the relationship between calf blood flow and L1-L4 BMD (A), estrogen and L1-L4 BMD (B), and estrogen and calf blood flow (C) in estrogen deplete (closed circles) and estrogen replete (open circles) physically active premenopausal women.