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*a randomized controlled trial*

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# Combined bioavailable isoflavones and probiotics improve bone status and estrogen metabolism in postmenopausal osteopenic women: a randomized controlled trial

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## ABSTRACT

**Background:** Female age-related estrogen deficiency increases the risk of osteoporosis, which can be effectively treated with the use of hormone replacement therapy. However, hormone replacement therapy is demonstrated to increase cancer risk. Bioavailable isoflavones with selective estrogen receptor affinity show potential to prevent and treat osteoporosis while minimizing or eliminating carcinogenic side effects.

**Objective:** In this study, we sought to determine the beneficial effects of a bioavailable isoflavone and probiotic treatment against postmenopausal osteopenia.

**Design:** We used a novel red clover extract (RCE) rich in isoflavone aglycones and probiotics to concomitantly promote uptake and a favorable intestinal bacterial profile to enhance isoflavone bioavailability. This was a 12-mo, double-blind, parallel design, placebo-controlled, randomized controlled trial of 78 postmenopausal osteopenic women supplemented with calcium (1200 mg/d), magnesium (550 mg/d), and calcitriol (25  $\mu$ g/d) given either RCE (60 mg isoflavone aglycones/d and probiotics) or a masked placebo [control (CON)].

**Results:** RCE significantly attenuated bone mineral density (BMD) loss at the L2–L4 lumbar spine vertebra ( $P < 0.05$ ), femoral neck ( $P < 0.01$ ), and trochanter ( $P < 0.01$ ) compared with CON (–0.99% and –2.2%; –1.04% and –3.05%; and –0.67% and –2.79, respectively). Plasma concentrations of collagen type 1 cross-linked C-telopeptide was significantly decreased in the RCE group ( $P < 0.05$ ) compared with CON (–9.40% and –6.76%, respectively). RCE significantly elevated the plasma isoflavone concentration ( $P < 0.05$ ), the urinary 2-hydroxyestrone (2-OH) to 16 $\alpha$ -hydroxyestrone (16 $\alpha$ -OH) ratio ( $P < 0.05$ ), and equol-producer status ( $P < 0.05$ ) compared with CON. RCE had no significant effect on other bone turnover biomarkers. Self-reported diet and physical activity were consistent and differences were nonsignificant between groups throughout the study. RCE was well tolerated with no adverse events.

**Conclusions:** Twice daily RCE intake over 1 y potentially attenuated BMD loss caused by estrogen deficiency, improved bone turnover, promoted a favorable estrogen metabolite profile (2-OH:16 $\alpha$ -OH), and stimulated equol production in postmenopausal women with osteopenia. RCE intake combined with supplementation (calcium, magnesium, and calcitriol) was more effective than supplementation

alone. This trial was registered at clinicaltrials.gov as NCT02174666. *Am J Clin Nutr* 2017;106:909–20.

**Keywords:** isoflavone, bone turnover, bioavailability, probiotics, osteopenia, postmenopause, equol, bone density

## INTRODUCTION

The natural decline in endogenous estrogen during menopause reduces bone mineral density (BMD) and incurs negative changes to bone microarchitecture, and this increases the risk of osteoporosis and fracture in women (1, 2). Menopausal women lose on average 2–5% BMD/y, and after the final menstrual period they lose 1–3% BMD/y (3, 4). Calcium and vitamin D (CaD) are the first-choice prophylactic treatment of clinicians in the management of postmenopausal osteopenia. The National Osteoporosis Foundation (NOF) recommends intakes of 1200 mg Ca/d and 20–25  $\mu$ g vitamin D/d in these patients. Meta-analyses have shown that CaD alone can reduce total- and hip-fracture risk by 15% and 30%, respectively, in older patients ( $\geq 65$  y of age)

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Supplemental Tables 1 and 2 are available from the “Online Supporting Material” link in the online posting of the article and from the same link in the online table of contents at <http://ajcn.nutrition.org>.

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Abbreviations used: BMC, bone mineral content; BMD, bone mineral density; BP, blood pressure; CaD, calcium and cholecalciferol; CDM, calcium, vitamin D, and magnesium; CON, control; CTx, collagen type 1 cross-linked C-telopeptide; DXA, dual-energy X-ray absorptiometry; ER, estrogen receptor; ET, estrogen therapy; FN, femoral neck; L2–L4, lumbar spine vertebra L2–L4; NOF, National Osteoporosis Foundation; PAL, physical activity level; RANKL, receptor activator of nuclear factor  $\kappa$ -B ligand; RC, red clover; RCE, red clover extract; RCT, randomized controlled trial; TC, total cholesterol; UHPLC-MS/MS, ultra HPLC-tandem mass spectrometry; 2-OH, 2-hydroxyestrone; 16 $\alpha$ -OH, 16 $\alpha$ -hydroxyestrone.

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(5, 6). Randomized controlled trials (RCTs), observational studies, and meta-analyses suggest that a ratio of calcium to magnesium of 2.8:1 is most optimal for bone health (7). Large RCTs support the hypothesis that estrogen therapy (ET) is also effective in the prevention and treatment of estrogen-deficient osteoporosis in postmenopausal women (8, 9). ET use remains controversial because it has been associated with cancer risk in estrogen receptor (ER)- $\alpha$  rich tissues (i.e., breast, ovaries, and endometria) (10). Meta-analyses indicate that the RR of ovarian cancer with ET use ranges from 1.19 to 1.46 and that ET use for <5 y has a RR of 1.43 (11–14). ET cessation leads to rapid loss of the osteoprotective effects against fracture (15, 16). Considering the treatment duration for maintenance of bone health during postmenopause and the associated risks of prolonged ET use, it is of clinical value to develop effective treatments with minimal side effects that are suitable for longer-term use. Isoflavones naturally occur in legumes, such as soy and red clover (RC) (17). Whereas estrogen acts as an agonist with binding affinity for ER $\alpha$  (highly expressed in cancer-sensitive tissues) and ER $\beta$ , isoflavones retain selective affinity for ER $\beta$ , which presents in tissues requiring certain stimulation by estrogen to function normally (e.g., bone tissue, bone marrow, adipose, brain, kidney, endothelial cells, and liver) (18, 19). Thus, isoflavones can adopt the regulatory roles of estrogen without incurring equivalent side effects in sensitive tissues (20). Isoflavones have a favorable safety profile in humans and are staple components in Indo-Asian diets (21). Analysis of dietary isoflavone intake and breast cancer risk in a multiethnic cohort of 84,450 women showed no significant association, and a 13-y follow-up indicated a protective effect in the highest intake quartile (20.3–178.7 mg isoflavone/d) (22). Isoflavones may also regulate hepatic cytochrome P450 enzymes and promote favorable estrogen metabolism by upregulating the ratio of 2-hydroxyestrone (2-OH) to 16 $\alpha$ -hydroxyestrone (16 $\alpha$ -OH) metabolites (23, 24). 16 $\alpha$ -OH is potentially more genotoxic and estrogenic than its 2-OH counterpart, which has minimal estrogenic potential and may retain cancer protective effects (25). Various isoflavones have distinct bioactivity and RC offers a unique profile of isoflavones (formononetin and biochanin A) (26). Enzymatic methods and probiotics can be used to increase isoflavone uptake, and fermented isoflavone preparations (aglycone rich) have demonstrated higher bioavailability than unfermented (glycoside rich) (27–29). In vivo trials in female rats with induced bone loss (ovariectomized) support the theory that formononetin in particular may have anti-resorptive and anabolic properties (30–36). Human RCTs have also shown promising effects of isoflavones for reducing bone resorption markers (37–39). We have previously executed a 3-mo RCT with healthy menopausal women receiving an aglycone-rich RC isoflavone (37 mg/d) and combined probiotic RC extract (RCE). The results indicated that RCE attenuated BMD loss at the lumbar spine vertebra L2–L4 (L2–L4) region (40). The present clinical trial aims to determine whether long-term RCE is more effective than traditional CaD treatment of postmenopausal osteopenia. The primary endpoint was the effect of RCE against BMD loss. Secondarily, we determined the effects of RCE on bone turnover markers, estrogen metabolites, plasma isoflavone concentration, equol-producer status, plasma lipid concentrations, and blood pressure (BP).

## METHODS

### Participants and study design

This study was a 12-mo, parallel-design, placebo-controlled, double-blind RCT. A total of 121 postmenopausal women with established osteopenia were screened and recruited from a community in northern Denmark (Hospital Vendsyssel, Hjoerring, Denmark). The clinical aspects of the study were carried out from November 2013 to February 2015, including screening, recruitment, and the trial period. Participants were recruited through advertisements (local newspaper, flyers, posters, and local radio) or referred to the project by general practitioners in the local area. Potential candidates were required to contact the project team over the phone for prescreening, at which point contact information and pre-eligibility information were collected. To be considered, participants had to be female,  $\geq 1$  y postmenopause, and have an established T score of  $-1$  to  $-2.5$  and an age of 60–85 y. Approved participants were sent documents containing participant information about the study, participants' rights in a biomedical research project (prepared by the Danish Scientific Research Ethics Committee), and a written consent form; they were then given  $\geq 2$  wk to read the information. Thereafter, participants were invited to an oral assessment and screening day (week  $-2$ ); participants were required to provide a previous dual-energy X-ray absorptiometry (DXA) scan indicating osteopenia and their habitual medicine. Candidates were informed that participation was voluntary and were given a comprehensive briefing of the study. After the oral briefing, eligibility assessment, and obtaining of participant consent, basal parameters were collected, including anthropometric measurements (waist circumference, BP, and BMI), and a DXA scan. All participants consented to keep habitual diet and exercise constant for the duration of the study.

Eighty-five participants were found to be eligible and were included in the study, and these participants were randomized into either the control (CON) extract group or the RCE treatment group by a computer-generated code (simple randomization). At the hospital during the baseline visit (at week 0), participants collected their supplements and extract, undertook 24-h ambulatory BP, submitted fasted blood and urine samples for analysis, completed physical activity level (PAL) questionnaires, and were given 3-d diet diaries with instructions and a return envelope addressed to the hospital. Inclusion criteria were as follows: postmenopausal women aged 60–85 y, osteopenia (T score:  $-1$  to  $-2.5$ ), and a BMI (in  $\text{kg/m}^2$ ) of 20–40. Exclusion criteria were as follows: medicinal treatment of osteoporosis within the last 3 mo (bisphosphonates, parathyroid hormone, strontium ranelate, or denosumab), treatment with estrogen or hormone therapy in the last 3 mo, treatment with estrogen agonist or antagonist products (raloxifene or tamoxifen), T score  $\leq -3$  or  $\geq -1$ , a diet rich in or supplementation with isoflavones, vitamin K supplementation, participation in other clinical trials within the past 3 mo, severe cardiovascular, psychiatric, neurological, or kidney disease, a history of cancer, a history of alcohol or substance abuse, high BP ( $>160/110$  mm Hg), metabolic disorders, and pregnancy or breastfeeding. A total of 78 participants successfully completed the study (see **Figure 1** for consort flow diagram). The trial was executed in accordance with guidelines laid down by the Declaration of Helsinki and

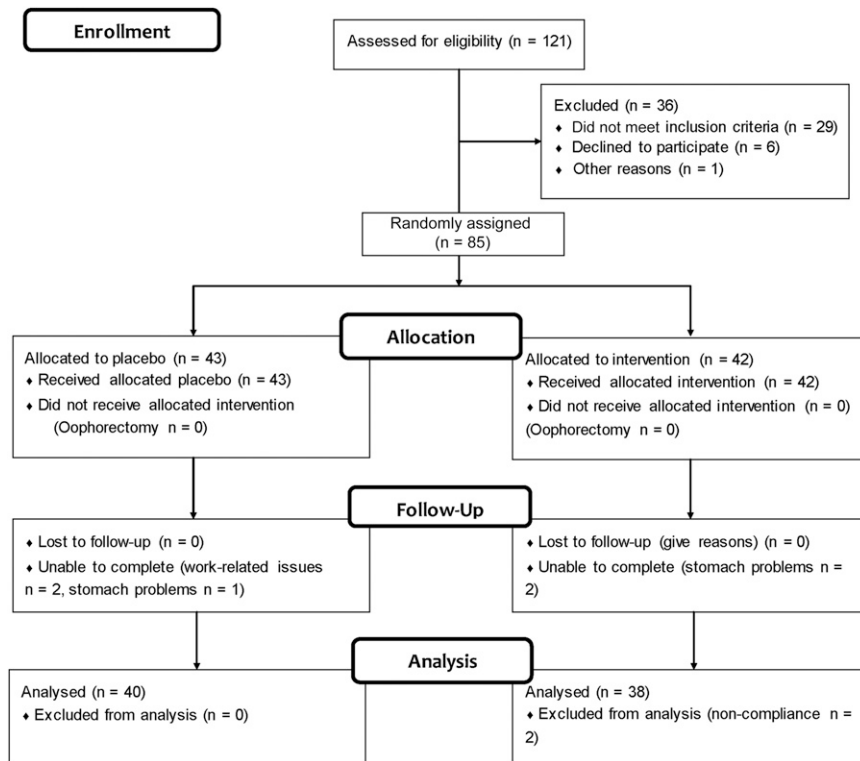


FIGURE 1 Consort participant flowchart of participation through the present study.

was approved by the Danish Research Ethics Committee (no. 1–16-02-466-13) and the Danish Data Protection Agency. In line with the International Committee of Medical Journal Editors, the study protocol was registered at clinicaltrials.gov (NCT02174666). This intervention study was a double-blind, head-to-head RCT with participants treated either with twice daily RCE ( $2 \times 95 \text{ mL} = 60 \text{ mg}$  isoflavones/d total) plus vitamin and mineral tablets containing 1040 mg Ca, 487 mg Mg, and  $25 \mu\text{g}$  vitamin D (CDM)/d or masked placebo extract plus CDM/d for 12 mo (Figure 2).

### RCE and CON extracts

The RCE was produced by Herrens Mark ApS. RC plants for the production of RCE were grown by Herrens Mark ApS. A

heterogeneous culture (proprietary) of probiotic lactic acid bacteria was added to pressed RCE to facilitate cold fermentation and improve bioavailability. Standardization of postfermentation aglycone content was determined by HPLC coupled with diode-array detection and mass spectrometry (DB Laboratory A/S). To mask taste and appearance of the RCE, stevia and a natural sugar-free raspberry or orange flavoring was added. For each batch, 90 L of either water (placebo) or RCE were sweetened with 18 g stevia and 6.3 L of sugar-free raspberry or orange flavoring. The placebo extract comprises 90 L of water mixed with 250 g brown food coloring (ammoniated caramel) (Kavli) to achieve likeness in appearance to the RCE. Portions of extract (95 mL each) were sealed in identical aluminum sachets, and these were consumed twice daily with a meal. The RC and placebo sachets were packed

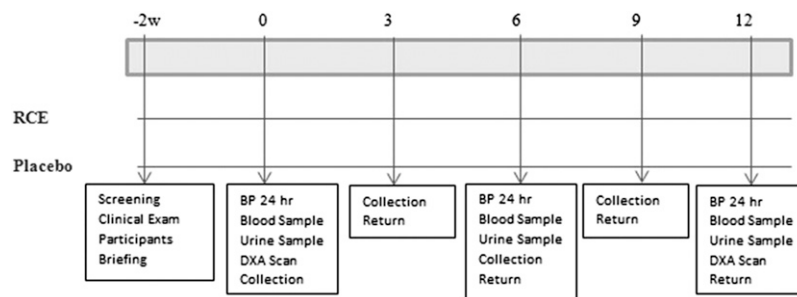


FIGURE 2 A schematic representation of the study design. The far left shows 2 groups: those receiving RCE and those receiving the control (placebo). Screening and briefing are scheduled at 2 wk prior (–2) to project and include assessment of basal parameters. Postscreening and postrandomization administration of either placebo or RCE took place at 0 (baseline). DXA scans took place at 0 and 12 mo. At months 0, 6, and 12, fasted blood and urine samples were taken and analyzed, along with BP. Extract and supplement collection took place at 0, 3, 6, and 9 mo; return of empty pill containers and extract sachets took place at 3, 6, 9, and 12 mo for compliance. BP, blood pressure; DXA, dual-energy X-ray absorptiometry; RCE, red clover extract; w, weeks.

in identical, sealed, white cardboard boxes and coded with 1 or 2 denoting groups. All participants returned empty containers, which were collected by the research team and recorded to monitor compliance (set to 95%). All participants and the research team were blinded and had no knowledge of the content of the boxes throughout the course of the study, the actual contents of the boxes with 1 and 2 codes were revealed at completion of the study by an independent third party from Herrens Mark ApS. Boxes were handed out at baseline and at 3, 6, and 9 mo.

### **Isoflavone quantitation by liquid chromatography-mass spectrometry and CaD supplements**

Isoflavone composition and quantitation analysis of the RCE was performed by DB Laboratory A/S. Quantitation was assessed by HPLC coupled with diode-array detection and mass spectrometry on a Summit LC/MS system consisting of a quaternary pump (P680 LPG), autosampler (ASI 100T), column oven (TCC-100) UV detector (PDA-100), and MS detector (Surveyor MSD Plus), all from Dionex. Four standards of the primary isoflavones (genistein, daidzein, formononetin, and biochanin A) and 1 glycoside derivative (Ononin) were obtained from Sigma Aldrich Denmark A/S and used for the determination of isoflavones in RCE. The RCE was diluted 10 times (from 50 to 5 mL) by methanol before analysis (for content, see **Supplemental Table 1**). Calcium and multivitamin supplements comprising the CDM were obtained from Fitness Pharma (for contents, see **Supplemental Table 2**). Participants were required to take 1 calcium supplement, 1 multivitamin, and either 95 mL of extract (RCE group) or placebo with no isoflavones (CON group) in the morning and evening with a meal; in all, this amounted to a total daily intake of 57.45 mg isoflavones (55.84 mg aglycones/d) for the RCE group and 0 mg isoflavones/d for the CON group.

### **Blood and urine samples**

Fasting blood and urine samples were collected from participants between 0800 and 0900 at baseline and at 6 and 12 mo. On collection, all EDTA plasma and urine samples were frozen and kept at  $-80^{\circ}\text{C}$  (Department of Clinical Biochemistry, Hospital Vendsyssel) until analysis.

### **Plasma lipids**

Blood plasma lipid concentrations were determined with the use of absorbance photometry by using the Roche Cobas C111 Clinical Chemistry Analyzer (Roche Diagnostics, GmbH). Total cholesterol (TC), HDL, and triglyceride concentrations were determined with the use of direct and enzymatic assay (Roche Diagnostics, GmbH). Final LDL concentrations were calculated with the use of the Friedewald equation [i.e.,  $\text{LDL} = \text{TC} - \text{HDL} - \text{VLDL}$  (estimated as  $\text{triglycerides}/50$ )].

### **Dual-energy X ray absorptiometry**

DXA was performed by the same health care professionals with the use of an XR 800 DXa scanner (Illuminatus software, version 4.2.4; Norland, CooperSurgical) at the L2–L4 region, femoral neck (FN), and trochanter sites at baseline and 12 mo. DXA was used to determine bone mineral content (BMC), BMD, and T score at each site.

### *Biomarkers of bone turnover*

Measurements of biomarkers of bone turnover were performed on freshly thawed aliquots by a single analyst blinded to clinical status. Plasma concentrations of collagen type I cross-linked C-telopeptide (CTX), osteocalcin, and procollagen type I N-terminal propeptide were measured on an automated analyzer according to the manufacturer's instructions. Plasma concentrations of free non-osteoprotegerin-bound receptor activator of nuclear factor  $\kappa$ -B ligand (RANKL) and plasma concentrations of undercarboxylated osteocalcin were measured by ELISA according to the kit manufacturer's instructions. The manufacturer-supplied analytic variation coefficients were as follows: CTX, procollagen type I N-terminal propeptide, and osteocalcin (Roche Diagnostics):  $<6\%$ ,  $<4\%$ , and  $<2\%$ , respectively; undercarboxylated osteocalcin (Takara Bio)  $<10\%$ , and RANKL (Biomedica)  $<3\%$ . Analyses were carried out by the same technicians at Aalborg University Hospital. The signaling marker osteoprotegerin (R&D Systems) had a manufacturer-coefficient of  $6\%$  and was performed by a single technician at Odense University Hospital.

### **Urinary estrogen metabolites**

Concentrations of the estrogen metabolites  $16\alpha$ -OH and 2-OH were determined in triplicate for urine samples by enzyme immunoassay (Estramet 2/16 ELISA; IBL International). Before analysis, glucuronides and sulfates were hydrolyzed enzymatically, and the procedure was performed according to the manufacturers' instructions (41). The obtained interbatch precision for 2-OH and  $16\alpha$ -OH was  $10.9\%$  and  $9.1\%$ , respectively. Analyses were carried out by the same technicians at Aalborg University Hospital.

### **Plasma isoflavone concentration**

The concentrations of the isoflavones in the plasma samples were determined by ultra-HPLC–tandem mass spectrometry (UHPLC-MS/MS) (Agilent) at Odense University Hospital. Plasma EDTA samples ( $250\ \mu\text{L}$ ) were mixed in tubes with  $250\ \mu\text{L}$  buffer (1 mL acetic acid glacial, 4.103 g sodium acetate trihydrate, and 500 mL water) at pH 4.9 containing 0.00625 g ( $250\ \text{U}$ ) sulphatase (Sigma Aldrich). Thereafter,  $25\ \mu\text{L}$  ( $2500\ \text{U}$ )  $\beta$ -glucuronidase (Sigma Aldrich) was added and vortexed. Samples were incubated for 4 h at  $37^{\circ}\text{C}$  in an incubator. After removal from the incubator,  $250\ \mu\text{L}$  of HPLC-grade acetonitrile (Sigma Aldrich) was added to precipitate proteins. Samples were then centrifuged at  $10,000 \times g$  for 10 min, and the supernatant extracted was transferred by syringe and filtered ( $0.22\text{-}\mu\text{m}$  pore filter) into HPLC vials. Purified ( $\geq 99.9\%$ ) formononetin, biochanin A, daidzein, genistein, and equol standards (Sigma Aldrich) were acquired for generating calibration curves. Plasma isoflavone concentration was determined as the sum of formononetin, biochanin A, daidzein, genistein and equol concentrations detected in plasma by UHPLC-MS/MS.

UHPLC-MS/MS was performed on a 1290 Infinity UHPLC composed of a pump (G4220A), an autosampler (G4226A), a thermostat (G1330B), and a column oven (G1316C) and hyphenated with a 6460 triple-quadrupole mass spectrometer (Agilent). The system was controlled by MassHunter Workstation software (version B.08.00, build 8.0.8023.0). Separation of the analytes was achieved on an Agilent Eclipse Plus C18 Rapid

Resolution High Definition column (1.8  $\mu\text{m}$ , 2.5  $\times$  50 mm). The column oven temperature was adjusted to 60°C. Solvent A was 0.1% (volume:volume) formic acid in water, and solvent B was 0.1% (volume:volume) formic acid in acetonitrile. The flow rate was set to 0.4 mL/min. The injected sample volume was 0.5  $\mu\text{L}$ . The gradient elution profile was as follows: linear gradient from 1% B at 0 min to 95% B at 15 min, then a return to 1% B from 15 to 16 min, followed by an isocratic post-run equilibration at 1% B. The Agilent Jet Stream electrospray ionisation source of the mass spectrometer was operated in the positive mode. The source parameters were as follows: gas temperature: 350°C, gas flow: 10 L/min, nebulizer: 241.32 kPa, sheath gas temperature: 400°C, sheath gas flow: 12 L/min, and the capillary: 4500 V and 32 nA. The experiments were conducted in dynamic multiple reaction monitoring. Nitrogen was used as collision gas. Authentic standards of the isoflavones formononetin, biochanin A, daidzein, genistein, and equol were used for identification and quantification.

### Equol-producer status

The time-resolved fluoroimmunoassay (Labmaster, Ltd.) for equol determination in plasma was used at baseline and 6 mo. The kit required the use of 200  $\mu\text{L}$  of plasma. Plasma samples were treated with enzymatic hydrolysis in acetate buffer (0.1 M, pH 5 + 0.2 U/mL glucuronidase + 0.2 U/mL sulphatase) incubated at 37°C overnight. Thereafter, equol was extracted in 1.5 mL diethyl ether, transferring equol from the polar plasma or water phase to the apolar ether phase. The sample was frozen and ether extracted, water was then thawed and re-extracted with ether, and this was then combined with the previously extracted ether and finally evaporated. A total of 200  $\mu\text{L}$  of assay buffer containing lyophilized europium-labeled equol was added to the tubes, and 20  $\mu\text{L}$  of the sample was taken for time-resolved fluoroimmunoassay. Europium-labeled equol and sample equol competed for goat anti-rabbit IgG on low-fluorescence microtiter plates that bind anti-equol antibody-forming fluorescent chelates. Samples were analyzed by an EnVision multimode plate reader (PerkinElmer), subsequent data were assessed with the use of WorkOut 2.5 (PerkinElmer). Equol-producer status in this study was defined as a plasma concentration of  $\geq 15$  nmol/L in accordance with Nettleton et al. (42).

### Ambulatory BP

Twenty-four-hour BP measurements were taken at baseline and at 12 mo with the use of the SpaceLab monitor (SpaceLabs Medical) and data was assessed by SpaceLabs software. Mean arterial pressure, heart rate, and systolic and diastolic BP were taken at specified time intervals. Participants were required to abstain from medium and heavy exercise for the duration of 24-h testing and to note down when they woke up and went to sleep.

### Three-day dietary intake

Habitual diets were collected with the use of a 3-d diet diary (3 consecutive days, including 1 weekend day) at baseline and at 6 and 12 mo. Participants were instructed verbally and given a guide (including examples) by the research team specifying how to fill in the diary over the 3 d. Before consumption, all food items

were weighed individually with the use of household scales. To minimize under-reporting or oversight, participants were instructed to record continuously. Completed diaries were handed in to the research team and analyzed with the use of MADLOGVita (MADLOG ApS; <https://www.madlogvita.dk/da/>), an online diet and nutrition calculation program. MADLOGVita was used to estimate the following: intake; energy; macronutrients (protein, carbohydrates and fat); and micronutrients (vitamin D, vitamin K, calcium, and magnesium). This program collects nutritional data from FoodComp and Danish food manufacturers; it is Denmark's most complete database with >14,500 specific Danish food products.

### PAL questionnaire

PAL questionnaires were completed at baseline and at 6 and 12 mo. It comprises 4 central multiple choice questions regarding current training and daily physical activity. Scores for each range from 1 to 4, where higher scores indicate lower PAL.

### Compliance and adverse events

Participants were required to collect and return empty extract sachets and supplement containers at 3, 6, 9, and 12 mo. These sachets and containers were counted by the research team, and compliance was calculated from the number returned. At least 90% of empty sachets and containers were required for participants to fulfill the compliance criteria. At the end of the study, participants achieved a mean ( $\pm$ ) compliance rate of 96.4%  $\pm$  3.64%. Adverse events were reported by participants via the project telephone number or during hospital visits. The RCE was well tolerated and there were no significant adverse events.

### Statistics

Statistical analysis included all participants who completed the study. Data were analyzed with the use of StataIC statistical software (version 11.2). Graphs were created by GraphPad Prism, version 4 (GraphPad Software, Inc.). All data were tested for normal distribution by way of visual inspection (quantile-quantile plots, histograms, and box plots) and by the D'Agostino-Pearson test. Between group differences in baseline and intergroup change over the duration of the study were determined for each parameter with the use of unpaired Student's *t* tests. Paired Student's *t* tests were used to assess intragroup differences from baseline to 12 mo. For parameters with >2 groups, 2-way and repeated-measures (within-subject) ANOVA were used. When significant main effects were detected ( $P < 0.05$ ), the Bonferroni multiple comparisons test was applied to compare data. All absolute and ratio data are presented in text and tables as means  $\pm$  SEMs, change data from baseline to 12 mo are presented as means (95% CIs). In all cases, a  $P$  value  $< 0.05$  was considered significant.

### Power calculation

To determine the required number of participants, the following equation was used:

$$N = (Z \alpha + Z \beta) 2 \times s^2 / \text{MIREDF}^2 (I)$$

The minimum relevant difference was set at 1.7;  $\alpha$  was 5%,  $\beta$  was 20%, and the spread (*s*) was set to 2.5. The minimum

number of participants required to complete the study was 68 (34 in each group). Due to the long duration of the study, 25% was added to the final *N* value to account for dropouts and losses, and the final total number of participants to include was 85. Effect size was based on BMD data of postmenopausal women after 1 y of soy treatment from Lydeking-Olsen et al. (43).

## RESULTS

### Baseline characteristics

Except for significantly higher ( $P < 0.034$ ) BMC at the trochanter in the CON group ( $8.46 \pm 0.27$  g) compared with the RCE group ( $7.67 \pm 0.28$  g), there were no significant intergroup differences in any of the baseline parameters listed in **Table 1**. A total of 78 participants successfully completed the trial and were included in all analyses (CON group:  $n = 40$ ; RCE group:  $n = 38$ ).

### DXA

The change in BMD ( $P = 0.043$ ) and T score ( $P = 0.045$ ) showed a significantly greater decrease in the lumbar spine of the CON group [BMD:  $-0.022$  g/cm<sup>2</sup> ( $-0.032$ ,  $-0.012$  g/cm<sup>2</sup>);

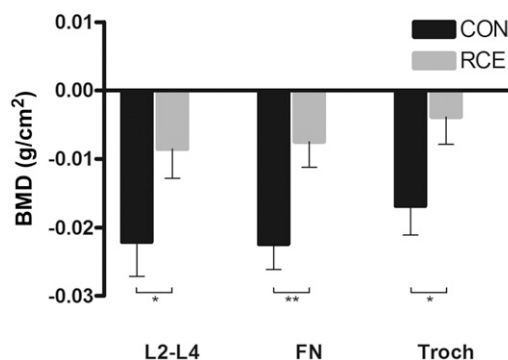
**TABLE 1**  
Baseline characteristics of 78 participants that completed the study<sup>1</sup>

	CON ( $n = 40$ )	RCE ( $n = 38$ )	$P^2$
Age, y	$62.85 \pm 0.99$	$60.84 \pm 1.07$	0.17
Height, m	$1.63 \pm 0.01$	$1.64 \pm 0.01$	0.87
Weight, kg	$71 \pm 2.15$	$66.61 \pm 1.81$	0.12
BMI, kg/m <sup>2</sup>	$26.65 \pm 0.81$	$24.84 \pm 0.62$	0.08
Waist, <sup>3</sup> m	$0.92 \pm 0.02$	$0.86 \pm 0.02$	0.06
Ambulatory blood pressure (24 h)			
Systolic, <sup>3</sup> mm Hg	$125.7 \pm 1.88$	$121.8 \pm 1.66$	0.13
Diastolic, mm Hg	$76.47 \pm 1.29$	$76.16 \pm 1.10$	0.52
DXA			
L2–L4 BMD, g/cm <sup>2</sup>	$0.86 \pm 0.10$	$0.87 \pm 0.13$	0.82
L2–L4 BMC, g	$39.47 \pm 1.05$	$39.77 \pm 1.06$	0.98
L2–L4 T score	$-1.97 \pm 0.16$	$-1.96 \pm 0.18$	0.82
FN BMD, g/cm <sup>2</sup>	$0.74 \pm 0.01$	$0.72 \pm 0.01$	0.46
FN BMC, g	$3.58 \pm 0.07$	$3.55 \pm 0.07$	0.74
FN T score	$-1.55 \pm 0.09$	$-1.64 \pm 0.09$	0.47
Troch BMD	$0.60 \pm 0.01$	$0.58 \pm 0.01$	0.26
Troch BMC	$8.46 \pm 0.27$	$7.67 \pm 0.28$	<0.05
Lipids			
Total cholesterol, mmol/mL	$5.64 \pm 0.16$	$5.54 \pm 0.14$	0.63
LDL, mmol/mL	$3.37 \pm 0.14$	$3.28 \pm 0.14$	0.65
HDL, mmol/mL	$1.82 \pm 0.08$	$1.81 \pm 0.07$	0.92
Triglycerides, <sup>3</sup> mmol/mL	$1.38 \pm 0.10$	$1.16 \pm 0.06$	0.12
Phytoestrogen			
Plasma isoflavones, ng/mL	$629.0 \pm 120.2$	$714.0 \pm 133.3$	0.64
Urinary estrogen metabolites			
2-OH, <sup>3</sup> ng/mL	$10.44 \pm 1.17$	$12.11 \pm 2.15$	0.84
16 $\alpha$ -OH, <sup>3</sup> ng/mL	$4.45 \pm 0.95$	$4.91 \pm 1.00$	0.54
2-OH:16 $\alpha$ -OH ratio, <sup>2</sup> ng/mL	$3.13 \pm 0.27$	$2.77 \pm 0.19$	0.60

<sup>1</sup>Data are presented as means  $\pm$  SEMs. BMC, bone mineral content; BMD, bone mineral density; CON, control; DXA, dual-energy X-ray absorptiometry; FN, femoral neck; L2–L4, lumbar spine vertebra L2–L4; RCE, red clover extract; Troch, trochanter; 2-OH, 2-hydroxyestrone; 16 $\alpha$ -OH, 16 $\alpha$ -hydroxyestrone.

<sup>2</sup> $P$  values for intergroup comparisons were determined by unpaired Student's *t* tests.  $P < 0.05$  was considered significant.

<sup>3</sup>Log-transformed data.



**FIGURE 3** Means  $\pm$  SEMs of change in BMD of the CON ( $n = 40$ , black) and treatment (RCE;  $n = 38$ , gray) groups at L2–L4, FN, and Troch. Significant differences were determined by unpaired Student's *t* test: \*\* $P < 0.01$ . BMD, bone mineral density; CON, control; FN, femoral neck; L2–L4, lumbar spine vertebra L2–L4; RCE, red clover extract; Troch, trochanter.

T score:  $-0.2$  ( $-0.29$ ,  $-0.11$ )] than the RCE group [BMD:  $-0.0085$  g/cm<sup>2</sup> ( $-0.017$ ,  $0.00006$  g/cm<sup>2</sup>); T score:  $-0.08$  ( $-0.16$ ,  $0.0001$ )] after 12 mo of treatment (**Figure 3**). Similar results were found at the FN, where BMD ( $P = 0.0059$ ) and T score ( $P = 0.0061$ ) showed a significantly greater decrease than the CON group [BMD:  $-0.022$  g/cm<sup>2</sup> ( $-0.03$ ,  $-0.015$  g/cm<sup>2</sup>); T score:  $-0.19$  ( $-0.25$ ,  $-0.12$ )] than the RCE group [BMD:  $-0.008$  g/cm<sup>2</sup> ( $-0.015$ ,  $0.00003$  g/cm<sup>2</sup>); T score:  $-0.06$  ( $-0.13$ ,  $-0.0001$ )]. A significantly greater reduction from baseline to 12 mo was also seen in the trochanter for BMD ( $P = 0.03$ ) and BMC ( $P = 0.034$ ) in the CON group [BMD:  $-0.017$  g/cm<sup>2</sup> ( $-0.025$ ,  $-0.008$  g/cm<sup>2</sup>); BMC:  $-0.54$  g ( $-0.79$ ,  $-0.3$  g)] compared with the RCE group [BMD:  $-0.004$  g/cm<sup>2</sup> ( $-0.01$ ,  $0.004$  g/cm<sup>2</sup>); BMC:  $-0.23$  g ( $-0.39$ ,  $-0.07$  g)]. There were no significant intergroup differences in the change in BMC at the lumbar spine or the FN.

### Bone biomarkers

A significant reduction ( $P = 0.045$ ) in bone resorption marker plasma CTx concentration was found in the RCE group [ $-0.04$  ng/mL ( $-0.09$ ,  $0.01$  ng/mL)] compared with the CON group [ $0.03$  ng/mL ( $-0.02$ ,  $0.07$  ng/mL)]. There were no significant differences between groups in any of the other bone biomarkers (**Table 2**). Both groups saw a significant increase in osteoprotegerin concentration ( $P < 0.001$ ) from baseline: the RCE group increased by  $480.1$  ng/mL ( $-138$ ,  $1273$  ng/mL), and the CON group by  $420.8$  ng/mL ( $-278$ ,  $1094$  ng/mL).

### Plasma isoflavone concentration

The isoflavone concentration was significantly elevated ( $P = 0.0094$ ) in the RCE group [ $3933$  ng/mL ( $627.6$ ,  $7238$  ng/mL)] from baseline to 12 mo of treatment (**Figure 4**) compared with the CON group [ $-322.7$  ng/mL ( $-608.1$ ,  $-37.23$  ng/mL)].

### Equol-producer status

At baseline, the mean equol concentrations for the CON and RCE groups were  $2.94 \pm 0.64$  nmol/L and  $3.37 \pm 0.42$  nmol/L, respectively. None of the participants were found to be equol producers at baseline, and at 6 mo, there was a significant increase ( $P < 0.0001$ ) in equol concentration for the RCE group

**TABLE 2**  
Bone turnover markers and estrogen metabolites<sup>1</sup>

Biomarkers	Baseline	12 mo	Change (95% CI)	P <sup>2</sup>
CON CTx, <sup>3</sup> ng/mL	0.40 ± 0.03	0.43 ± 0.19	0.03 (−0.02, 0.07)	
RCE CTx, <sup>3</sup> ng/mL	0.44 ± 0.03	0.39 ± 0.16	−0.04 (−0.09, 0.01)	<0.05
CON PINP, ng/mL	47.59 ± 2.49	48.29 ± 3.05	0.71 (−5.45, 6.87)	
RCE PINP, ng/mL	47.77 ± 2.96	49.33 ± 3.49	1.47 (−3.88, 6.83)	0.85
CON OPG, pg/mL	1249 ± 60.4	1670 ± 67.78 <sup>4</sup>	420.8 (−278, 1094)	
RCE OPG, pg/mL	1212 ± 47.2	1692 ± 65.0 <sup>4</sup>	480.1 (−138, 1273)	0.44
CON RANKL, pg/mL	2.07 ± 0.19	1.81 ± 0.17	−0.26 (−0.51, −0.02)	
RCE RANKL, pg/mL	1.77 ± 0.17	1.77 ± 0.18	−0.002 (−0.35, 0.34)	0.22
CON uOC, <sup>3</sup> ng/mL	4.60 ± 0.58	4.36 ± 0.51	−0.24 (−1.13, 0.65)	
RCE uOC, <sup>3</sup> ng/mL	3.52 ± 0.31	3.63 ± 0.38	0.13 (−0.46, 0.73)	0.35
CON OC, <sup>3</sup> ng/mL	23.61 ± 1.28	24.30 ± 1.50	0.69 (−1.77, 3.15)	
RCE OC, <sup>3</sup> ng/mL	24.01 ± 1.35	23.98 ± 1.64	−0.03 (−2.40, 2.33)	0.67
CON estradiol, nmol/L	0.03 ± 0.01	0.04 ± 0.01	0.002 (−0.007, 0.01)	
RCE estradiol, nmol/L	0.03 ± 0.01	0.04 ± 0.01	0.01 (−0.009, 0.03)	0.42
CON 2-OH, <sup>3</sup> ng/mL	10.44 ± 1.17	10.54 ± 1.43	0.10 (−1.78, 1.98)	
RCE 2-OH, <sup>3</sup> ng/mL	12.11 ± 2.15	12.64 ± 1.33	0.54 (−2.25, 3.33)	0.14
CON 16α-OH, <sup>3</sup> ng/mL	4.45 ± 0.95	4.44 ± 0.94	−0.02 (−0.95, 0.91)	
RCE 16α-OH, <sup>3</sup> ng/mL	4.91 ± 1.00	4.63 ± 1.19	−0.29 (−1.25, 0.67)	0.96
CON 2-OH:16α-OH, <sup>3</sup> ng/mL	3.13 ± 0.27	2.98 ± 0.17	−0.16 (−0.67, 0.29)	
RCE 2-OH:16α-OH, <sup>3</sup> ng/mL	2.77 ± 0.19	3.41 ± 0.23	0.64 (0.36, 1.00)	<0.05

<sup>1</sup> Absolute data are presented as means ± SEM; change data are shown as means (95% CIs). CON, control; CTx, collagen type 1 cross-linked C-telopeptide; OC, osteocalcin; OPG, osteoprotegerin; PINP, procollagen type I N-terminal propeptide; RANKL, receptor activator of nuclear factor κ-B ligand; RCE, red clover extract; uOC, undercarboxylated osteocalcin; 2-OH, 2 hydroxysterone; 16α-OH, 16α-hydroxysterone.

<sup>2</sup> P values for intragroup comparisons were determined by paired Student's *t* tests. *P* < 0.05 was considered significant.

<sup>3</sup> Log-transformed data.

<sup>4</sup> Within-group paired significance of *P* < 0.01 from baseline to 12 mo.

[36.43 nmol/L (4.7, 68.2 nmol/L)] compared with the CON group [0.08 nmol/L (−1.4, 1.55 nmol/L)]. At 6 mo, the CON group retained a mean total equol concentration of 3.02 ± 0.67 nmol/L, and the RCE group showed a mean total equol concentration of 39.80 ± 15.68 nmol/L (**Figure 5**). By 6 mo, 55% (*n* = 21) of the participants in the RCE group were identified as equol producers.

### Urinary estrogen metabolites

The concentration ratio of 2-OH to 16α-OH estrogen metabolites (**Figure 6**) was significantly increased (*P* = 0.026) in the RCE group [0.64 ng/mL (0.36, 1.00 ng/mL)] compared with the CON group [−0.16 ng/mL (−0.67, 0.29 ng/mL)]. There were no significant differences in the intergroup change of 2-OH or 16α-OH alone between the RCE and CON groups (Table 2).

### Dietary intake

Dietary intake does not include nutrients provided by supplementation in this study (**Table 3**). There were no significant intergroup differences in habitual dietary intakes found for any of the nutrients between any of the groups at any point during the study. A significant effect of time on dietary calcium (*P* < 0.001), magnesium (*P* < 0.001), and protein (*P* < 0.05) intakes was found. Bonferroni post hoc tests revealed no significant between-group differences in nutrient intakes throughout the study. Overall, there was a significant decrease from baseline in dietary intakes of calcium, magnesium, and protein in both groups. These amounted to mean decreases of −159 and −143 mg for calcium and −43.7

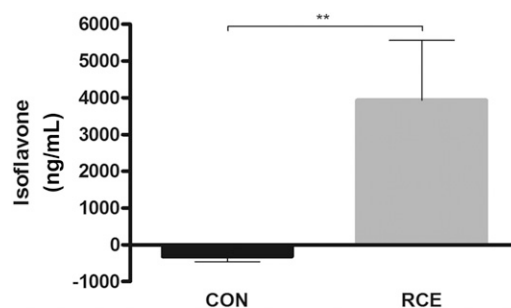
and −37.6 mg for magnesium in the CON and RCE groups, respectively.

### Plasma lipids

There were no significant differences found for inter- or intra-group plasma concentrations of TC, HDL, LDL, or triglycerides (**Table 4**) throughout the study.

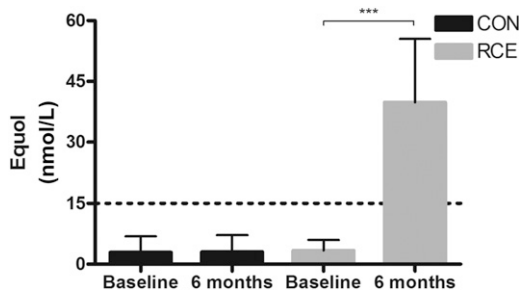
### BP

There were no significant differences found for systolic or diastolic BP change between the RCE or CON groups (Table 4). BP was well regulated in both groups throughout the study.



**FIGURE 4** Means ± SEMs of change in plasma isoflavone concentrations of the CON (*n* = 40, black) and RCE (*n* = 38, gray) groups. Significant differences were determined by unpaired Student's *t* test: \*\**P* < 0.01. CON, control; RCE, red clover extract.





**FIGURE 5** Means  $\pm$  SEMs of plasma equol concentrations of the CON ( $n = 40$ , black) and RCE ( $n = 38$ , gray) groups at baseline and at 6 mo with a 15-nmol/L equol-producer threshold (dotted line). Paired Student's  $t$  tests of log-transformed data found a significant increase at 6 mo within the RCE group alone. \*\*\* $P < 0.001$ . CON, control; RCE, red clover extract.

### Physical activity

Physical activity data are shown in **Table 5**. There was a significant effect of time on self-reported daily activity ( $P < 0.01$ ), and Bonferroni post hoc tests revealed no significant intergroup differences during the study. There was no significant difference found in daily training between groups at any point during the study.

### Compliance

At the end of the study, the total number of sachets and pill containers returned after 12 mo indicated that 97.5% ( $n = 78$ ) of all participants ( $N = 80$ ) achieved a compliance of  $\geq 95\%$  at study completion. The mean compliance rate was  $96.44\% \pm 0.40\%$ . There were no significant intergroup differences in compliance rates.

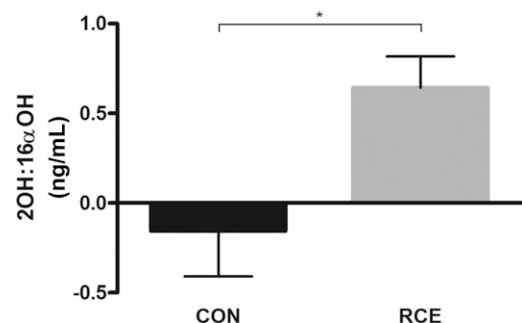
### Adverse events

Participants in the CON ( $n = 1$ ) and RCE groups ( $n = 2$ ) reported gastrointestinal problems during the study. Out of the 85 included participants 3.5% ( $n = 3$ ) dropped out of the study due to gastrointestinal issues, and there was no significant differences between groups. There were no other reported adverse events, and 12-mo daily intake of RCE was well tolerated.

## DISCUSSION

In this 12-mo. double-blind RCT of osteopenic postmenopausal women, RCE exerted a potent physiological effect against estrogen-deficient bone loss in postmenopausal women with osteopenia. Moreover, RCE combined with CDM was found to be more effective than CDM supplementation alone in preserving bone density in women with estrogen deficiency. Groups achieved high baseline equivalence, because there were no significant intergroup differences in any of the baseline characteristics apart from BMC at the trochanter where the CON group was significantly higher (Table 1). All postmenopausal participants were estrogen deficient throughout the study, as indicated by the low plasma estrogen concentrations ranging from 0.03 to 0.04 nmol/L. DXA scans found that 12 mo of treatment with RCE was more effective at attenuating expected postmenopausal bone resorption at the FN, L2–L4 region, and trochanter than CDM alone (Figure 3). The BMD loss from baseline to 12 mo was  $\sim 2$ -fold lower at the FN in the RCE group ( $-0.99\%$ ) than the CON group ( $-2.2\%$ ); at the L2–L4 region, the CON group lost  $\sim 3$ -fold more BMD

( $-3.05\%$ ) than the RCE group ( $-1.04\%$ ); and at the trochanter, there was a 4-fold greater loss in BMD in the CON group ( $-2.79\%$ ) than in the RCE group ( $-0.67\%$ ). There were no significant intergroup differences in BMC change at any skeletal regions apart from the trochanter, where the RCE group had significantly less BMC loss than the CON group. This finding is likely influenced by the significantly higher BMC of the CON group at the trochanter at baseline. Together, these data suggest that in certain skeletal locations, RCE improved both bone mineralization and density, whereas in other locations, the bone formed was of a higher density, with no significant effect on mineralization. The significant effects on BMC at other sites may have been possible with a higher number of participants (44). The beneficial effects of isoflavone treatments against estrogen-deficient BMD loss, but not BMC loss, have been documented in previous RCTs (45). Observational data support that cortical (denser) bone loss has a greater dependency on estrogen deficiency than does trabecular (more porous) bone loss; to test this, a 4-y RCT investigated the effects of ET in postmenopausal women assessed by high-resolution peripheral quantitative computed tomography and found that cortical bone was more responsive to ET than trabecular bone (46). This may also be the case in the present study, where a greater response to isoflavone treatment was found at the FN (which is richer in cortical tissue) than in the lumbar spine. We have previously demonstrated short-term attenuative effects of a fermented RCE (37 mg isoflavone aglycones/d) and probiotic treatment on BMD at the L2–L4 region in healthy menopausal women (40). The spine comprises mostly trabecular bone, and we previously postulated that a longer treatment duration and a higher dose may be required to achieve attenuation of BMD loss in cortical skeletal locations (47). The data from the present study support this assertion, because BMD loss at the FN and trochanter was blunted in the RCE group compared with the CON group after 12 mo at a higher dosage. Improvements to BMD in postmenopausal women given 1 y of ET or bisphosphonates have been shown to range from  $\sim 0$ – $2.5\%$  and  $\sim 4$ – $6\%$ , respectively, at the spine and  $\sim 0$ – $0.5\%$  and  $\sim 0.8$ – $3\%$ , respectively, at the FN (48, 49). Although the effect of size in the present study is slightly lower than ET or bisphosphonates, the safer profile and minimal side effects of RCE support its use in wider patient groups and its suitability for long-term use. The beneficial effects of RCE on BMD were further corroborated by plasma CTx



**FIGURE 6** Means  $\pm$  SEMs of the change in urinary 2-OH to 16 $\alpha$ -OH concentration ratios of the CON ( $n = 40$ , black) and RCE ( $n = 38$ , gray) groups from baseline to 12 mo. Unpaired Student's  $t$  tests of log-transformed data found a significant increase in 2-OH to 16 $\alpha$ -OH concentration ratio in the RCE group compared with the CON group. \* $P < 0.05$ . CON, control; RCE, red clover extract; 2-OH, 2-hydroxyestrone; 16 $\alpha$ -OH, 16 $\alpha$ -hydroxyestrone.

**TABLE 3**  
Three-day dietary intake<sup>1</sup>

	Group	Baseline	6 mo	12 mo	Interaction <i>P</i> <sup>2</sup>	Time <i>P</i> <sup>2</sup>	Treatment <i>P</i> <sup>2</sup>
Energy, kJ	CON ( <i>n</i> = 40)	6893 ± 233.4	6954 ± 291	6442 ± 265.2	0.44	0.08	0.68
	RCE ( <i>n</i> = 38)	6795 ± 223.4	7104 ± 237.6	6796 ± 254.1			
Protein, g	CON ( <i>n</i> = 40)	73.27 ± 2.51	72.94 ± 2.84	68.45 ± 2.71	0.92	<0.05	0.91
	RCE ( <i>n</i> = 38)	72.67 ± 2.45	73.70 ± 2.86	69.11 ± 2.93			
Carbohydrate, g	CON ( <i>n</i> = 40)	199.3 ± 6.75	192.7 ± 8.11	185.4 ± 7.33	0.69	0.20	0.71
	RCE ( <i>n</i> = 38)	197.8 ± 8.42	199.9 ± 8.13	192.7 ± 10.09			
Lipid, g	CON ( <i>n</i> = 40)	57.17 ± 3.15	59.36 ± 3.61	53.79 ± 3.31	0.42	0.35	0.94
	RCE ( <i>n</i> = 38)	54.63 ± 2.94	57.94 ± 3.63	57.04 ± 2.54			
Vitamin D, <sup>3</sup> μg	CON ( <i>n</i> = 40)	4.01 ± 0.68	4.98 ± 0.91	3.31 ± 0.56	0.44	0.14	0.54
	RCE ( <i>n</i> = 38)	3.62 ± 0.63	4.14 ± 1.01	3.22 ± 0.68			
Vitamin K, <sup>3</sup> μg	CON ( <i>n</i> = 40)	96.71 ± 19.45	77.33 ± 11.39	73.05 ± 11.01	0.51	0.36	0.43
	RCE ( <i>n</i> = 38)	86.18 ± 14.76	86.16 ± 12.52	89.55 ± 19.68			
Calcium, mg	CON ( <i>n</i> = 40)	866.0 ± 49.33	880.9 ± 50.27	707.0 ± 49.34	0.74	<0.001	0.55
	RCE ( <i>n</i> = 38)	836.5 ± 52.42	811.7 ± 61.03	693.9 ± 45.35			
Magnesium, mg	CON ( <i>n</i> = 40)	286.8 ± 11.14	273.3 ± 10.62	242.9 ± 9.96	0.048	<0.001	0.61
	RCE ( <i>n</i> = 38)	266.1 ± 9.99	289.7 ± 13.13	228.5 ± 11.51			

<sup>1</sup> Absolute data of dietary nutrient intake are presented as means ± SEMs. These data do not include nutrient intake from supplements. CON, control; RCE, red clover extract.

<sup>2</sup> Main effects (treatment and time) and interactions were determined by 2-way repeated-measures ANOVA. *P* < 0.05 was considered significant. Bonferroni multiple comparison tests were used to compare means when the main effects or interactions were significant.

<sup>3</sup> Log-transformed data.

(the reference standard from bone resorption) concentrations (Table 2). After 12 mo, CTx concentrations were significantly decreased in the RCE group (−9.40%) compared with the CON group (6.76%) (50). No other changes in biomarkers were significant between groups. Osteoprotegerin concentrations significantly increased from baseline in both groups. Although there was a tendency for higher osteoprotegerin concentrations in the RCE group than in the CON group, the differences did not achieve significance. Vitamin D was previously shown to modulate osteoblastic osteoprotegerin and RANKL secretion through the nuclear vitamin D receptor, and thus vitamin D supplementation in this trial may have contributed to increases in osteoprotegerin concentrations in both groups, thereby partially obscuring the

potential RCE-mediated effects on osteoprotegerin (51). Isoflavones exhibit anti-inflammatory, anti-osteoporotic, and anti-oxidative bioactivity to varying degrees. The primary isoflavone constituent in the RCE is formononetin, which has demonstrated promising capability for these bioactivities (30, 35, 52). A recent in vitro trial found that formononetin dose-dependently inhibited RANKL-induced proinflammatory cytokines (TNF-α, IL-1β, and IL-6) and chemokines (macrophage inflammatory factor-1α, monocyte chemoattractant protein-1, and regulated upon activation, normal T-cell expressed and secreted) in primary bone marrow-derived precursors. Formononetin also suppressed RANKL-induced osteoclastogenesis by downregulating extracellular signal-regulated kinases, c-Jun N-terminal kinases,

**TABLE 4**  
Plasma lipids and blood pressure<sup>1</sup>

	Group	Baseline	12 mo <sup>2</sup>	Change (95% CI)	<i>P</i> <sup>3</sup>
Total cholesterol, mmol/mL	CON ( <i>n</i> = 40)	5.64 ± 0.16	5.50 ± 0.14	−0.15 (−0.39, 0.10)	0.81
	RCE ( <i>n</i> = 38)	5.54 ± 0.14	5.44 ± 0.17	−0.11 (−0.33, 0.12)	
HDL, mmol/mL	CON ( <i>n</i> = 40)	1.82 ± 0.08	1.70 ± 0.08	−0.13 (−0.22, −0.03)	0.80
	RCE ( <i>n</i> = 38)	1.81 ± 0.07	1.67 ± 0.09	−0.14 (−0.23, −0.05)	
LDL, mmol/mL	CON ( <i>n</i> = 40)	3.37 ± 0.14	3.08 ± 0.14	−0.30 (−0.51, −0.08)	0.28
	RCE ( <i>n</i> = 38)	3.28 ± 0.14	3.15 ± 0.13	−0.13 (−0.34, 0.08)	
Triglycerides, mmol/mL	CON ( <i>n</i> = 40)	1.38 ± 0.10	1.31 ± 0.09	−0.06 (−0.22, 0.10)	0.11
	RCE ( <i>n</i> = 38)	1.16 ± 0.06	1.26 ± 0.07	0.10 (−0.02, 0.21)	
Systolic BP, mm Hg	CON ( <i>n</i> = 40)	125.7 ± 1.88	125.6 ± 1.94	−0.13 (−2.08, 1.82)	0.84
	RCE ( <i>n</i> = 38)	121.8 ± 1.66	121.4 ± 1.79	−0.42 (−2.56, 1.71)	
Diastolic BP, mm Hg	CON ( <i>n</i> = 40)	76.47 ± 1.29	75.18 ± 1.01	−1.29 (−2.6, 0.02)	0.12
	RCE ( <i>n</i> = 38)	76.16 ± 1.10	76.39 ± 1.10	0.24 (−1.23, 1.71)	

<sup>1</sup> Absolute data are presented as means ± SEMs; change data are shown as means (95% CIs). BP, blood pressure; CON, control; RCE, red clover extract.

<sup>2</sup> There were no significant differences in intergroup changes from baseline. Intragroup comparisons were determined by paired Student's *t* tests. *P* < 0.05 was considered significant.

<sup>3</sup> *P* values for intergroup comparisons determined by unpaired Student's *t* tests. *P* < 0.05 was considered significant.

**TABLE 5**  
Physical activity and training<sup>1</sup>

	Group	Baseline	6 mo	12 mo	Interaction <i>P</i> <sup>2</sup>	Time <i>P</i> <sup>2</sup>	Treatment <i>P</i> <sup>2</sup>
Daily activity	CON ( <i>n</i> = 40)	2.16 ± 0.2	2.56 ± 0.2	1.94 ± 0.19	0.20	<0.01	0.19
	RCE ( <i>n</i> = 38)	1.86 ± 0.17	2.21 ± 0.15	1.97 ± 0.18			
Training	CON ( <i>n</i> = 40)	2.10 ± 0.13	1.98 ± 0.14	2.19 ± 0.14	0.09	0.12	0.83
	RCE ( <i>n</i> = 38)	1.93 ± 0.13	2.21 ± 0.15	2.22 ± 0.16			

<sup>1</sup> Absolute data of physical activity and training are presented as means ± SEMs. CON, control; RCE, red clover extract.

<sup>2</sup> Main effects (treatment and time) and interactions were determined by 2-way repeated measures ANOVA. *P* < 0.05 was considered significant. Bonferroni multiple comparison tests were used to compare means when the main effects or interactions were significant.

protein kinase B, and p38 mitogen-activated protein kinase signaling, as well as inhibiting nuclear factor of activated T cells-1 activation, c-Fos, and nuclear factor  $\kappa$ B; thus suggesting non-genomic and genomic effects (53). A study of female ovariectomized rats supported that formononetin has the potential to distinctly influence bone turnover, finding treatment to reduce osteoclastogenesis by decreasing urinary CTx concentration, upregulating messenger RNA expression of osteoprotegerin genes, and decreasing RANKL messenger RNA (30). Oxidized free radical species also play a role in the activation of RANKL, induction of osteoclastogenesis, and osteoblast apoptosis; a study of ovariectomized rats by Mu et al. (52) demonstrated that formononetin increases glutathione peroxidase, superoxide dismutase, and catalase, decreasing lipid peroxidation. The present study supports the effects of formononetin-rich RCE and probiotic treatment on bone turnover, although not all mechanisms reported in previous studies manifested because there was no effect on osteocalcin or RANKL. 16 $\alpha$ -OH estrogen metabolites are considered more genotoxic and estrogenic than 2-OH estrogen metabolites, which have minimal estrogenic potential and may retain cancer-protective properties (25). RCE treatment in this study was found to increase the urinary 2-OH to 16 $\alpha$ -OH ratio compared with the CON, thereby promoting more favorable estrogen metabolism and potentially reducing cancer risk. These data suggest that isoflavones exhibit capabilities to encourage a less carcinogenic estrogen metabolism (23, 24). Three-day diet diaries found a significant effect of time on calcium (<0.001), magnesium (<0.001), and protein (<0.05). There were no differences detected between groups in any dietary nutrients at any point during the study, supporting that dietary nutrient intake differences had minimal influence on the outcomes of the study (Table 3). To put this into context, a change in dietary calcium amounted to a percentile change in the NOF-recommended calcium intake of -13.25% for the CON group and -11.88% for the RCE group. The calculated optimal magnesium intake based on NOF calcium recommendations at a 2.8:1 ratio is 428.57 mg; the change at the end of the study in magnesium amounted to a decrease of -10.06% for the CON group and -8.75% for the RCE group. Combining the contribution of supplementary magnesium and calcium with the dietary intake of these minerals shows that both the CON group and the treatment group were well above recommended intakes throughout the duration of trial. Overall, the CON group had a mean total daily intake of 1747 mg Ca and 729.9 mg Mg, and the RCE group had a mean daily intake of 1733 mg Ca and

715.5 mg Mg. The ratio of total calcium to magnesium intake ranged from 2.38:1 to 2.53:1 throughout the study. Self-reported exercise and training remained consistent throughout the study, and no difference was found between the 2 groups, supporting a negligible effect of changes in exercise on bone turnover in this study (54, 55). Equol is a daidzein (a derivative of formononetin) metabolite produced by anaerobic bacteria and has greater estrogenic potency than its precursor (42). None of the participants were found to be equol producers at baseline, and no participant in the CON group became a producer. After 6 mo of treatment, 55% of the participants in the RCE group became equol producers (*n* = 21). This suggests that the probiotics in the RCE positively influenced the intestinal bacterial milieu of participants, promoting a profile favorable for equol production. In Western countries, ~30% of the population is capable of equol production, and equol-producer status is shown to be higher in vegetarians, where ~60% are producers (56). Isoflavones typically occur as glycosides in plants and in most food products. Glycosides are hydrophilic and have higher molecular weights that prevent passive diffusion across the intestinal brush border; they require hydrolytic conversion by glycosidases to more apolar, lipophilic, and lower-molecular-weight aglycones to facilitate absorption (57, 58). Liquid chromatography-mass spectrometry analyses have determined that the fermented RCE given to participants in the present study provided mostly isoflavone aglycones (~97.1%) and found the mean isoflavone plasma concentrations to increase from 714 to 4647 ng/mL after 12 mo in the RCE group. An RCT of postmenopausal women receiving a higher dose than the present study of 100 mg aglycone equivalents (glycosides)/d for 30 d showed mean plasma isoflavone concentrations of 1251 ng/mL; in contrast, in the present study, we found >3-fold higher plasma concentrations of isoflavone when given a dose of 57.45 mg/d of isoflavones (59). These findings support the important role of isoflavone molecular form and the intestinal microbiome in isoflavone uptake, metabolism, and ultimately therapeutic efficacy.

### Limitations

A study duration of  $\geq 2$  y would incorporate  $\geq 3$  complete bone remodeling cycles; this would further strengthen the evidence provided by DXA in this trial. The use of high-resolution peripheral quantitative computed tomography in this study would have facilitated a more comprehensive and greater distinction of isoflavone effects on cortical compared with trabecular bone (46).

Including other biomarkers of bone turnover would provide a more comprehensive overview of bone-turnover mechanisms, particularly sclerostin (an ER $\beta$ -mediated pre-resorptive marker produced by osteocytes) and bone-specific alkaline phosphatase from osteoblasts (37–39, 60). Methods determining food intake, although comprehensive, are subject to bias and only cover a small window of the total duration of the study (9 d). Self-report measures in this study are subjective and open to various biases, especially recall bias and response bias. The specific participant type (postmenopausal) and number could represent a limitation of this study with regards to generalizability. In the case of plasma lipids or ambulatory BP, all participants in this study were normotensive and were not overweight; hypertensive and obese participants would be better suited to investigate potential effects. Interactions of multiple treatment components with the same biomarker obscure the beneficial effects of single components.

In conclusion, this study demonstrates that bioavailable RC isoflavones combined with probiotics potently attenuated estrogen-deficient BMD loss and improved bone turnover in postmenopausal osteopenic women. Bioavailable RCE enhanced the efficacy of traditional CaD (with magnesium) treatment against bone loss. RCE was also found to increase the equol-producing capabilities of participants. The bone-sparing properties of isoflavones found in this study appear to be enhanced when barriers to uptake are overcome by maintaining isoflavones in aglycone form through fermentation and by providing probiotics to improve intestinal uptake and isoflavone metabolism. Moreover, RCE promoted more favorable estrogen metabolism by increasing the 2-OH to 16 $\alpha$ -OH metabolite ratio, thereby indicating the potential to reduce cancer risk.

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