

Eva Lydeking-Olsen  
Jens-Erik Beck-Jensen  
Kenneth D. R. Setchell  
Trine Holm-Jensen

## Soymilk or progesterone for prevention of bone loss

### A 2 year randomized, placebo-controlled trial

■ **Summary** *Background* Given concerns over the use of hormone replacement therapy (HRT), women are seeking natural alternatives to cope with the symptoms and effects of menopause. The bone sparing effects of soy protein and its isoflavones is well established in animal studies, while 5 previous human studies on soy and bone have

yielded variable outcomes due in part to their short duration of study. Progesterone has been suggested as a bone-trophic hormone, but the effect of long-term, low dose transdermal progesterone is unknown. *Aim* The aim of the study was to compare for the first time the long-term effects of soymilk, with or without isoflavones with natural transdermal progesterone, or the combination, on bone mineral density in the lumbar spine and hip. *Methods* Postmenopausal, Caucasian women with established osteoporosis or at least 3 risk-factors for osteoporosis, were randomly assigned, double-blind to one of four treatment-groups: soymilk containing isoflavones (soy+, n = 23), transdermal progesterone (TPD+, n = 22), or the combination of soy+ and TPD+, (n = 22) or placebo (isoflavone-poor soymilk, soy÷ and progesterone-free-cream TPD÷, n = 22). All subjects received comparable intakes of calcium, minerals and vitamins. Bone mineral content (BMC) and density (BMD) were measured in lumbar spine and hip

by using dual-energy X-ray absorptiometry (DEXA) at baseline and after 2 years. *Findings* The percentage change in lumbar spine BMD and BMC respectively, did not differ from zero in the soy+ group (+1.1 %, +2.0 %) and TDP+ group (÷1.1 %, +0.4 %) but significant bone loss occurred in the control group (÷4.2 %, ÷4.3 %) and the combined treatment group (÷2.8 %, ÷2.4 %). No significant changes occurred for femoral neck BMD or BMC. *Interpretation* Daily intake of two glasses of soymilk containing 76 mg isoflavones prevents lumbar spine bone loss in postmenopausal women. Transdermal progesterone had bone-sparing effects but when combined with soy milk a negative interaction between the two treatments occurs resulting in bone-loss to a greater extent than either treatment alone.

■ **Key words** soy – isoflavones – genistein – daidzein – equol – transdermal progesterone – bone density – bone conservation – postmenopausal women

Received: 8 October 2003  
Accepted: 18 February 2004  
Published online: 14 April 2004

E. Lydeking-Olsen (✉) · T. Holm-Jensen  
Institute for Optimum Nutrition  
Teglgaardstraede 4, 1st. floor  
1452 Copenhagen K, Denmark  
Tel.: +45-33324486  
Fax: +45-33324481  
E-Mail: forskning@optinut.dk

J.-E. Beck-Jensen  
The Osteoporosis and Metabolic Bone Unit  
Dept. of Endocrinology  
Copenhagen University Hospital  
Hvidovre, Denmark

K. D. R. Setchell  
Clinical Mass Spectrometry  
Children's Hospital and Medical Center  
Cincinnati (OH), USA

## Introduction

Current therapies for prevention and treating osteoporosis include estrogen and hormone replacement therapy (ERT and HRT), the selective estrogen receptor

modulator (SERM, i. e. raloxifene), bisphosphonates and calcitonin.

The increased risk of breast and uterine cancer with long-term ERT and HRT [1] and unwanted side effects account for poor compliance and loss of treatment effect [2, 3]. Recent results from the Women's Health Initiative

showing a lack of cardioprotective effect of HRT [4, 5] should heighten interest in natural alternatives to acute and chronic menopausal symptomatology.

Soy protein and its constituent isoflavones have become a focus of much interest for their potential bone-sparing effects. Several lines of circumstantial evidence warrant clinical trials of soy foods as a natural alternative to HRT. First, the non-steroidal isoflavones which bind selectively to estrogen receptors [6, 7] have been found in vitro to stimulate osteoblasts and inhibit osteoclast activity, effects that are consistent with reduced bone turnover [8–11]. Second, animal studies using rodent models of postmenopausal bone loss have consistently found bone-sparing effects of soy protein or isoflavones [12]. Third, short-term human studies have provided tantalizing evidence that isoflavones can reduce bone loss as measured by surrogate markers of bone turnover and changes in bone mineral density in postmenopausal women [13–17]. And finally, epidemiological studies from Japan and China have found that postmenopausal women with the highest intake of isoflavone-rich soy foods have the highest BMD in the lumbar spine compared with women with low intakes. Somekawa et al. showed that women in the two +50 mg/d groups of isoflavone-intake had a 7–9% higher lumbar spine BMD – both in the early and late postmenopausal group [18], whereas Mei found a 6.4% difference in lumbar spine BMD and 8.4% difference in wards triangle BMD between the highest and lowest tertile of isoflavone intake [19].

Based on these observations a number of dietary intervention studies have been performed to determine the effectiveness of soy protein in preventing bone loss in postmenopausal women [13–17, 20, 21]. These have yielded variable outcomes but all have been of a duration of no more than 12 months, making it difficult to observe significant changes in bone mineral density given the slow rate of bone turnover. A potential confounder in human isoflavone research is the heterogeneity in bioavailability and metabolism of the isoflavones – thus the equal-status clearly separates subjects in two distinct groups who are likely to have markedly different responses to soy food feeding, 30–50% being equal-producers in various populations [37]. Equol is a non-steroidal metabolite of one of the major isoflavones, daidzein, produced by the gut bacterial flora [37]. It is much less researched than the other main isoflavone genistein, due to not being available in purified form for in vitro and animal research. We now report the first long-term double-blinded randomized controlled study of 2-year duration in which soy protein with and without isoflavones was compared for its effects on bone by measurement of bone mineral density and content as the primary end-points. A second and increasingly popular alternative therapy used by postmenopausal women has been the use of natural transdermal progesterone

creams. Progesterone is considered to be a bone-trophic agent [22–24]. An open longitudinal case-series found that progesterone added to a conventional treatment program of healthy lifestyle and low-dose ERT (Premarin) actually increased bone mineral density (BMD) by up to 22.4% over 3 years [25]. However, the only controlled trial of transdermal progesterone showed no effect on BMD after one year but it did improve vasomotor symptoms [26].

If isoflavone-rich soy foods and/or progesterone prevent bone loss then these interventions could serve as alternative or adjuvant intervention during menopause and in older age for women who are poor candidates for or choose not to receive traditional HRT. Soy foods could also serve as a cheap and available non-pharmacological intervention in parts of the world where pharmacological treatment is less available due to socio-economic or other factors.

The purpose of this study was to examine the hypothesized bone-sparing effect of isoflavone-rich soymilk, transdermal progesterone or both, on bone mineral density (BMD) and bone mineral content (BMC) of the lumbar spine and hip in post-menopausal women. Biochemical markers of bone formation [serum-type I procollagen-N-terminal-peptide (PINP)] and bone resorption [serum type I C-terminal telopeptide (ICTP)] were also measured.

---

## Subjects and methods

### ■ Subjects

Subjects were recruited through newspaper advertisements and feature articles in local newspapers and magazines. Potential subjects were screened initially via telephone to ensure that they were at least one year postmenopausal, with a maximum age of 75 years, had not received any bone-active medication for at least 2 years previously and had at least 3 risk-criteria for osteoporosis: early menopause < 45 years of age, low-energy bone-fractures with typical localization, smoking, low body weight (BMI < 19 kg/m<sup>2</sup>), low physical activity level, low intake of calcium/vitamin D, heritage of osteoporosis, previous systemic steroid treatment ≥ 6 month duration or had been diagnosed as osteoporotic by DEXA scanning or due to fractures.

Exclusion criteria were drug or alcohol addiction, malignant disease, immobility, current steroid treatment, osteomalacia, diabetes, unstable thyroid disease, severe osteoarthritis in lumbar spine/hip, chronic inflammatory diseases, active liver and kidney disease.

Of 507 responders, 290 passed the telephone screen and 177 (61%) were assessed for eligibility. Of these 57 were not-eligible and 13 withdrew before randomization (11 for social reasons and 2 for other health rea-

sons), leaving 107 for inclusion. Before baseline testing an information meeting was conducted at which the subjects were assured that participation was completely voluntary and after oral and written information, signed a consent form.

The women came to the institute every 3 months the first year and every 6 months (or as needed) the second year for testing, teaching, group sessions and supply replenishment.

Of 107 women entered in the study 7 withdrew within 3 months (3 for disliking the soymilk, 4 for reasons unrelated to the study), six developed intolerance to soymilk, two developed intolerance to the skin cream and 3 were self-reported non-compliant due to aversion to the soy milk, leaving 89 for analysis. The protocol and consent forms were approved by the Regional Ethical Committee for Copenhagen and Frederiksberg, Denmark (J.no 02-131/1997), The Danish Medicines Agency (J.no. 5312-240-1997) and The Danish Data Protection Agency (J.no. 1998-1200-113).

## Research design and treatment

Participants were randomly assigned double blind to two years intervention in the following groups: Isoflavone-rich soymilk (soy+,  $n=23$ ), transdermal progesterone (TPD+,  $n=22$ ), combined (soy+, TDP+,  $n=22$ ) or placebo (isoflavone-poor soymilk, soy- and progesterone-free cream TDP-,  $n=22$ ). Subjects were supplied with a total of 500 ml of soymilk/d supplying 17.5 g protein/d as divided servings – one glass in the morning and evening, respectively, with an aglycone isoflavone content of 76.0 mg/day for 500 ml. The agly-

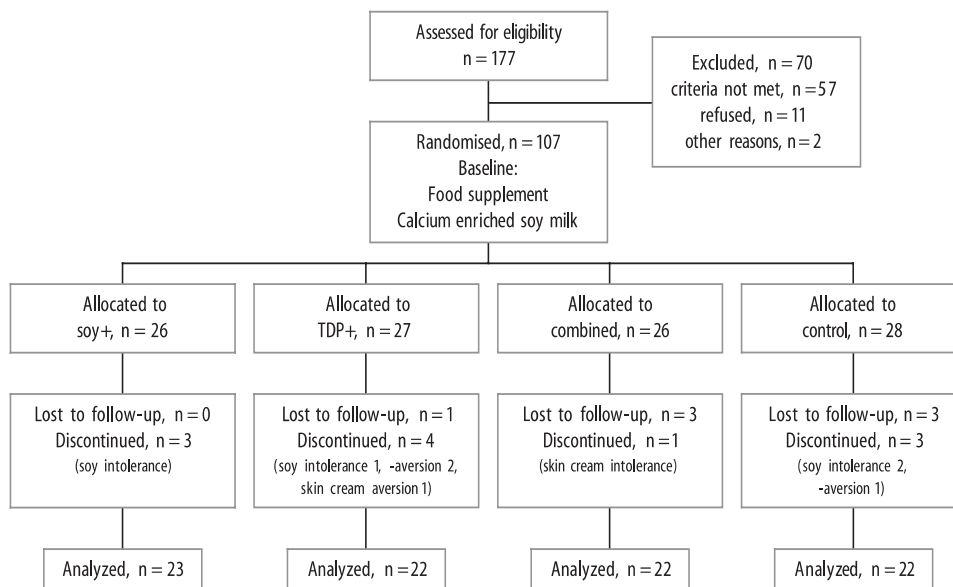
cone isoflavones are the unconjugated absorbable forms of the isoflavones.

The soymilk was produced in Belgium from selected isoflavone-rich soybeans (soy+) or alcohol washed soy concentrate providing only 1.0 mg isoflavones/day (soy-). The soymilk provided ~ 0.8 MJ (200 Kcal) and was shelf stable for 1 year. Subjects were instructed to incorporate the soymilk in their meals while cutting down on cow's milk products. Suggestions for using soymilk in different ways were provided in written form and 99% of subjects participated in group meetings and cooking classes every 3 months in the first year to ensure long-term compliance. The soymilk was enriched with calcium to a level of ~ 150 mg/100 ml and all subjects were provided a commercial non-prescription food supplement (Osforte®) containing 680 mg calcium (citrate and carbonate), 300 mg magnesium (aminochelate), 20 mg silicium (sodium metasilicate), 15 mg zinc (aminochelate), 6 mg manganese (aminochelate), 3 mg boron (proteinate), 2 mg copper (aminochelate), 200 mg Vit. C, 40 mg Pyridoxin, 200 iu Vit. D3 and 1 mg Vit. K1.

The total calcium-intake from diet, soymilk and food supplement was in the 1,500 mg-range.

Skin cream (either containing progesterone or not) was applied cyclically at ~ 30 g (1 oz)/3 week followed by a one week break, thus supplying 540 mg micronized progesterone/3 week cycle equivalent to 25.7 mg/d. Skin cream was applied to skin surfaces like the inner thigh, inner arms or face, according to manufacturer's instruction. Each batch of soymilk and skin cream was analyzed to ensure their contents of isoflavones and progesterone, respectively. Study flow is summarized in Fig. 1.

Fig. 1 Flow diagram



## ■ Data collection and measurements

Individual information on health, medical, reproductive and menopausal history, physical activity and lifestyle was obtained for all subjects by interview, aided by semi-quantitative questionnaires.

The questionnaire included information on bone health, family history and heritage, weight and dieting history, environmental exposures and general health.

Estrogen exposure (in years) was calculated for each subject by subtracting her age at menarche from her age at last menstrual cycle. Lifetime use of cigarettes was expressed as pack-years.

Subjects underwent a normal general physical examination with weight (in underwear) and height measured using a precision scale (Seca) to the nearest 0.1 kg and 0.5 cm respectively. Body mass index (BMI) was calculated as weight in kilograms divided by height in m<sup>2</sup> (kg/m<sup>2</sup>).

Nutrition and lifestyle history was used to elicit usual intake patterns of milk products, grains, fish, caffeine-containing drinks and food-items, alcohol, special diets, fats and oils as well as vitamin and mineral supplement use.

Seven-day weighed food records were obtained at baseline, one and two years, after thorough instruction and demonstration of the supplied electronic scale (Soehnle Domino, limits of 2000 g). Weights were controlled for correct measurement by weighing items of known weight in each round of food recording. Food recording covered all seven days of the week and subjects provided food-labels and recipes of mixed dishes to ensure correct coding of items.

Diet records are considered the best method for assessing usual dietary intake [27] and were used to assess typical mean intakes of energy, macronutrients, dietary fiber, alcohol, caffeine, vitamins, minerals and fatty acids at baseline and throughout the study. Food records were analyzed by 3<sup>rd</sup> year nutrition students and B.Sc in nutrition staff members, using DANKOST 3000 computerized nutrient database program (Danish Catering Center, Inc, Denmark) based on the national Danish composition of foods table (The Veterinary and Food Administration, fourth edition, 1996).

These analyses did not include vitamin and mineral supplements at baseline or those we provided the subjects with. Information on nutrient content of vitamin and mineral supplements was obtained from manufacturers and/or the VFA administration database and recorded for each subject. Total intakes reported are dietary plus supplemented levels.

Physical activity level was recorded using both lifetime and actual levels of work and leisure activity, according to The Nordic Nutrition Recommendation [28] – guidelines to a physical activity level (PAL) used for calculating an estimate of dietary energy need based on

basal metabolic rate (BMR) calculations with the appropriate age, weight and PAL for each subject (NRR 1996).

Bone mineral density was measured at baseline and at 2 years by dual x-ray absorptiometry (Norland xR 26 Mark II), and expressed as areal density (gHA/cm<sup>2</sup>) or content (g HA). The lumbar spine, L<sub>2</sub>-L<sub>4</sub> and the femoral neck were assessed by the same two trained research assistants. Equipment was calibrated each week with a phantom and the laboratory's long-term within-subject in vivo reproducibility CVs of lumbar spine BMD and BMC are 2.0 % and 1.8 %, respectively. The corresponding CVs for the hip are 3.5 % and 3.3 %. No attempt were made to drop subjects after their DEXA results was known as we also collected cross-sectional data on psychological profiles, pH measurements in the stomach and duodenum, heavy metal profiles in hair and homocysteine values.

Fasting blood samples were collected at baseline and 1 and 2 years to measure safety parameters, lipids, isoflavones and bone markers.

Safety parameters performed were routine hematological counts, serum-liver and -kidney biochemistry (ALAT, ASAT, alkaline phosphatase, calcium, potassium, sodium, uric acid, creatinine), thyroid stimulating hormone and se-cobalamin. All analyses were performed in a GLP-certified laboratory (Nova Medical Medi-Lab, Copenhagen, Denmark).

Serum and plasma for bone-marker and isoflavone analysis were stored frozen at -20°C for subsequent analysis. Bone-markers measured were type-I C-terminal telopeptide (ICTP) and type-I procollagen N-terminal peptide (PINP) – both with radioimmunoassay kits (Orion Diagnostica, Finland) according to the manufacturer's instruction. The intra-assay variability was 4.8 % for ICTP and 8.6 % for PINP and the corresponding inter-assay variability was 5.7 % and 5.1 %.

## Analysis of soymilk for isoflavones by HPLC

Isoflavones and their glycosidic conjugates were extracted from soymilk (5 mL) by refluxing for 1 hour in 80 % methanol (50 mL). After filtering the sample through Whatman No.1 filter paper, the aqueous methanolic phase was then accurately made up to 100 mL in a graduated volumetric. An internal standard, equilenin (60 µg), was added to three replicate 1.0 mL (1/100<sup>th</sup>) portions of this extract which was then taken to dryness under a stream of nitrogen, and resuspended in 10 mL of 0.5M sodium acetate buffer (pH 4.5). Isoflavone glycosides were hydrolyzed at 37°C overnight by addition of β-glucosidases present in *Helix pomatia* digestive juice (0.1 mL; Sigma Chemical Company, St. Louis, MO). Isoflavones, now converted to their aglycones, were then extracted by passage of the hydrolysate through a precharged C<sub>18</sub>-Bond Elut solid-phase cartridge and recovered by elution with methanol (5 mL).

The metanolic extract was then taken to dryness under a stream of nitrogen gas and resuspended in the HPLC mobile phase for analysis by HPLC (100  $\mu$ L). The conditions for separation of individual isoflavones have been described in detail previously [29]. The sample size injected was 10  $\mu$ L, flow rate 1.0 mL/min and the UV absorbance was monitored at 260 nm using a Waters 2487 UV detector. Separation of the individual isoflavones was accomplished on a 250  $\times$  4.6 mm ODS (C<sub>18</sub>) reversed phase HPLC column (Keystone Scientific, Bellefonte PA) and the isocratic mobile phase consisted of methanol:ammonium acetate (75/25, v/v). Quantification of total daidzein, genistein and glycitein as their respective aglycones was achieved by comparing the peak area responses given by these values against calibration standards of each isoflavone.

#### Determination of isoflavones in plasma and urine by gas chromatography-mass spectrometry

The concentration of daidzein, genistein and equol was measured by GC-MS using stable isotopically labeled internal standards. These internal standards were added to the plasma sample prior to its extraction and work-up. Total and individual isoflavones were determined after extraction and enzymatic hydrolysis of the conjugates with a combined sulfatase and glucuronidase enzyme preparation. The plasma (0.5 mL) was equilibrated with 50 ng of the internal standards [<sup>13</sup>C]daidzein, and [<sup>13</sup>C]genistein, and [<sup>13</sup>C]equol, diluted with 10 vol of 0.5 M triethylamine sulfate (pH 5.0) and heated to 64 °C before passage through a pre-wetted solid-phase C<sub>18</sub>-Bond Elut cartridge. The solid-phase cartridge was then washed with distilled water (10 mL) and isoflavones and their conjugates were recovered by elution with methanol (5 mL). The methanol extract was evaporated to dryness under nitrogen, reconstituted in 10 mL of 0.5 M acetate buffer (pH 4.5), and hydrolyzed at 37 °C overnight with a solution of 10,000 Fishman Units of a mixed  $\beta$ -glucuronidase/sulfatase (*Helix pomatia*, Sigma Chemicals Inc.) in 0.5 M sodium acetate buffer, pH 4.5, that had been previously filtered through a cartridge of C<sub>18</sub>-Bond Elut to remove naturally occurring isoflavones present in this enzyme preparation. After hydrolysis, isoflavones were isolated by solid-phase extraction on a C<sub>18</sub>-Bond Elut cartridge as described above. The sample was taken to dryness under a stream of nitrogen gas and isoflavones converted to their *tert*-butyldimethylsilyl (tBDMS) ether derivatives for analysis by GC-MS with selected ion monitoring. tBDMS ethers were prepared by addition of acetonitrile (100  $\mu$ L) and *N*-methyl-*N*-*t*-butyldimethylsilyltri-flouroacetamide in 1% *t*-butylmethylchlorosilane (100  $\mu$ L) and the sample was heated at 65 °C for 2 h. The reagents were removed by evaporation in a stream of nitrogen and the derivatives dissolved in hexane (100  $\mu$ L).

Isoflavone tBDMS ethers were separated and quantified by gas chromatography-mass spectrometry with selected ion monitoring. Chromatographic separation was achieved on a DB-1 fused silica capillary column (30 m  $\times$  0.25 mm i. d., 0.25  $\mu$  film thickness; j & W Scientific Inc., Folsom CA) using helium as the carrier gas (flow-rate approx. 2 mL/min) and with a temperature program from 260–310 °C with increments of 10 °C/min. Selected ion monitoring GC-MS of specific and characteristic ions in the electron ionization (70 eV) spectra of the tBDMS ether derivatives of each isoflavone permitted highly sensitive and specific quantification. The following ions were monitored: *m/z* 425 (daidzein), *m/z* 426 ([<sup>13</sup>C]daidzein), *m/z* 470 (equol), *m/z* 471 ([<sup>13</sup>C]equol), *m/z* 555 (genistein), and *m/z* 556 ([<sup>13</sup>C]genistein). The individual isoflavones were quantified by comparing the peak area in the specific ion channels at the correct retention time determined from authentic compounds, with the peak area response for the internal standard. This area ratio was then interpolated against calibration curves constructed for known amounts (0–200 ng) of the individual isoflavones. Concentrations were expressed as ng/mL or  $\mu$ mol/L for individual plasma isoflavones

#### Statistical analysis and randomization

Power calculations were based on an alpha-level of 0.05 and a beta-level (risk of type II error) of 15%, based on an expected overall difference between groups of 5%, equivalent to 0.5 SD with a detection limit of 1%. The overall power of 85% required approx. 22 subjects in each group and to allow for drop-outs the protocol called for 25 in each arm.

Statistical analysis was performed with PC SAS, version 8.2 (SAS Institute, Inc. Cary, NC, USA). Descriptive statistics include means for normally distributed data (i. e. age, BMI, energy intake, menopausal age, dietary energy, percents of protein, carbohydrates and fats), medians for data not normally distributed (i. e. calcium and vit D intake, exercise, ALAT, cobalamin) and frequencies (bone, reproductive and smoking history).

Analysis of variance (ANOVA) was used to determine differences between groups. To determine whether changes over the intervention period were different from zero, paired *t*-tests were performed. Changes in bone markers were analyzed using a multiple linear regression model with log transformation of not-normally distributed data. An alpha-level of 5% was used in all statistical tests. Randomization was done centrally, generated by a table of random numbers and subjects entered consecutively at recruitment. The randomization code was not broken until final statistical analysis had been performed.

## Results

### ■ Subjects

The mean age of these postmenopausal women was 58.2 y, with a mean time since menopause of 10.9 y. Based on lumbar spine T-scores at baseline, comparing the bone mass to that of normal, young women, 14 subjects were classified as being osteoporotic (T-score  $< \div 2.5$  SD, equivalent to a bone mass  $\sim 25\%$  below normal) and of these 13 completed the study. 58 were osteopenic (T-score between  $\div 2.49$  and  $\div 1.0$  SD) and of these 52 completed the study. 23 subjects had normal values (T-score between  $\div 1.0$  and  $+1.0$  SD) and 19 completed the study. Five had T-scores above 1.0 SD and all completed the study. 25 subjects reported of earlier, low-energy fractures with typical osteoporotic localization but only 7 were previously diagnosed by their physician as being osteoporotic. Three had tried bisphosphonate treatment years ago, but quit either due to side effects ( $n = 1$ ) or lack of effect ( $n = 2$ ). 25 had more than 2 years previously received HRT, usually during menopausal transition, but did not wish to continue.

Four of the women were taking medication for hypothyroidism at baseline (two in soy+ group, one each in the control and combined groups) but they were well controlled and euthyroid. Fourteen cases of cobalamin deficiency were found and treated at baseline ( $n = 12$ ) and at one year ( $n = 2$ ), respectively. Three of these were non-completers, four were in the TDP+, three in the soy+, two in the combined and one in the control groups, respectively. The treatment of cobalamin deficiency did not affect the outcomes in the trial. Four were treated for hypertension with diuretics at baseline (two in the control and one each in the combined and TDP+ groups) and this treatment was maintained during the study. Dietary and lifestyle assessment showed a mean energy intake of 7.5 MJ/d (SD 1.8) with protein, fat and carbohydrate energy percentages of 14.7%, 31.9% and 49.6% respectively, leaving 3.8% of the energy-intake to alcohol. Median alcohol intake was 3 servings per week (range 0–23). None of the groups differed from these overall values, nor did they change significantly over time.

Daily caffeine intake was 290.0 mg/d (SD 209.6). There was a small decrease of caffeine intake over time, to 258.4 mg/d representing a nonsignificant 11% difference over two years, with no significant group differences.

Physical activity levels were modest with median PAL at 1.5 (range 1.4–1.8) representing a generally sedentary lifestyle with limited recreational physical activity, although a subgroup bicycled to work and 1–2 subjects in each group had higher levels of leisure time exercise. Physical activity levels did not change over time.

There were 48.3% past smokers and 20.2% [ $n = 18$ ]

current smokers, reflecting the high prevalence of smoking among Danish women. Three of the smokers quit smoking for a period of less than 6 months during the study, but began smoking again; thus, the overall smoking status was the same in the end as at baseline.

During the study there was a non-significant weight gain of 1.4–1.9% of baseline weight (0.9–1.2 kg) in the soy+, combined and control groups, whereas the TDP+ group did not gain weight.

Additional baseline characteristics are shown in Table 1.

### ■ Compliance

Compliance was controlled by counting of left-over pills (food supplement), weighing of left-over skin cream and monthly diaries for the use of soymilk (liter/month) as well as measurements of plasma isoflavone levels.

Overall compliance with the intervention was in the range of 96.3–97.9% (SD 3.3–8.6) for the food supplement; 95.0–98.7% (SD 6.0–12.6) for the skin cream and 83.9–98.7% (SD 9.4–13.7) for the soymilk. There were no differences between the groups regarding compliance. Poor compliers, defined as reporting 25–75% compliance are included in the analysis and represent 2, 4, 3 and 3 subjects in the control, combined, TDP+ and soy+ groups, respectively.

Median plasma total isoflavone levels at baseline were 18.8 ng/ml (range 9.3–388.8 ng/ml) in the control group and 39.2 ng/ml (range 9.7–411.1 ng/ml) in the soy+ group due to the presence of 2 vs. 4 habitual soymilk users in the two groups ( $p = 0.03$ ). Total isoflavone level remained low in the control group, median 24.9 ng/ml (range 15.9–225.7 ng/ml) and increased seven to tenfold in the soy+ group to a median level of 281.9 ng/ml (range 63.0–1252.7 ng/ml),  $p = 0.0001$ , demonstrating good compliance.

### ■ Tolerance and side effects

Participants were requested to call if any undesired events occurred and intervention was stopped for a few days until complaints had cleared and then restarted one-by-one to clarify if the complaint was caused by the intervention. If so, the reaction was reproduced twice and a decision about cessation made. For the food supplement, 11% reported mild digestive upsets if taken on an empty stomach, but all tolerated the supplement if taken with food. One subject reduced the dosage due to loose stools.

For the skin cream 22% experienced mild side effects, not leading to cessation: skin irritation if used in face, but tolerated elsewhere (11%), breast tenderness of a few days' duration at the beginning of the study (9%),

**Table 1** Baseline characteristics of postmenopausal women, n = 89<sup>1</sup>

Measure/group	1. soy+ (n = 23)	2. TDP+ (n = 22)	3. Combined (n = 22)	4. Control (n = 22)
Age (y) <sup>2</sup>	57.8±8.4	59.4±7.0	59±7.4	56.3±6.7
Weight (kg) <sup>2</sup>	66.2±10.3	63.9±10.8	61.3±11.1	64.8±9.4
BMI (kg/m <sup>2</sup> ) <sup>2</sup>	24.0±4.2	24.4±3.9	23.3±4.2	23.7±3.5
Time since menopause (y) <sup>3</sup>	6.0 (1–29)	9.5 (1–26)	10.5 (1–30)	6.0 (1–26)
Diet and lifestyle:				
Percentage past smokers [n]	47.8 [11]	40.9 [9]	54.5 [12]	50 [11]
Percentage current smokers [n]	21.7 [5]	9.1 [2]	36 [8]	13.6 [3]
Mean pack-y, current smokers	27.3 (18–55)	18 (16–20)	13.6 (3–21.6)	20.4 (2–40)
Energy intake (MJ/d)	7.3±1.6	6.9±1.3	7.4±1.6	8.3±2.1
Total vit. D (µg) <sup>3,5</sup>	7.27 (2.75–17.83)	7.59 (4.20–20.88)	9.84 (1.53–31.15)	6.64 (0.88–25.11)
Exercise (h/week) <sup>3</sup>	2 (0–11.5)	1.9 (0–9)	2 (0–10)	2 (0–14)
Serum variables:				
TSH, mIU/l <sup>3</sup>	1.8 (0.63–7.1)	1.3 (0.38–6)	1.7 (0.33–5.8)	1.5 (0.63–12)
ALAT (U/l) <sup>3</sup>	23.0 (9–35)	21.5 (11–59)	27.5 (14–62)	28.0 (8–73)
ASAT (U/l) <sup>2</sup>	22.0±4.4	22.8±6.5	24.3±6.6	23.6±5.8
Alkaline Phosphatase <sup>3</sup>	151.0 (86–238)	180.0 (132–306)	179.0 (131–260)	185.5 (89–579)
se-creatinine (µmol/l) <sup>2</sup>	81.0±8.9	81.0±8.7	81.3±7.3	79.9±8.3

<sup>1</sup> There were no significant differences among the groups for these characteristics

<sup>2</sup> Values are mean ± SD

<sup>3</sup> Medians (range)

<sup>4</sup> Dietary intake calculated from 7d weighed record by Dankost 2000

<sup>5</sup> From diet and supplements

spotting of a few days' duration or a single menstrual bleeding (6%, evenly distributed among control and active groups) and mild hot flashes (3%). Two subjects stopped using the cream, one had severe local skin irritation, the other an aversion to the use, but no physical symptoms. For the soymilk, 29% experienced mild and temporary side effects, not leading to cessation: mild digestive trouble (nausea, bloating, flatulence 23%), undesired weight gain (fluid retention, 9%), mild throat irritation (4%), mild hot flashes (2%) and temporary joint pain (1%).

Six subjects (6%) had side effects leading to cessation: 2 subjects had severe digestive trouble with nausea, flatulence and diarrhea and 4 subjects had both digestive trouble and generalized symptoms of tiredness, fluid retention, feeling malaise, sleep disturbances, shortness of breath and joint pain. These reactions occurred 2–11 months into the study and cleared within days after stopping the soymilk intake. They were evenly distributed across the groups and cannot be attributed to the isoflavone level, but demonstrate intolerance to soy protein.

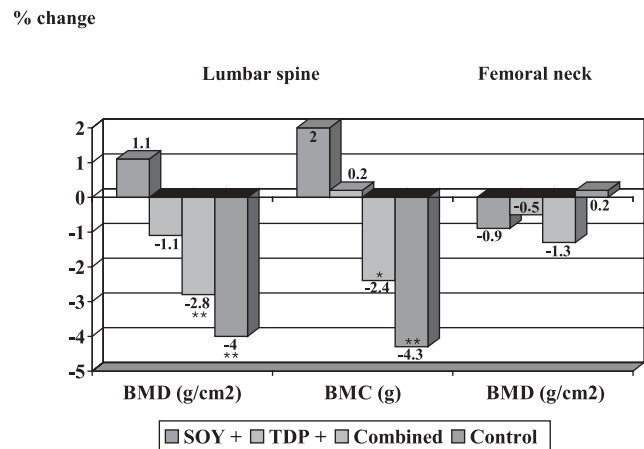
Hematological, liver and kidney parameters were normal and remained so throughout the study as well as TSH remained normal and unchanged with no group differences. We could not identify a vulnerable subgroup with respect to thyroid function, based on TSH screening, related to soy in this sample of women.

## ■ Bone measurement response to treatment

### Lumbar spine

Mean percentage change in lumbar spine BMD and BMC did not decline in the soy+ or the TDP+ group; however significant losses occurred in the combined group and the control group, shown in Fig. 2. Descriptive results for BMD and BMC are shown in Table 2.

Absolute values for bone measurements at baseline were not significantly different between the four inter-



**Fig. 2** Long-term effect of soymilk and progesterone on bone in postmenopausal women, n = 89, 2y, by DEXA. \* P = 0.05, \*\* P = 0.01

**Table 2** Lumbar spine bone mineral density BMD and content at baseline and post-treatment in postmenopausal women<sup>1</sup>

Measure/ treatment group	Baseline			Post treatment		
	Minimum	$\bar{X} \pm SD$	maximum	minimum	$\bar{X} \pm SD$	maximum
BMD (g/cm <sup>2</sup> )						
Control	0.528	0.865±0.190	1.380	0.404	0.835±0.212 <sup>a</sup>	1.416
Combined	0.614	0.821±0.153	1.205	0.627	0.796±0.138 <sup>b</sup>	1.101
TDP+	0.507	0.868±0.153	1.181	0.532	0.855±0.144	1.188
Soy+	0.478	0.925±0.260	1.475	0.500	0.933±0.265	1.504
BMC (g)						
Control	20.030	39.515±8.865	56.290	15.110	38.031±9.549 <sup>c</sup>	58.060
Combined	25.960	36.369±8.319	58.480	25.130	35.330±7.618 <sup>d</sup>	53.790
TDP+	21.780	37.657±7.100	54.000	23.200	37.569±6.622	52.760
Soy+	21.760	42.546±11.202	69.820	23.410	42.794±10.917	69.420

<sup>1</sup> Control isoflavone free soymilk n = 22

soy+ isoflavone rich soymilk group, n = 23; TDP+ transdermal progesterone cream group, n = 22; combined group combination of soy+ and TDP+, n = 22

<sup>a</sup> p = 0.006; <sup>b</sup> p = 0.003; <sup>c</sup> p = 0.005; <sup>d</sup> p = 0.03; all compared to baseline values

vention groups, but absolute BMD and BMC changed significantly in the control group (p = 0.006 for BMD and p = 0.005 for BMC) and in the combined group (p = 0.003 for BMD and p = 0.03 for BMC) and remained unchanged in the soy+ and TDP+ groups.

Results of ANOVA indicated that treatment had a significant effect on percentage change in BMD (p = 0.04) and BMC (p = 0.03).

Paired t-test for percentage change showed a significant loss in the control group (-4.2%, p = 0.01 for BMD and -4.3%, p = 0.01 for BMC) and in the combined group (-2.8%, p = 0.01 for BMD and -2.4%, p = 0.05 for BMC) whereas the soy+ and TDP+ groups remained unchanged at +1.1% and +2.0% in the soy+ group and -1.1% and +0.4% in the TDP+ group for BMD and BMC, respectively.

The difference between the groups was non-significant comparing control and combined groups and significantly different comparing control and soy+ (p = 0.009 for BMD and p = 0.006 for BMC). For control vs. TDP+ there was a significant difference for BMC percentage change (p = 0.03)

Response in relation to specific serum isoflavone levels showed that equol-producer status was associated with a better response, which did not reach statistical significance due to low numbers.

Subgroup analysis, using a cut off level of 10 ng/ml for plasma-equol divided the soy+ group into 10 Eq+ subjects and 12 Eq- subjects (one missing value) with mean Equol levels of 44.0 ng/ml (SD 25.20) and 3.2 ng/ml (SD 0.74) (p < 0.0001) and corresponding responses of +2.4% and +2.8% changes for BMD and BMC in the Eq+ group, compared to the Eq- group just maintaining bone mass at +0.6% and +0.3% respectively.

Due to the small numbers, results are not statistically significant but the result indicates that equol could be

the major isoflavone-metabolite responsible for a clinically important effect in bone.

Levels of the other isoflavones daidzein, genistein and total isoflavones did not differ significantly between the Eq+ and Eq- groups.

For TDP+ a subgroup of 4 women had bone gains ranging 4.3–12.9% for BMD indicating that there might exist a subgroup with special sensitivity to progesterone.

## Hip

There were no significant differences between groups for BMD and BMC measurements at the femoral neck, wards triangle or trochanter at baseline and no significant changes occurred with treatment. All four groups had minimal changes in femoral neck BMD with percentage changes 0.2% and -1.3% for control and combined groups; -0.9% and -0.5% for soy+ and TDP+ groups, indicating that a factor not differing between groups: Soy protein (regardless of isoflavone level) or the food supplement had a stabilizing effect.

## ■ Biochemical markers of bone formation

Values for serum PINP and ICTP were not normally distributed and were thus log-transformed for the statistical analysis. Baseline and posttreatment were not significantly different between the groups for PINP, ICTP or the PINP/ICTP ratio.

A multiple regression model was used to determine the effect of the bone markers on the bone measurement outcome in relation to treatment and known explanatory variables for bone status such as age, BMI, estrogen exposure time and baseline bone values.

There was a non-significant percentage change in the formation marker, PINP, in the two groups that lost bone

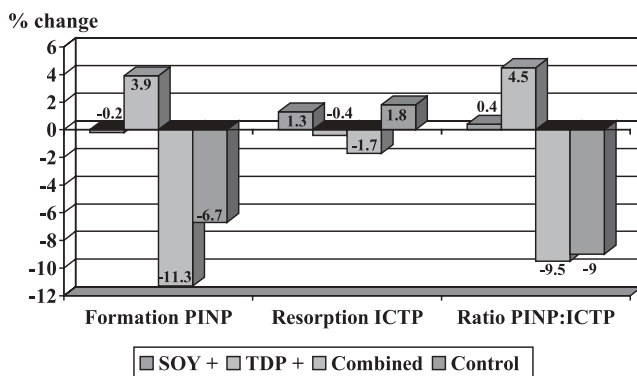


-6.7% (control) and -11.3% (combined), whereas the two groups that maintained bone had changes of +3.9% (TDP+) and -0.2% (soy+), see Fig. 3. The same trend was seen for the PINP/ICTP ratio with  $\pm 9.0\%$  and  $\pm 9.5\%$  decreases from baseline in the control and combined groups and +4.5% and +0.4% increases in the TDP+ and soy+ groups. Only minimal changes were seen in ICTP ( $\pm 1.8\%$ ) across the groups.

## Discussion

In this study we examined the hypothesized bone-sparing effect of isoflavone-rich soymilk, transdermal progesterone, or the combination on bone mineral density (BMD) and bone mineral content (BMC) of the lumbar spine and hip in postmenopausal women. Biochemical markers of bone formation, serum-type I procollagen-N-terminal-peptide (PINP), and bone resorption, serum type I C-terminal telopeptide (ICTP), were also measured.

This is the first study designed specifically to examine bone effects of soy and progesterone in postmenopausal women over several bone-remodelling cycles, showing a positive effect of soy and its isoflavones or progesterone on bone mass. Bone remodelling is the process by which bone is deposited, resorbed and formed through controlled functions of osteoblasts, osteoclasts and their associated activation factors and cofactors. A full, normal cycle is approx. 180 days but the effect of intervention in one cycle is small; thus, for testing clinically relevant changes, studies of at least 2 y duration are warranted. Results indicated that soymilk with isoflavones prevented bone loss in the lumbar spine, whereas the control group had a significant loss. Progesterone slowed bone loss and combined treatment had a negative interaction resulting in a greater bone loss than either treatment alone, although not as pronounced as placebo.



**Fig. 3** Bone marker changes over 2 years, % change from baseline (PINP procollagen type I, N-terminal polypeptide; ICTP collagen type I, C-terminal-telopeptide)

Isoflavones found predominantly in soy products are structurally and functionally estrogen-like substances similar to 17-beta estradiol. Isoflavones bind weakly to the estrogen-receptor-alpha (ER-alpha) [6, 7] dominant in uterine and breast-tissue and both genistein and equol, but not daidzein, exerts a strong binding to the estrogen-receptor-beta (ER-beta) dominant in bone [7]. Animal experiments and short-term studies indicated that soy has a positive effect on bone, both in peri- and postmenopausal women; findings that are in agreement with recent epidemiological studies. Animal studies have shown that soymilk-based diets increased calcium absorption in rats [30], and isoflavones in soy protein isolate were shown to prevent femoral [31, 32] and vertebral [32] bone loss in rats. Furthermore, soy protein isolate increased bone formation by stimulating insulin-like growth factor 1 messenger RNA synthesis in rats [33] or moderately increasing bone turnover in favor of bone formation [31-33]. In an ovariectomy-induced bone loss rat model it has been demonstrated that daidzein and estradiol were equally effective and better than genistein for prevention of bone loss [34], bearing in mind that all rats are equol producers. Mechanisms for genistein effects on bone are suggested to involve an increase in osteoprotegerin mRNA expression from osteoblasts, which in turn neutralizes the receptor-activator for NF-kB needed for osteoclast formation and bone resorption [35]. Likewise it has been shown in a rodent model that genistein exhibits estrogenic actions in bone and bone-marrow, but not in the uterus [36]. Similar studies have not been performed for daidzein or equol.

In humans three short-term human studies of 6-9 months duration have examined the effect of soy protein (40 g/d with 80-90 mg/d of isoflavones) on bone mineral density, two of them showing a bone-sparing effect in the lumbar spine [14, 15] in a post- and a perimenopausal group of women, respectively. Both used casein as the control product.

The study by Gallagher using soy as the control [20] did not show any effect in any measuring site, whereas the study by Clifton-Bligh (2001) using a cover-derived isoflavone preparation showed a significant increase in BMD in cortical bone (radius and ulna) with dosages ranging 57-85.5 mg/d, but not 28.5 mg/d [16]. The most recent study compared HRT with pure genistein 54 mg/d or placebo, for one year and demonstrated similar protective effects from genistein and HRT on BMD at lumbar spine, femoral neck and wards triangle with increases of 3 and 3.6% for genistein, 3.8 and 2.4% for HRT and -1.6 and -0.65% for placebo in the lumbar spine and femoral neck, respectively [17]. This is the first study done with an isolated isoflavone supplement, additionally demonstrating both reduced excretion of resorption markers (PYR and DPYR) as well as increased serum levels of bone-specific ALP and osteocalcin. In the HRT group findings were similar to genistein for the

resorption markers, but formation markers did not increase.

A variety of factors determine the presence and activity of the so far little known gut flora bacteria responsible for the conversion of daidzein to equol: intestinal flora acquired from childhood, dietary factors and antibiotic history and use, being the two most likely contributors. Due to the small numbers, results are not statistically significant but indicate that equol could be the major isoflavone metabolite responsible for a clinically important effect in bone.

We could, however, not identify any specific dietary compounds or patterns related to equol production, but Rowland found that equol-producers consumed less fat and more carbohydrate as percentage of energy ( $26 \pm 2.3\%$  vs.  $35 \pm 1.6\%$  for fat and  $55 \pm 2.9\%$  vs.  $47 \pm 1.7\%$  for carbohydrate) compared to non-equol producers [38] and Lampe likewise showed that equol producers consumed more carbohydrate, fiber and plant protein than non-equol producers [39].

Compliance of our subjects was excellent as reflected by isoflavone levels in blood and self-reported intake of soymilk. A limitation of the study was relatively small group sizes, large enough to show an overall effect, but too small to have enough statistical power for the subgroup analysis. Keeping the compliance level high in larger studies is an educational task whose importance should not be neglected.

The other 2y study by Vitolins examined whole body bone-mineral density with soy and two different isoflavone levels. There was no difference between the groups – all 3 groups lost less than 1% over 2 years indicating that soy protein itself has a calcium sparing effect, which is likely to be measured best in the total skeleton or the hip where cortical bone is dominant [21]. Earlier studies have found that women eating a vegetarian diet with a sufficient calcium intake (lacto-ovo vegetarians, eating a mixture of plant and cow's milk/egg protein) had the same bone mass as omnivores up to age 50, and that the omnivores at age 80 had lost 35% cortical bone, compared to the vegetarians losing only 18% [40, 41]. The mechanism for this finding was proposed to be the lower amount of sulphur-containing amino acids in plant proteins, tipping the acid-alkaline balance towards the alkaline side, thus sparing calcium loss [41]. One study found higher mid-radius BMC in vegetarians age 60–98 y [42], whereas others have found similar bone densities in caucasian vegetarians and omnivores, both pre- and postmenopausal [43, 44].

The role of progesterone in preventing bone loss was proposed by Prior [22, 23] who observed that female athletes developed spinal bone loss coincident with anovulatory cycles that had normal estrogen levels, but progesterone deficiency [22]. She suggested that prevention of osteoporosis begins with detection and treatment of amenorrhea and ovulation disorders [24]. Pre-

vious data on progesterone are few and conflicting as only a case series and one 1-year study has been published [25, 26].

For progesterone the difference between our study and the study by Leonetti showing no effect on bone is that our subjects had lower bone mass at baseline, that the progesterone was given cyclically with a one-week break every month, thus mimicking a natural cycle better, possibly maintaining receptor sensitivity, as well as this being a two-year vs. a one-year study [26].

Our results indicate that progesterone can have a role in the prevention of osteoporosis, but further studies are needed to clarify absorption kinetics from the transdermal preparations, dose-response relationships and to clarify if there is a subgroup with special responsiveness to progesterone, or if it is the combination of two natural hormones – Premarin and progesterone given together, that was responsible for the bone gains reported by Lee, rather than progesterone per se [25].

The negative interaction between soy+ and progesterone exists and may partly be influenced by the fact that the combined group had the largest proportion of smokers and the lowest body weight, although not significantly different from the other groups. The negative interaction persists after exclusion of the smokers and indicates that the two interventions should not be mixed. The negative interaction was similar in equol and non-equol producers. No previous studies on this issue exists.

Studies in pre-menopausal women have shown a ~45% decrease in total-cycle progesterone levels during a 1 l/d soymilk diet [46], whereas in post-menopausal women endogenous estrogens, thyroid, pancreatic and adrenal hormones have been studied, but not progesterone [47].

The negative interaction cannot fully be explained by existing studies; it could be the progesterone acting as an antiestrogen on the isoflavones or the opposite – the isoflavones lowering the circulating levels of the supplemented progesterone.

The minimal changes observed in the bone resorption marker, ICTP, across the groups suggests that soy protein may carry an antiresorptive effect, whereas the isoflavones and progesterone maintaining levels of the bone formation marker, PINP, suggest a different mechanism of action than conventional antiresorptive medications.

In summary the results of this study suggest that progesterone can spare bone loss and that soy foods with isoflavones can prevent bone loss of the lumbar spine in postmenopausal women, who may otherwise be expected to lose 1.5–3% of bone/y. This prevention of bone loss, particularly if continued into old age, could translate into a decrease in lifetime risk of osteoporosis and a lowering of fracture rates. Further and much larger scale studies are needed to address this issue, as

well as to clarify the possible calcium sparing effect of soy protein regardless of isoflavone level on [cortical] bone – especially in relation to cows milk.

In the planning of future studies it is important to examine the metabolism of the isoflavones, especially regarding equol status and antibiotic treatment history/current use, as we are apparently dealing with two distinct subgroups, where different clinical responses are to be expected.

Thus, data suggest that the inclusion of soy foods at a level providing at least 15 g protein/d could have a general sparing effect of postmenopausal calcium loss with the largest benefit expected to be in cortical bone, like the whole skeleton and hip. Choosing isoflavone-rich soy food varieties with isoflavones in the 50–90 mg range (aglycone equivalent) further adds to this by providing an endocrine effect, with the benefit targeted at the more endocrine-sensitive trabecular bone in the spine. This is in principle important on a general population level and specially important for women who are at high risk, are poor candidates for HRT, choose not to receive it, or live in areas of the world where access to pharmaceutical prevention is scarce or not generally available due to socio-economic factors. Further and larger studies are needed to address the long-term effects and possible side-effects of soy foods and their isoflavones, especially the risk of breast and uterine cancer. Further studies regarding gut flora metabolism (“bacterio-typing”) with respect to equol-producer status as well as fracture studies are now needed.

Our findings indicate that isoflavone-rich soy foods or progesterone prevent bone loss in the lumbar spine and offer a natural alternative, or adjuvant intervention, during menopause and in older age for women who are poor candidates for traditional HRT. The relatively simple inclusion of soy foods offers a cheap and non-pharmacological intervention for prevention of osteoporosis.

■ **Acknowledgements** We want to express our gratitude first of all to the study participants, who maintained compliance and commitment at a high level throughout the study. Also special thanks to the nutrition students and staff who undertook the diet-analysis and coding: Mette Munkholm, Jeanette Joergensen, Karna Riis Larsen and Louise Fisker as well as the research assistants conducting the bone scans: Ulla Falktoft and Vibeke Hellesen and Mia Damhus, who undertook randomization. Soy milk and placebo were provided by ALPRO, Belgium, Proderma® and placebo by Phillips Nutritionals, Nevada, USA and Osforte® by Genese A/S, Denmark. The study was supported by grants from The Danish Ministry of Health, Fondation Idella, Glunz and Jensen Foundation, Civilingenieur Frode V. Nyegaard and Wife's Foundation, Director E. Danielsen and Wife Foundation and IMK Foundation. The sponsors and the suppliers of the study had no role in the study design, data collection, data analysis, data interpretation or writing the report.

#### Contributors

Eva Lydeking-Olsen wrote the protocol and report with contributions from KDR Setchell and J-E Beck Jensen. E Lydeking-Olsen and T Holm-Jensen investigated subjects, J-E Beck Jensen's group conducted bone scans and bone marker analysis. E Lydeking-Olsen and J-E Beck Jensen performed statistical analyses. KDR Setchell analyzed isoflavone levels.

## References

- Scharbo-Dehaan M (1996) Hormone replacement therapy. *Nurse Pract* 21: 1–13
- Groeneveld FP, Bareman FP, Barentsen R, Doktor HJ, Drogendijk AC, Hoes AW (1994) Determinants of first prescription of hormone replacement therapy. A follow-up study among 1689 women aged 45–60 years. *Maturitas* 20:81–89
- de Lignieres B (1996) Hormone replacement therapy: Clinical benefits and side-effects. *Maturitas* 23(suppl): S31–S36
- Writing group for the Women's Health Initiative Investigators (2002) Risk and benefits of estrogen plus progestin in healthy postmenopausal women. *JAMA* 288:321–333
- Grady D, Harrington D, Bittner V, Blumenthal R, Davidson M, Hlatky M, et al. (2002) Cardiovascular disease outcomes during 6.8 years of hormone therapy. *JAMA* 288:49–57
- Kuiper GG, Enmark E, Peltö-Huikko M, Nilsson S, Gustafsson JA (1996) Cloning of a novel receptor expressed in rat prostate and ovary. *Proc Natl Acad Sci* 93:5925–5930
- Kuiper GG, Carlsson B, Grandien K, Enmark E, Haggblad J, Nilsson S, Gustafsson JA (1997) Comparison of the ligand binding specificity and transcript tissue distribution of estrogen receptors alpha and beta. *Endocrinology* 138: 863–870
- Williams JP, Jordan SE, Barnes S, Blair HC (1998) Tyrosine kinase inhibitor effects on avian osteoclastic acid transport. *Am J Clin Nutr* 68:1369S–1374S
- Rassi CM, Lieberherr M, Chaumaz G, Pointillart A, Cournat G (2001) Daidzein inhibits osteoclast differentiation and activity in vitro. *Ann Nutr Metab* 45:230 (Abstract)
- Cho Y, Chung S, Cho K, et al. (2002) Black bean (*Rhynchosia molubilis*, Yak-Kong) exerts a prominent estrogenic effect on the proliferation of human MG-63 osteoblastic cells. *J Nutr* 132:614S
- Chen X, Anderson JJB (2002) Effects of isoflavones on osteoblast proliferation and differentiation. *J Nutr* 132(3):616S (Abstract)
- Setchell KDR, Lydeking-Olsen E (2003) Dietary phytoestrogens and their impact on bone-evidence from in vitro and in vivo, human observational and dietary intervention studies. *Am J Clin Nutr* 78(suppl):593S–609S
- Scheiber MD, Liu JH, Subbiah MT, Rebar RW, Setchell KD (2001) Dietary inclusion of whole soy foods results in significant reductions in clinical risk factors for osteoporosis and cardiovascular disease in normal postmenopausal women. *Menopause* 8(5): 384–392
- Potter SM, Baum JA, Teng H, Stillman RJ, Shay NF, Erdman JW Jr (1998) Soy protein and isoflavones: Their effect on blood lipids and bone density in postmenopausal women. *Am J Clin Nutr* 68(suppl):1375S–1379S

15. Alekel DL, St Germain A, Peterson CT, Hanson KB, Stewart JW, Toshiya T (2000) Isoflavone rich soy protein isolate attenuates bone loss in the lumbar spine of perimenopausal women. *Am J Clin Nutr* 72:844–852
16. Clifton-Bligh PB, Baber RJ, Fulcher GR, Nery ML, Moreton T (2001) The effect of isoflavones extracted from red clover (Rimostil) on lipid and bone metabolism. *Menopause* 8:S259–S265
17. Morabitu N, Crisafulla A, Vergara C, Gaudio A, Lasco MA, Frisina N, D'Anna R, Corrado F, Pizzoleo MA, Cincotta M, Altavilla D, Ientile R, Squadrito F (2002) Effects of genistein and hormone-replacement therapy on bone loss in early postmenopausal women: A randomized double-blind placebo-controlled study. *J Bone Miner Res* 17(10):1904–1912
18. Somekawa Y, Chiguchi M, Ishibashi T, Aso T (2001) Soy Intake Related to Menopausal Symptoms, Serum Lipids, and Bone Mineral Density in Postmenopausal Japanese Women. *Obstet Gynecol* 97(1):109–115
19. Mei J, Yeung SS, Kung AW (2001) High dietary phytoestrogen intake is associated with higher bone mineral density in postmenopausal but not premenopausal women. *J Clin Endocrinol Metab* 86(11):5217–5221
20. Gallagher JC, Rafferty K, Haynatzka V, Wilson M (2000) The effect of soy protein on bone metabolism. *J Nutr* 130:667S (abstr)
21. Vitolins M, Anthony M, Lenschik L, Bland DR, Burke GL (2002) Does Soy Protein and Its Isoflavones Prevent Bone Loss in Peri- and Postmenopausal Women? Results of a Two-Year Randomized Clinical Trial. *J Nutr* 582S (abstr)
22. Prior JC, Vigna YM, Schechter MT, Burgess AE (1990) Spinal bone loss and ovulatory disturbances. *N Engl J Med* 323(18):S1221–S1227
23. Prior JC (1990) Progesterone as a Bone-Trophic Hormone. *Endocrine Rev* 11(2):S386–S398
24. Prior JC, Vigna YM, Alojado N (1991) Progesterone and the prevention of osteoporosis. *Can J Ob/Gyn & Women's Health Care* 3(4):S178–S184
25. Lee JR (1991) Is Natural Progesterone the Missing Link in Osteoporosis Prevention and Treatment? *Medical Hypotheses* 35:314–316
26. Leonetti HB, Longo S, Anasti JN (1999) Transdermal Progesterone Cream for Vasomotor Symptoms and Postmenopausal Bone loss. *Obstet Gynec* 94(2):225–228
27. Potosky AL, Block G, Hartman AM (1990) The apparent validity of diet questionnaires is influenced by number of diet-record days used for comparison. *J Am Diet Assoc* 90:810–813
28. Nordisk Ministerråd (1996) Nordiska näringsrekommendationer. Nord 28, Copenhagen
29. Setchell KD, Welsh MB, Lim CK (1987) High-performance liquid chromatographic analysis of phytoestrogens in soy protein preparations with ultraviolet, electrochemical and thermospray mass spectrometric detection. *J Chromatogr* 386:315–323
30. Omi N, Aoi S, Murata K, Ezawa I (1994) Evaluation of the effect of soybean milk peptide on bone metabolism in the rat model with ovariectomized osteoporosis. *J Nutr Sci Vitaminol (Tokyo)* 40:201–211
31. Arjmandi BH, Birnbaum R, Goyal NV, et al. (1998) Bonesparing effect of soy protein in ovarian hormone-deficient rats is related to its isoflavone content. *Am J Clin Nutr* 68:364S–368S
32. Arjmandi BH, Alekel L, Hollis BW, et al. (1996) Dietary soybean protein prevents bone loss in an ovariectomized rat model of osteoporosis. *J Nutr* 126:161–167
33. Arjmandi BH, Getlinger MJ, Goyal NV, et al. (1998) Role of soy protein with normal or reduced isoflavone content in reversing bone loss induced by ovarian deficiency in rats. *Am J Clin Nutr* 68(suppl):1358S–1363S
34. Picherit C, Coxam V, Bennetau-Peissero C, Kati-Coulibaly S, Davicco MJ, Lebecque P, Barlet JP (2000) Daidzein is more efficient than genistein in preventing ovariectomy-induced bone loss in rats. *J Nutr* 130(7):1675–1681
35. Viereck V, Grundker C, Blaschke S, Siggelkow H, Emons G, Hofbauer LC (2002) Phytoestrogen genistein stimulates the production of osteoprotegerin by human trabecular osteoblasts. *J Cell Biol* 84(4):725–735
36. Ishimi Y, Miyaura C, Ohmura M, Onoe Y, Sato T, Uchiyama Y, Ito M, Wang X, Suda T, Ikegami S (1999) Selective effects of genistein, a soybean isoflavone, on B-lymphopoiesis and bone loss caused by estrogen deficiency. *Endocrinology* 140(4):1893–1900
37. Setchell KDR, Brown N, Lydeking-Olsen E (2002) Equol – the forgotten isoflavone may be the clue to the clinical effectiveness of soy and its isoflavones. *J Nutr* 132(12):3577–3584
38. Rowland IR, Wiseman H, Sanders TA, Adlercreutz H, Bowey EA (2000) Interindividual variation in metabolism of soy isoflavones and lignans: influence of habitual diet on equol production by the gut flora. *J Nutr* 36:27–32
39. Lampe JW, Karr SC, Hutchins AM, Slavin JL (1998) Urinary equol excretion with a soy challenge: influence of habitual diet. *Proc Soc Exp Biol Med* 217:335–339
40. Marsh AG, Sanchez TV, Midkelsen O, Keiser J, Mayor G (1980) Cortical bone density of adult lacto-ovo-vegetarian and omnivorous women. *J Am Diet Assoc* 76(2):148–151
41. Marsh AG, Sanchez TV, Michelsen O, Chaffee FL, Fagal SM (1988) Vegetarian lifestyle and bone mineral density. *Am J Clin Nutr* 48(3 suppl):837–841
42. Tylavsky FA, Anderson JJ (1988) Dietary factors in bone health of elderly lactoovovegetarian and omnivorous women. *Am J Clin Nutr* 48(3S):842–849
43. Tesar R, Notelovitz M, Shim E, Kauwell G, Brown J (1992) Axial and peripheral bone density and nutrient intakes of postmenopausal vegetarian and omnivorous women. *Am J Clin Nutr* 56(4):699–704
44. Lloyd T, Schaeffer JM, Walker MA, Demers LM (1991) Urinary hormonal concentrations and spinal bone densities of premenopausal vegetarian and nonvegetarian women. *Am J Clin Nutr* 54(6):1005–1010
45. Spence LA, Lipscomb ER, Cadogan J, Martin BR, Peacock M, Weaver CM (2002) Effect of soy isoflavones on calcium metabolism in postmenopausal women. *J Nutr* 581S (abstr)
46. Lu L-JW, Anderson KE, Grady JE, Kohen F, Nagamani M (2000) Decreased Ovarian Hormones during a Soya Diet: Implication for Breast Cancer Prevention. *Cancer Research* 60(8):4112–4121
47. Persky VW, Turyk ME, Wang L, Freels S, Chatterton Jr R, Barnes S, Erdman Jr J, Sepkovic DW, Bradlow HL, Potter S (2002) Effect of soy protein on endogenous hormones in postmenopausal women. *Am J Clin Nutr* 75:145–153