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Contribution of Serum Inflammatory Markers to Changes in Bone Mineral Content and Density in Postmenopausal Women: A 1-Year Investigation

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Abstract

Bone formation and resorption are influenced by inflammatory processes. We examined the relationships among inflammatory markers and bone mineral content and density (BMC, BMD) and determined the contribution of inflammatory markers to 1-year changes in BMC and BMD in

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healthy postmenopausal women. This analysis included 242 women at baseline from our parent Soy Isoflavones for Reducing Bone Loss (SIRBL) project who were randomly assigned to one of three treatment groups: placebo, 80 mg/d soy isoflavones, or 120 mg/d soy isoflavones. BMD and BMC from the lumbar spine (LS), total proximal femur (hip), and whole body were measured by dual energy x-ray absorptiometry (DXA) and the 4% distal tibia (DT) by peripheral quantitative computed tomography (pQCT). Serum inflammatory markers (C-reactive protein (CRP), interleukin (IL)-1β, IL-6, tumor necrosis factor-alpha (TNF-α), and white blood cell count (WBC)) were measured at baseline, 6 and 12 months. Due to attrition or missing values, data analysis at 12 months includes only 235 women. Significant associations among Il-6, TNF-α, and WBC were observed with percent change in LS, hip, and whole body BMC and BMD. Multiple regression analysis indicated that in combination inflammatory markers accounted for 1.1% to 6.1% of the variance to the observed 12 month changes in BMC and BMD. Our results suggest that modifying inflammatory markers, even in healthy postmenopausal women, may possibly reduce bone loss.

Keywords

Cytokines; Bone mineral content and density (BMC, BMD); Inflammatory markers; Postmenopausal women

Introduction

Bone is a dynamic tissue and is continually being remodeled. In adult humans, it is estimated that about 10% of total bone mass is replaced per year (1). Bone turnover is regulated by a plethora of systemic factors, such as estrogen, serum calcium, vitamin D status, and physical activity, as well as genetics (2). The activity of the bone matrix is governed by bone forming osteoblasts and bone resorbing osteoclasts, which are both equally important in maintaining bone homeostasis. An imbalance in bone turnover due to excessive osteoclastogenesis can lead to bone loss and osteoporosis (3).

Osteoimmunology examines the interaction between bone and the immune system (4). Immune and bone cells share developmental pathways that arise from hematopoietic stem cells derived from the bone marrow. The differentiation of hematopoietic stem cells is regulated by bone and immune cell interactions. Immune cells may alter the balance of osteoclast and osteoblast cells by secreting immunoregulatory cytokines that affect the differentiation of bone precursor cells (5). The inflammatory disease rheumatoid arthritis is a classic example of this interaction. Macrophages and T-cells secrete inflammatory cytokines that activate osteoclasts, which leads to joint destruction and bone loss in rheumatoid arthritis patients (6).

During and after menopause, women lose a significant percentage of bone due to reduced estrogen production. Estrogen plays an important role in regulating the production and activity of inflammatory cytokines, such as interleukin (IL)-1β, IL-6, and tumor necrosis factor-alpha (TNF-α) (7). In an estrogen deficiency state or condition, these cytokines typically increase and activate osteoclastogenesis, thus leading to bone loss. Serum inflammatory cytokines have been shown to be higher in postmenopausal women compared to premenopausal women (8). Additionally, short term or acute inflammation as measured by c-reactive protein (CRP), compared to chronic inflammatory processes responsible for cytokine production, may also influence the degree or rate of bone loss during menopause.

Because of the lack of estrogen production and high turnover of bone in postmenopausal women, understanding the relationship between inflammation and bone is important in

clinical conditions such as osteoporosis. The focus of this analysis was to examine the relationship of serum inflammatory markers and white blood cell (WBC) count to bone mineral content (BMC) and density (BMD) in healthy postmenopausal women not receiving hormone therapy. The serum inflammatory markers examined were interlukin (IL)-1β, IL-6, TNF-α, C-reactive protein (CRP), and WBCs. We included WBCs to account for the likelihood of concomitant infection which may result in an acute inflammatory response.

Methods

Subjects

We enrolled healthy postmenopausal women (45.8 – 65.0 years of age) as part of a randomized, double-blind, placebo-controlled multi-center (Iowa State University [ISU], Ames, IA and University of California at Davis [UCD], Davis, CA) clinical trial. The parent study (**S**oy **I**soflavones for **R**educing **B**one **L**oss; SIRBL) was designed to examine the effect of two doses of isoflavones extracted from soybeans on bone loss during the course of three years in at-risk postmenopausal (e.g., less than 10 years since their last menses) women. Details of the parent project have been previously reported (9). Serum samples were obtained from women who were enrolled in the parent project. Subjects were recruited throughout the Sacramento region in California and the state of Iowa through direct mailing lists, articles in local newspapers, local/regional radio advertisements, local television stations, community announcements, and other recruitment avenues. Details of the screening and selection process as well as enrollment and randomization have been reported (9). Briefly, telephone respondents numbered 5,255; women who met the initial selection criteria via telephone interview (n=677) were invited to the clinic for further evaluation from which another 422 were excluded for not meeting eligibility criteria. Ultimately 255 were randomly assigned to placebo, low (80 mg/d) or high 0120 mg/d) dose of soy isoflavone tablets in the parent project. Women were deemed healthy based on clinical chemistry profile, renal, liver, and thyroid function as well a lipid panel. Each woman was also required to have a physical exam by her primary care physician, gynecological exam, mammogram, and a signed medical release.

We measured height and weight to confirm body mass index (BMI) (kg/m^2) status. We used dual energy x-ray absorptiometry (DXA) to determine areal BMD of the LS and left total proximal femur (hip). Women with evidence of osteopenia or osteoporosis based on T-score of the LS and/or proximal femur BMD (using >1.5 SD below the young adult mean as cut-off) and women with evidence of previous or existing spinal fractures were excluded. We also excluded women with spine and/or femur BMD >1.0 SD above the mean. The focus of the parent project was disease prevention rather than disease treatment, therefore women with T-scores for lumbar spine or proximal femur BMD that were 1.5 SD below the young adult mean or > 1.0 SD above the mean were excluded from the study. For this ancillary project, we examined serum CRP, cytokines, and WBCs from 242 women enrolled in the parent SIRBL study at baseline. Due to attrition and/or missing serum samples, data from 235 women were available for analysis at 6 and 12 months.

The study protocol, consent form, and subject-related materials for the parent project were approved by the respective Institutional Review Boards (IRB) at UCD (ID# 200210884-2) and ISU (ID# 02-199). Approvals for the DXA and peripheral quantitative computed tomography (pQCT) procedures were obtained from each institution's IRB and appropriate safety boards. Written informed consent was obtained from all women at the start of pre-baseline screening.

Questionnaires—At the pre-baseline visit to ensure the health status of participants, trained interviewers administered health and medical history and reproductive history

questionnaires. These data were used to calculate age and time since menopause (time since last menses - TSLM).

Anthropometric Measurements—Body weight (to the nearest 0.1 kg) was measured with women wearing minimal clothing, using a balance beam scale (Abco Health-o-meter; Health-o-meter Inc.; Bridgeview, IL) at ISU and an electronic scale (Circuits & Systems, Inc.; E. Rockaway, NY) at UCD. Height was measured using a wall-mounted stadiometer (Ayrton stadiometer, Model S100; Ayrton Corp., Prior Lake, MN) and recorded to the nearest 0.1 cm. Women, without shoes, were instructed to stand erect, place hands on their hips, and inhale maximally. Standing height was measured at maximal inspiration; BMI was calculated for each woman.

Bone Measurements—Total proximal femur (hip) [subregions: femoral neck (FN), trochanter (Troc)], and LS (L1–L4 in the anteroposterior projection) BMC (g) and BMD (g/cm^2) were assessed via a Delphi W DXA (Hologic, Inc; Bedford, MA) instrument. The coefficient of variation (CV) for DXA measurements at both ISU and UCD sites has been previously reported (9); the within-subject *in vivo* CV for areal BMD were 1.1% and 0.9% at the spine and 0.7% and 0.8% for the hip and 0.8% and 1.0% for whole body at ISU and UCD, respectively. In addition to DXA measurements, pQCT measurements were made at the 4% distal tibia; specifics of this procedure as well as measurement details (including CVs) at the respective sites have been previously reported (10). Due to improper alignment of the anatomic reference line at the distal tibia site, 51 UCD women and 1 ISU woman were excluded, resulting in 66 UCD and 121 ISU subjects (N=187) available for tibia analysis. Compared to LS or hip sites measured by DXA, pQCT measurements at the distal tibia represent a site that is predominantly trabecular bone. Matching instruments at each geographic site and daily calibration ensured that pQCT and DXA instruments provided comparable results. One operator at each geographic site performed pQCT and DXA scans. Cross-training for pQCT and DXA scanning between sites has ensured comparable quality control. Laboratory personnel at each site were trained by the manufacturers' technicians and received further training on pQCT software analysis (Bone Diagnostic, Inc.; Fort Atkinson, WI). A research assistant at UCD performed all pQCT scan analyses following guidelines provided by Bone Diagnostic, Inc. The ISU DXA operator performed all DXA scan analyses following Hologic guidelines for BMD using software version 12.3:7. DXA operators at both sites had more than 10 years experience assessing BMD in human clinical trials. Additionally, the DXA operator at the UCD site, by state law, was a licensed DXA technician. At the Iowa site the operator was initially trained by Hologic with 2 additionally continuing education unit training session for DXA operators during the past 10 years.

Blood Collection and Analyses—Blood was collected following an overnight fast. Whole blood was sent to the respective certified clinical laboratories for complete blood count with differentials. White blood cell count was obtained and values used as a marker of acute infection and thus a potential surrogate marker of inflammation as the result of infection. We isolated serum from whole blood by centrifuging for 15 min (4°C) at 1000 × g and stored aliquots at −80°C until analyzed for inflammatory markers.

Inflammatory marker measurements—Serum CRP concentration was determined in duplicate with a high-sensitivity sandwich enzyme-linked immunosorbent assay kit (ALPCO Diagnostics; Salem, NH) using a microtiter plate reader (ELx808; Bio-Tek Instruments, Inc.; Winooski, VT). The sensitivity of the CRP was 12.9 ng/mL; the intra-assay CV was 3.7% and inter-assay CV was 6.0%. The concentrations of IL-1β, IL-6, and TNF-α were determined in serum with a high-sensitivity human cytokine multiplex assay (LINCOplex kit; LINCO Research; St. Charles, MO) using a Luminex 100 (Luminex Corporation;

Austin, TX). Using Luminex technology, the lowest detectable value for each cytokine was: IL-1β – 0.01 pg/mL; IL-6 – 0.03 pg/mL; TNF-α – 0.48 pg/mL. The intra-assay CVs for IL-1β, IL-6, and TNF-α, respectively, were 14.5%, 6.0%, and 5.8%. The inter-assay CVs for IL-1β, IL-6, and TNF-α, respectively, were 5.4%, 3.6%, and 6.0%.

Statistical Analysis

For the parent project, women participated in serial testing during three years of treatment. However, this ancillary project examined women at baseline, 6 and 12 months and the changes in inflammatory markers and BMC and BMD during a 1 year period. Descriptive statistics included mean and standard deviation for age, time since last menses (TSLM), body size, body composition, and bone to characterize the research subjects. The inflammatory marker data (CRP, IL-1β, IL-6, TNF-α) were examined for normality and log-transformed prior to the regression analysis because they did not follow a normal distribution, causing a violation of assumptions. We have reported median (range) values for inflammatory markers as well as WBCs because they were not normally distributed. Pearson correlation analysis was used to assess the relationship among physical characteristics, inflammatory markers, and percent change (1 year) in BMC and BMD. Percent change was defined as baseline value minus 6 month and 12 month values. Multiple regression analysis with stepwise selection was used to evaluate the contribution of inflammatory markers to the change in BMC and BMD. The following variables were used in the model: TSLM, age, BMI, CRP, IL-1 β, IL-6, TNF-α, and WBCs. Data at 0, 6, and 12 months were available for 235 of the 242 women in the parent SIRBL study. However, bone data from the pQCT for the distal tibia were available for 187 women. All models included site as an obligatory variable to account for potential differences across study sites. Statistical analyses were conducted using the SAS software, version 9.1. Results were considered statistically significant at the $p \leq 0.05$ level.

Results

Descriptive characteristics of the women as well as inflammatory marker values and bone parameters at baseline are presented in Table 1. Median values for inflammatory markers were within the range reported in the literature. However, 65 women had low WBC (<4.5 but >2.3 × 109/L) and none of the women had elevated WBC. Fifty women (20.6%) had non-detectable values for IL-1β and four women (1.7%) had non-detectable values for IL-6. Hence, we replaced these nondetectable values with 0.01 pg/mL (lowest detectable value was 0.01 for IL-1β and 0.03 for IL-6) to retain all data in the regression models subsequent to log transformation for regression analysis. Pearson correlation coefficients were used to assess the relationship of inflammatory markers with age, height, weight, TSLM, and 1 year percent change in BMC and BMD for femoral neck, hip, LS, distal tibia, and whole body; correlations are presented in Table 2. Only variables that had a significant correlation with bone parameters are shown. IL-6 and TNF-α exhibited significant associations with percent change in BMC and BMD across a majority of bone sites. WBC, a marker indicative of infection and which may be a surrogate marker of inflammation, was significantly associated with percent change in BMC at 2 sites: hip and femoral neck. No significant associations were found among any inflammatory marker and pQCT measures of BMC or BMD.

In the parent project lumbar spine, hip or whole body showed no significant effect of soy isoflavones on BMD except for a modest effect on percent change in femoral next BMD with 120 mg/d soy isoflavones. This positive effect, however, was observed only after controlling for age, whole body fat mass and serum C-Tx, a marker of bone resorption. So, to better understand the possible contribution of inflammatory markers to changes in bone, we developed models using stepwise multiple regression analysis to evaluate their combined

contribution to the percent change in BMC or BMD at the hip, LS, femoral neck, trochanter, whole body, and distal tibia (Table 3). Variables used in the regression models included: age, BMI, TSLM, CRP, IL-1β, IL-6, TNF-α, and WBC. Site was included in all models as an obligatory variable to account for potential differences across sites. With the exception of the femoral neck, a combination of inflammatory markers contributed to the 12 month percent change in BMC or BMD at the hip, LS, trochanter, and whole body. CRP, an acute phase protein, was a significant contributor to the percent change in BMC or BMD at the LS, whole body, and trochanter. TNF-α and IL-1β were significant contributors to the percent change at the hip and WBC was a significant contributor at the trochanter site. Overall, the combined contribution of the inflammatory markers accounted for 1.1 to 6.1% of the variance (R^2) in the regression models for the percent change in BMC or BMD at these specific sites. As expected, based on Pearson correlations, no combination of inflammatory markers was found to contribute significantly to the 1-year changes in BMC or BMD measured by pQCT.

In summary, using multiple regression modeling, we found that markers of inflammation made small, but important contributions, ranging from 1.1 to 6.1% of the variance, to the 1-year percent change in BMC or BMD across a variety of bone sites assessed by DXA.

Discussion

Inflammatory markers are key players in bone biology and are involved in the regulation of osteocytes; as a result, the dynamic balance of bone formation and resorption are influenced by inflammatory markers. Of the serum inflammatory markers we examined, we found IL-6 and TNF-α to be the cytokines most often associated with percent change in BMC and BMD across a variety of bone sites. Additionally, the use of stepwise multiple regression analysis allowed us to evaluate the proportion of the change in BMC or BMD that could be attributed to these inflammatory markers. After accounting for other factors such as TSLM in the regression models, CRP was the inflammatory marker that most often (4 of 6 bone sites) contributed to percent change in BMC or BMD.

C-reactive protein is generally viewed as an acute phase marker of systemic inflammation that may have a relationship with bone. A large human study (N=7,000) by Tomiyama et al. (12) showed that elevated plasma CRP was associated with early stages of osteopenia. Other studies have shown that serum CRP was associated with higher bone turnover rates and was significantly higher in women with osteoporosis and osteopenia (13,14). In our study with 235 healthy women, CRP was also a significant contributor to bone changes at the LS, trochanter, and whole body. This is somewhat surprising because our women were recruited as healthy postmenopausal women, free of any chronic illness thought to affect bone metabolism. As we have noted previously (11), these women were deemed healthy (not known to be in an inflammatory state), as evidenced by their average cytokine and acute phase protein values (Table 1), but some women exhibited higher concentrations of these markers. We have reported (11) that at baseline, 39% of women had CRP above 1.5 mg/L cutoff values. Less than 1% had TNF-α above 15.0 pg/mL, whereas 41% had IL-6 above 12.5 pg/mL and 10% had IL-1β above the 5.0 pg/mL cutoff values. Our findings are corroborated by the literature indicating that menopause is associated with increased pro-inflammatory markers (15), particularly IL-6 (16). However, the ranges reported in the literature, except for CRP listed in Table 1, are for adults in general and not specifically for postmenopausal women, perhaps explaining why some of our participants did not fall within these ranges.

An interesting observation from our data was that the contribution of inflammatory markers to the variability in BMC and BMD were observed only for measurements obtained from

DXA; not pQCT. This is somewhat surprising; however, our smaller sample size for the pQCT measurements (N=187) compared to the DXA measurements (N=235) may have impacted our power to detect significant bone effects using pQCT.

In conclusion, it is important to emphasize that since we enrolled healthy women who were not osteoporotic, with BMD T-scores between −1.5 to 1.0, which may explain why we were unable to detect consistent relationships among systemic cytokines, as markers of chronic inflammation, and BMD. However, in healthy postmenopausal women, acute phase responses s, as assessed by CRP and WBC, did play a small but significant role in the decline in DXA-measured BMC and BMD across a variety of bone sites. Furthermore, our systemic cytokine values may not be an accurate reflection of cytokines effect at the molecular level. Additionally, our results suggest that modifying inflammatory markers, even in healthy postmenopausal women, may possibly decrease osteoporotic risk by reducing the rate of bone loss. Further research is needed to examine the interrelationship among cytokines and other markers of inflammation and bone measurements to determine whether treatment to reduce inflammation would be an appropriate approach for reducing the rate of bone loss in postmenopausal women.

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Table 1

Descriptive Statistics of Subjects at Baseline

	Mean ± SD	Median (min – max)[b]
Subject Characteristics		
Age (y)	54.4 ± 3.3	
Time since last menses (y)	3.4 ± 2.0	
Weight (kg)	67.5 ± 9.3	
Height (cm)	164.6 ± 6.3	
BMI (kg/m^2)	24.9 ± 3.1	
Bone Measurements		
Lumbar Spine BMC (g)	58.49 ± 7.66	
BMD (g/cm^2)	0.991 ± .080	
Hip BMC (g)	31.44 ± 3.64	
BMD (g/cm^2)	0.909 ± 0.074	
Femoral Neck BMC (g)	3.77 ± 0.41	
BMD (g/cm^2)	0.745 ± .068	
Whole Body BMC (g)	2143 ± 232	
BMD (g/cm^2)	1.137 ± .076	
Tibia Trabecular BMC (mg)[a]	172.5 ± 35.1	
BMD (mg/cm^3)[a]	231.9 ± 31.9	
Inflammatory Markers[b]		
CRP (mg/dL)	1920 ± 2794	1.0 (0.01 – 29.9)
IL-1β (pg/mL)	2.0 ± 6.7	0.6 (0 – 106.8)
IL-6 (pg/mL)	17.3 ± 25.9	8.3 (0 – 255.4)
TNF-α(pg/mL)	4.7 ± 7.7	3.9 (0.5 – 121.0)
WBC (×10^9/L)	5.1 ± 1.1	5.0 (2.3 – 8.4)

[a] number of subjects for pQCT was 187

[b] median reference ranges as reported in Courtney et al. Menopause 15: 619–627, 2008 (ref #11).

CRP = C-reactive protein

IL-1β = interleukin 1β

Il-6 = interleukin 1

TNF-α = tumor necrosis factor α

WBC = white blood cell count

BMC = bone mineral content

BMD = bone mineral density

BMI = body mass index

Table 2

Associations Among 1-Year Changes in Bone Mineral Content and Density and Inflammatory Markers

% Change in BMC and BMD	IL-6	TNF-α	WBC
Lumbar Spine BMC	0.136 (0.03)	0.124 (0.05)	NS
Lumbar Spine BMD	0.136 (0.03)	0.127 (0.04)	NS
Hip BMC	0.131 (0.04)	0.126 (0.04)	0.152 (0.02)
Hip BMD	0.131 (0.04)	0.126 (0.05)	NS
Femoral Neck BMC	0.129 (0.04)	NS	0.154 (0.02)
Femoral Neck BMD	0.128 (0.04)	NS	NS
Trochanter BMD	0.133 (0.03)	0.129 (0.04)	NS
Whole Body BMC	0.126 (0.04)	0.125 (0.05)	NS
Whole Body BMD	0.132 (0.04)	0.128 (0.04)	NS
Tibia BMC	NS	NS	NS
Tibia BMD	NS	NS	NS

NS = non-significant

Values are correlation coefficients and (p values)

Il-6 = interleukin-6

TNF-α = tumor necrosis factor-α

WBC = white blood cell count

Table 3

Stepwise Regression Models for Contributions of Inflammatory Markers to 1-Year Change in BMC and BMD at Specific Sites

% Change	Hip BMC	Hip BMD	Lumbar BMD	Whole Body BMC	Whole Body BMD	Trochanter BMC
Parameter Estimate	**N = 235**	**N = 235**	**N = 235**	**N = 235**	**N = 235**	**N = 235**
Constant	−0.716	−0.120	0.262	0.717	0.646	2.991
Site	1.222 P = 0.000	0.417 P = 0.008	−0.133 P = 0.53	0.195 P = 0.35	−0.649 P = 0.001	0.444 P = 0.49
IL-6	0.007 P = 0.18	0.006 P = 0.068	−0.001 P = 0.75	0.004 P = 0.37	0.001 P = 0.88	0.004 P = 0.76
TNF-α	0.081 P = 0.05	0.054 P = 0.048	−0.028 P = 0.44	−0.024 P = 0.50	−0.023 P = 0.47	0.17 P = 0.13
CRP	0.0000006 P = 0.88	−0.0000002 P = 0.93	−0.000079 2P = 0.037	−0.0000763 P = 0.041	−0.0000951 P = 0.005	0.0 P = 0.03
WBC	0.008 P = 0.95	−0.02 P = 0.79	0.086 P = 0.39	0.034 P = 0.74	0.073 P = 0.42	−0.663 P = 0.037
IL-1β	−0.105 P = 0.027	−0.075 P = 0.016	0.037 P = 0.38	0.036 P = 0.38	0.021 P = 0.56	−0.227 P = 0.08
R^2	0.061	0.017	0.011	0.02	0.043	0.021

Values are regression coefficients with probability levels.