Breast Safety and Efficacy of Genistein Aglycone for Postmenopausal Bone Loss: A Follow-Up Study

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Context: Genistein aglycone improves bone metabolism in women. However, questions about the long-term safety of genistein on breast as well as its continued efficacy still remain.

Objective: We assessed the continued safety profile of genistein aglycone on breast and endometrium and its effects on bone after 3 yr of therapy.

Design: The parent study was a randomized, double-blind, placebo-controlled trial involving 389 osteopenic, postmenopausal women for 24-months. Subsequently, a subcohort (138 patients) continued therapy for an additional year.

Patients and Interventions: Participants received 54 mg of genistein aglycone daily (n = 71) or placebo (n = 67). Both treatment arms received calcium and vitamin D_3 in therapeutic doses.

Main Outcomes: Mammographic density was assessed at baseline, 24 and 36 months by visual classification scale and digitized quantification. BRCA1 and BRCA2, sister chromatid exchange, and endometrial thickness were also evaluated. Lumbar spine and femoral neck bone mineral density were also assessed. Secondary outcomes were biochemical levels of bone markers.

Results: After 36 months, genistein did not significantly change mammographic breast density or endometrial thickness, BRCA1 and BRCA2 expression was preserved, whereas sister chromatid exchange was reduced compared with placebo. Bone mineral density increases were greater with genistein for both femoral neck and lumbar spine compared to placebo. Genistein also significantly reduced pyridinoline, as well as serum carboxy-terminal cross-linking telopeptide and soluble receptor activator of NF-κB ligand while increasing bone-specific alkaline phosphatase, IGF-I, and osteoprotegerin levels. There were no differences in discomfort or adverse events between groups.

Conclusions: After 3 yr of treatment, genistein exhibited a promising safety profile with positive effects on bone formation in a cohort of osteopenic, postmenopausal women. **(*J Clin Endocrinol Metab* 93: 4787–4796, 2008)**

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Abbreviations: B-ALP, Bone-specific alkaline phosphatase; BMD, bone mineral density; CI, confidence interval; CTX, carboxy-terminal cross-linking telopeptide; DPYR, deoxypyridinoline; ER, estrogen receptor; HFC, high frequency cell; IMI, image mean index; PYR, pyridinoline; ROI, region of interest; sRANKL, soluble receptor activator of NF-κB ligand.

A variety of plant compounds may counteract the menopausal loss of estrogen and mitigate some symptoms associated with menopause due to specific interactions with estrogen receptors (1–4).

Asiatic women with high intake of isoflavones from soy products have been shown to have decreased risk of osteoporosis, cardiovascular disease, breast and uterine cancer, and climacteric symptoms (5, 6). These epidemiological data do not necessarily clarify the real impact of isoflavones on the physiological events surrounding menopause. Although frequent reports demonstrate positive influences of isoflavones on human health, the question remains as to whether these compounds share some of the adverse effects of classical hormone replacement therapy including an increased incidence of breast and endometrial cancer (7–9). In particular, there is limited knowledge of the safety and efficacy of relatively pure isoflavone preparations, such as genistein aglycone, when administered to a significant number of postmenopausal women.

Genistein aglycone is an isoflavone found in low concentrations in soybeans and in elevated amounts in certain soy foods, whereas genistin, the glucoside form of genistein, is much more abundant in unprocessed soybeans and other soy products. As a natural selective estrogen receptor (ER) modulator (10), genistein aglycone was recently shown to regulate bone metabolism positively, significantly increasing femoral neck and lumbar spine bone mineral density (BMD), without the harmful estrogenic activity in the uterus over a 2-yr period (11, 12). The aim of this study was to evaluate, in a subcohort of osteopenic, postmenopausal women (n = 138) remaining from the original study (11), the continued safety profile of genistein aglycone (54 mg/d) on breast and uterine tissue and its effects on bone metabolism after 3 yr of therapy.

Subjects and Methods

Clinical trial design

The protocol followed the principles of the Declaration of Helsinki and was conducted according to good clinical practice and under local institutional review board oversight. Study subjects gave a further written informed consent and were recruited among those participating in the 2-yr study (11). The initial study population consisted of 389 women. Of these, 304 subjects (placebo group, n = 154; genistein group, n = 150) completed the 2-yr program, and 138 subjects chose to continue the study through 3 yr (placebo group, n = 67; genistein group, n = 71; Fig. 1).

Participants and interventions

All characteristics of the study subjects were previously reported (11). Specifically, we inserted in the exclusion criteria the treatment with any drug that could affect the skeleton in the preceding year and the use of oral or transdermal estrogen, progestin, androgen, or other steroids, cholesterol-lowering therapy, or cardiovascular medications (including antihypertensive drugs) in the preceding 6 months before the beginning of the trial. The subjects remaining in the 3-yr extension of the original study (11) for both arms were not rerandomized. The parent study demonstrated that isoflavone intake was typical of a Western diet (13). The diet (11) was continued, and dietary intake and body mass index were also assessed during the third year extension.

The purity of genistein was greater than 98%. Placebo and genistein tablets appeared exteriorly similar and had a similar taste. The tablets contained the following other components: calcium carbonate (500 mg) and vitamin D_3 (400 IU) (11).

Variables and statistical analysis

It was determined previously that to obtain 80% power using two-sided tests with an expected difference between the two groups of at least 20% with α of 5%, the study sample had to have at least 97 subjects in each group (11). All primary as well as secondary variables were tested for normality using the Kolmogorov-Smirnov test as previously reported (11), and, as a result, no transformation was indicated for any variable and all analyses were conducted on raw scores.

Data are given as means ± SD, and 95% confidence intervals (CIs) are given where appropriate. The significance of difference was assessed by a two-way repeated measures ANOVA followed by *post hoc* analyses where indicated. A P value of 0.05 or less was considered statistically significant, and 95% CIs were provided wherever possible. Statistical analysis was performed using SPSS for Windows release 6.0 (SPSS Inc., Chicago, IL), and SAS version 9.1 (SAS Institute, Inc., Cary, NC).

Safety parameters

Mammographic breast density

Mammograms (Mammo Diagnost Uc Philips, Best, The Netherlands) were obtained at baseline and after 2 and 3 yr of treatment to determine breast density and to evaluate any abnormalities. Mammography examinations for each patient comprised mediolateral oblique and craniocaudal views of both breasts. Only the mediolateral oblique view of the left breast was used for the visual classifications of breast density. Previous studies have shown very little difference between the left and right breast in the response to treatment (14). All mammograms were assessed and discussed by two independent radiologists who were blinded to treatments. Finally, all mammograms were read in progression and then compared between them.

Visual classification

Mammographic density of all coded films was classified according to the Wolfe classification (15) in four categories: N1, essentially normal breast with a parenchyma composed primarily of fat and with, at most, a few fibrous connective tissue strands; P1, prominent ductal pattern in up to one fourth of the breast volume; P2, prominent ductal pattern in more than one fourth of the breast volume; and DY, extremely dense parenchyma, which usually denotes connective tissue hyperplasia.

Digitized assessment of breast density

A computer-based quantitative assessment and classification were performed. The identifying data were removed from the films, and the operator was unaware of the patients' identities and duration of treatment.

The mammographic images on traditional support (radiography) were acquired by using the film scanner VIDAR VXR-12 with linear look-up table and elaborated with the commercial software Image Pro-Plus (Media Cybernetics Inc., Gleichen, Germany), using *ad hoc* software.

For every investigated subject, two series were created, one for the right and one for the left breast, based on the baseline and the control images after 2 and 3 yr, respectively. For each image, the same procedure was followed, consisting of the identification and delimitation of the same region of interest (ROI) for each image; the differentiation of gray levels into 10 classes; the evaluation of the extension area for each class; the normalization of the ROI *vs.* the isoareas; the setting up of the histogram representing the distribution of calculated intensities of gray levels for each isoarea; and the determination of image mean index (IMI), in accordance with the following algorithm: $IMI = \Sigma_i\, a_i\, p_i/A$ where a_i represents the calculated value of the area of the single class; p_i represents the weight assigned to that class; and A represents the sum of all isoareas of the ROI. The evaluation of IMI gave one value, expressed in arbitrary units, for each series of images at different time points (baseline, 24 and

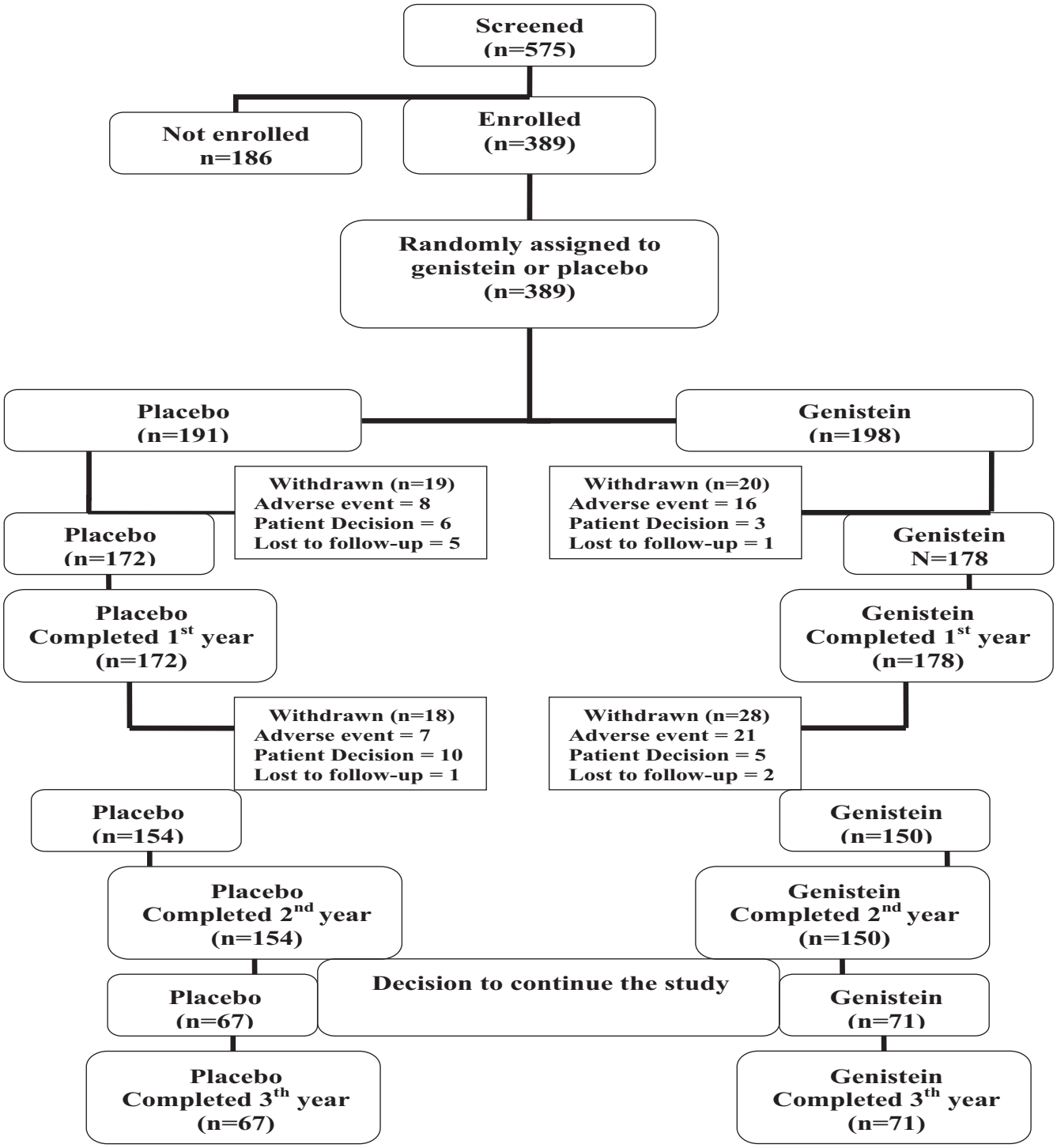


FIG. 1. Flow chart describing the progress of the participants during parent study in the third year extension of that trial (11).

36 months). Two-way ANOVA with *post hoc* comparisons were carried out to assess the main effect of treatment, time, and treatment × time interaction; $P < 0.05$ was considered to be statistically significant. The study had 80% power to detect a 1% change in IMI at a significance level of 5%.

BRCA1 and BRCA2 mRNA levels

Breast cancer type 1 susceptibility (BRCA1) and breast cancer type 2 susceptibility (BRCA2) mRNA were assayed as previously described (16). Briefly, total RNA was extracted from whole blood and reverse transcribed to cDNA, and the amount of BRCA1 and BRCA2 (TaqMan probes were purchased from Applied Biosystems, Foster City, CA) was evaluated by real-time RT-PCR. The results for the target gene were expressed as an n-fold difference relative to the endogenous control gene (relative expression levels).

Sister chromatid exchange

Sister chromatid exchange was evaluated as previously described (17). The percentage of high frequency cells (HFCs) for each individual was estimated using the pooled distribution of all sister chromatid exchange cell measurements. For each subject, the expected number of HFCs (x) was calculated by the following expression: $x = 1.645$ [*np*

$(1 - p) + n(1 - p) + 0.5]^{1/2}$ where n is the number of cells scored and p is equal to 0.95. An individual was considered an outlier if the number of HFCs obtained was greater than x. Slides from each culture were randomly numbered and scored "blind" in a numerical fashion. The mitotic index values were calculated as the percentage of cells in mitosis on the microscopy slides used for cytogenetic analysis.

Endometrial thickness

Ultrasonographic endometrial thickness evaluation was assessed by one operator who was blinded to treatments at baseline and after 2 and 3 yr. The endometrial thickness, at sagittal incidence, was measured from a basal layer to the other. If the endometrial thickness was at least 8 mm or if uterine bleeding occurred during the study, a hysteroscopic examination and an endometrial biopsy were performed. Data analysis included effects derived by treatment, time, and interaction of these two terms.

General safety

Several blood parameters of general safety, such as prothrombin time, partial thromboplastin time, hemoglobin, total serum protein, urinary creatinine, hepatic and pancreatic enzymes, were evaluated using routine methods.

Genistein aglycone serum levels

Genistein aglycone levels were measured in serum samples by using a time-resolved fluorescence immunoassay (Labmaster TR-FIA; Labmaster, Ltd., Turku, Finland) (18). The fluorescence was read by using a Victor 1420 Multilabel Counter (PerkinElmer, Waltham, MA). Intraassay and interassay coefficients of variation for genistein vary from 3.2–4.1% and 4.5–5.3%, respectively.

Bone metabolism

Primary outcomes

BMD. We measured the anteroposterior lumbar spine and femoral neck BMD by dual-energy x-ray absorptiometry (Hologic QDR4500 W; Hologic Technologic Srl, Turin, Italy) at baseline and after 2 and 3 yr of treatment, as already described (11). The coefficient of variation of our instrument is 0.5% with the standard phantom; *in vivo*, we calculated a coefficient of variation of 1.1% for the lumbar spine and 1.5% for the femoral neck.

Secondary outcomes

Markers of bone resorption/formation. Bone-specific alkaline phosphatase (B-ALP; normal range, 8.5–17.9 μg/liter) and IGF-I (normal range, 74–162 μg/liter) were measured by an immunoenzymatic assay (Pantec s.r.l., Torino, Italy) to assess the effect of genistein aglycone on bone formation. Carboxy-terminal cross-linking telopeptide (CTX) was measured by using Serum CrossLaps ELISA (Nordic Biosciences, Herlev, Sweden). CTX values in postmenopausal women are between 0.142 and 1.351 ng/ml.

Pyridinium cross-links [pyridinoline (PYR) and deoxypyridinoline (DPYR)] were measured at baseline and after 2 and 3 yr of treatment, following a 2-h morning fast before each collection. PYR (normal range, 25–91 pmol/μmol of urinary creatinine) and DPYR (normal range, 3–21 pmol/μmol of urinary creatinine) were measured by HPLC (Bio-Rad Laboratories, Hercules, CA).

Osteoprotegerin and soluble receptor activator of NF-κB ligand. Osteoprotegerin (OPG; normal range, 2.5–4.5 pmol/liter in postmenopause) was measured with a commercially available kit (Pantec). This assay detects monomeric, dimeric, and ligand-bound forms of OPG (lower detection limit, 0.14 pmol/liter). Serum levels of soluble receptor activator of NF-κB ligand (sRANKL; normal range, 0.35–0.39 pmol/liter in female) were measured by an immunoenzymatic kit (Pantec) that detects free sRANKL, but not sRANKL complexed to OPG (lower detection limit, 0.08 pmol/liter).

TABLE 1. Baseline characteristics of 138 postmenopausal women in the two randomized groups

Characteristics	Placebo (n = 67)	Genistein (n = 71)
Age (yr)	53.5 ± 2.0	53.8 ± 2.9[a]
Body mass index	25.6 ± 4.6	24.29 ± 2.8[a]
Months since menopause	42.8 ± 26.3	42.9 ± 35.9[a]
Lumbar spine BMD (g/cm^2)	0.847 ± 0.11	0.850 ± 0.08[a]
Femoral neck BMD (g/cm^2)	0.675 ± 0.06	0.660 ± 0.05[a]

Values are mean ± SD. NS, Not significant.

[a] NS *vs.* placebo.

Results

Patient characteristics

The baseline characteristics of the subcohort of postmenopausal women (n = 138) are shown in Table 1. No statistically significant differences were observed between groups. The mean femoral neck BMD value at baseline in our randomized groups was lower than 0.795 g/cm^2, suggesting significant bone loss.

Safety parameters

Digitized assessment of breast density

No significant difference in IMI was detected between groups at the beginning (Fig. 2A) or after 2 and 3 yr of treatment. A significant reduction from baseline in IMI was observed in both groups at the end of the study ($P < 0.001$; Fig. 2A).

Mammographic breast density-visual classification

Using Wolfe classification, a greater percentage of genistein aglycone recipients were rated as having improved breast density after 3 yr of treatment, although there was no significant difference observed between groups at different time points (Fig. 2B).

BRCA1 and BRCA2 mRNA levels

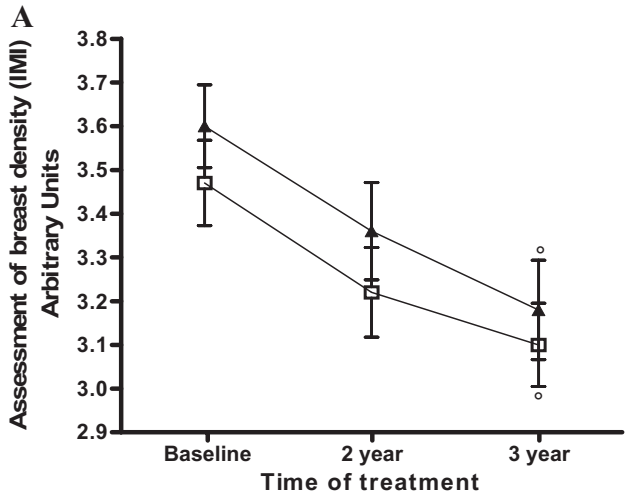
A significant difference in BRCA1 and BRCA2 mRNA levels was observed between groups after 2 and 3 yr of treatment, but no significant change was observed within the genistein group (Fig. 2, C and D). Moreover, a treatment × time interaction was also highly significant ($P < 0.0001$; respectively).

Sister chromatid exchange

Between-group analyses revealed that genistein aglycone significantly decreased sister chromatid exchange (Fig. 3A) after 3 yr compared with placebo ($P < 0.001$). Moreover, a treatment × time interaction was also highly significant ($P < 0.0001$; Fig. 3A).

Endometrial thickness

No significant difference in mean endometrial thickness was detected between groups at the beginning of the study or after 3 yr (Fig. 3B). A significant reduction from baseline in endometrial thickness was observed in both groups at the end of the study ($P < 0.001$; Fig. 3B).



B

Wolfe classification	Basal		2nd year		3rd year	
	Placebo	Genistein	Placebo	Genistein	Placebo	Genistein
N1	N=57	N=53	N= 63	N=64	N= 66	N=69
P1	N= 10	N=16	N= 4	N=7	N= 1	N=2
P2	N=0	N=2	N=0	N=0	N=0	N=0
DY	N=0	N=0	N=0	N=0	N=0	N=0

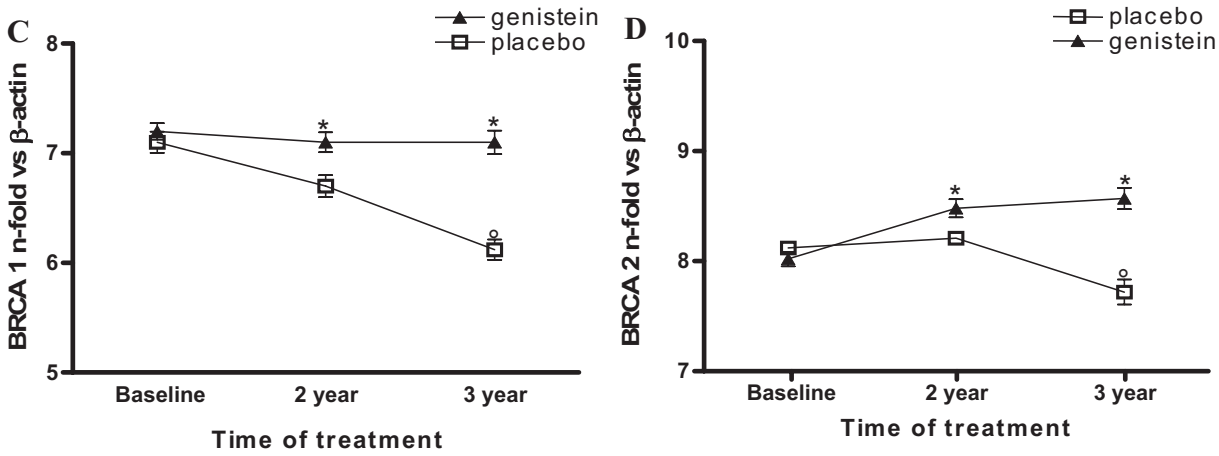


FIG. 2. A, Mean (95% CI) at different time-points in IMI breast density. No significant difference in IMI breast density was observed between groups at different time-points (°, $P < 0.001$ *vs.* placebo and genistein baseline). B, Values present the number of women with corresponding Wolfe score. No significant difference in proportions was observed between and within groups at different time points. C, Mean (95% CI) at different time-points in BRCA1 mRNA levels, and D, in BRCA2 mRNA levels (all *, $P < 0.001$ *vs.* placebo). No significant difference in BRCA1 and BRCA2 mRNA was observed within genistein-treated group (all °, $P < 0.001$ *vs.* placebo baseline).

General safety and genistein levels

There were no significant changes in parameters of general safety (Table 2). No subjects discontinued therapy because of adverse events. However, three placebo recipients and five genistein aglycone recipients reported mild gastrointestinal discomfort in this follow-up study. Genistein levels have also been evaluated in these recipients, as reported in Table 2.

Bone

Primary outcomes

After 3 yr of therapy, genistein aglycone significantly increased BMD at femoral neck and lumbar spine compared with placebo in the subcohort of this follow-up study (all $P < 0.001$; Fig. 4, A and B) beyond that seen after 2 yr of product administration (11), although no significant difference was found between 2 and 3 yr of therapy in the treatment arm. Moreover, a treatment × time interaction was also highly significant ($P < 0.0001$, respectively).

Secondary outcomes

B-ALP showed a significant increase over time for the genistein group compared with baseline, but no significant change for the placebo group. Between-group differences were significant for each of these variables at both time points ($P < 0.001$). Moreover, a treatment × time interaction was also highly significant ($P < 0.0001$; Fig. 5A).

IGF-I showed a significant increase over time for genistein recipients when compared with placebo (second year, $P < 0.05$; third year, $P < 0.001$), which remained unchanged after 3 yr (Fig. 5B).

In placebo recipients, urinary excretion of PYR and DPYR markedly increased throughout the study. Genistein aglycone treatment reduced PYR compared with placebo ($P < 0.01$, Fig. 5C). DPYR excretion, however, showed a nonsignificant decrease over a 3-yr period for genistein aglycone compared with placebo, which showed no clinical trends.

In all postmenopausal women treated with genistein, CTX decreased significantly over the 3-yr period compared with baseline ($P < 0.001$) and second year ($P < 0.01$). There was no

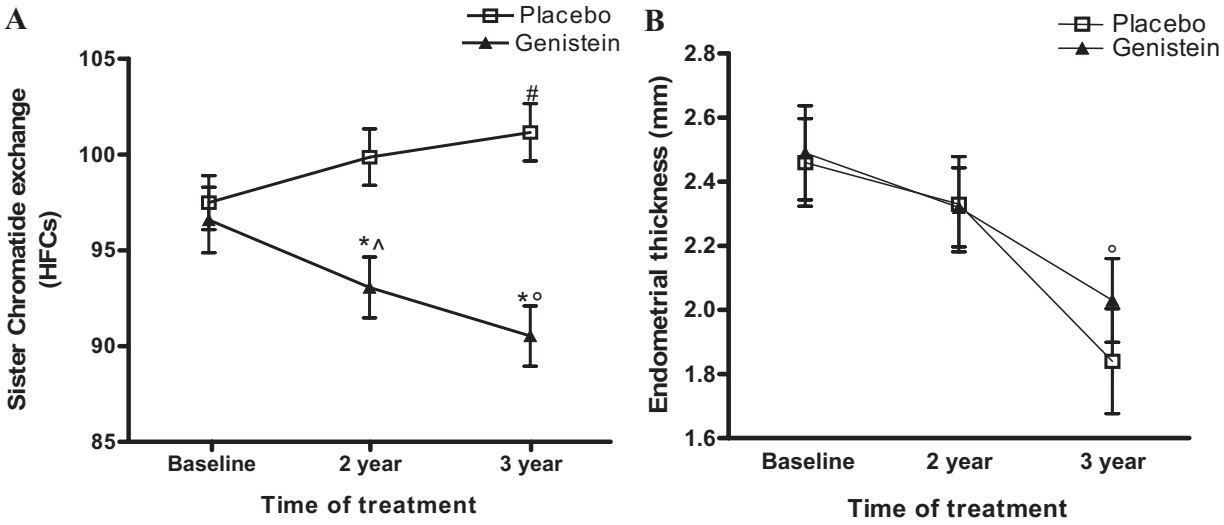


FIG. 3. A, Mean (95% CI) at different time-points in chromatid sister exchange (*, $P < 0.001$ *vs.* placebo; °, $P < 0.001$ *vs.* genistein baseline; ^, $P < 0.01$ *vs.* genistein baseline; #, $P < 0.01$ *vs.* placebo baseline). B, Mean (95% CI) at different time-points in endometrial thickness. No significant difference in endometrial thickness was observed between the two groups at different time-points. (°, $P < 0.001$ *vs.* placebo and genistein baseline).

significant decrease of CTX at 2 and 3 yr for the placebo group (Fig. 5D). Between-group analyses showed that genistein aglycone significantly decreased CTX at 2 and 3 yr compared with placebo ($P < 0.001$). Moreover, a treatment × time interaction was also highly significant ($P < 0.0001$; Fig. 5D).

OPG was significantly increased after 2 ($P < 0.01$ *vs.* baseline) and 3 yr ($P < 0.001$ *vs.* baseline) of therapy for the genistein aglycone group, whereas there was no significant change for the placebo group. Between-group differences were significant for each of these variables at 2 and 3 yr ($P < 0.001$; Fig. 6A).

sRANKL was significantly decreased at 2 and 3 yr for genistein *vs.* baseline ($P < 0.001$; Fig. 6B) and second year ($P < 0.05$; Fig. 6B), whereas there was no significant change for placebo. Between-group analyses revealed that genistein significantly decreased sRANKL at 2 and 3 yr compared with placebo ($P < 0.001$). Moreover, a treatment × time interaction was also highly significant ($P < 0.0001$; Fig. 6B). Table 3 summarizes all the above described measurements.

Discussion

The relationship between soy isoflavones and reproductive cancers is not simple. In fact, a significant number of factors, including the timing of exposure, individual differences in metabolism, hormonal milieu, and different modalities of isoflavone intake might play a crucial role in these effects (19). Most case-control studies have indicated some protective effect of soy (19–21), whereas some others have failed to show any relationship between total intake and breast cancer development (22, 23). Antineoplastic effects have been shown in several *in vitro* studies for isoflavones such as genistein aglycone via enzymatic inhibition, antiangiogenesis effects, stimulation of the immune system, and potent antioxidant capacity (1). Several markers for breast cancer, mutagenesis, carcinogenesis, as well as specific measurements of breast density and endometrial hyperplasia were taken on a 138-subject subcohort of an initial larger study (11) over a 3-yr period. Based on these findings, we did not observe any negative effects on breast and endometrium after 3 yr of treatment. However, this study does not establish a safety profile for other well-known isoflavones and/or their metabolites. Moreover, although these findings are very promising in this subcohort of postmenopausal women, a bigger population size and a longer follow-up period will be needed to address more satisfactorily the chronic safety and efficacy issues concerning the health effects of genistein aglycone in humans.

Genistein aglycone treatment noticeably preserved BRCA1 and BRCA2 mRNA levels compared with the placebo group in

TABLE 2. General safety profiles by group and visit

	Genistein group (n = 71)			Placebo group (n = 67)		
Variable	**Baseline**	**2 yr**	**3 yr**	**Baseline**	**2 yr**	**3 yr**
Creatinine (mg/dl)	0.73 ± 0.13	0.74 ± 0.1	0.75 ± 0.08	0.76 ± 0.12	0.78 ± 0.1	0.81 ± 0.1
AST (IU/liter)	21.86 ± 6.51	22.42 ± 5.64	22.08 ± 5.05	21.26 ± 5.8	22.99 ± 6.62	25.16 ± 5.95
ALT (IU/liter)	23.32 ± 6.35	22.83 ± 6	22.71 ± 4.77	23.29 ± 5.55	24.49 ± 6.67	26.17 ± 4.88
GGT (IU/liter)	21.65 ± 7.92	21.62 ± 6.75	20.9 ± 5.89	24.65 ± 7.51	26.09 ± 8.28	28.05 ± 7.71
Amylase (IU/liter)	86.62 ± 20.89	84.97 ± 18.84	77.09 ± 19.1	74.37 ± 19.07	79.55 ± 18.06	82 ± 16.85
Lypase (IU/liter)	32.03 ± 11.75	32.44 ± 8.86	30.16 ± 10	35.08 ± 7.24	36.46 ± 8.01	38.25 ± 7.71
Genistein (μmol/liter)	0.149 ± 0.020	0.75 ± 0.08[a]	0.78 ± 0.05[a]	0.146 ± 0.018	0.142 ± 0.02	0.14 ± 0.03

Values are mean ± SD. AST, Aspartate aminotransferase; ALT, alanine aminotransferase; GGT, γ -glutamyltransferase.

[a] $P < 0.001$ *vs.* placebo.

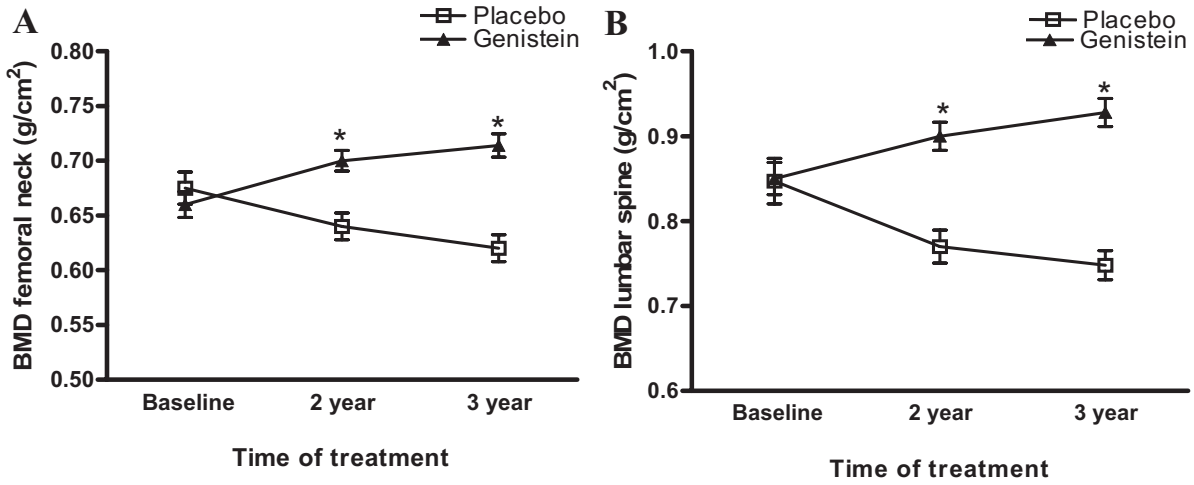


FIG. 4. A and B, Mean (95% CI) at different time-points in BMD of the femoral neck (*, $P < 0.001$ *vs.* placebo) and lumbar spine (*, $P < 0.001$ *vs.* placebo) in the two randomized groups.

our osteopenic, postmenopausal women. It was recently shown that BRCA1 and BRCA2 are involved in the maintenance of genomic integrity and transcriptional regulation in breast cells (24). BRCA1 is primarily involved in DNA repair functions as well as control of meiotic sex chromosome inactivation (25). Additionally, BRCA1, but not mutant BRCA1, inhibits estradiol up-regulation of extracellular signal-related kinase, which is specifically implicated in breast cancer cell proliferation via the interaction of estrogen with ERα (26). BRCA2, on the other hand, is part of the homologous recombination machinery that helps to maintain genomic integrity, but the role of estrogen and its effect on BRCA2 mutations is unclear (25). The fall of estrogens at

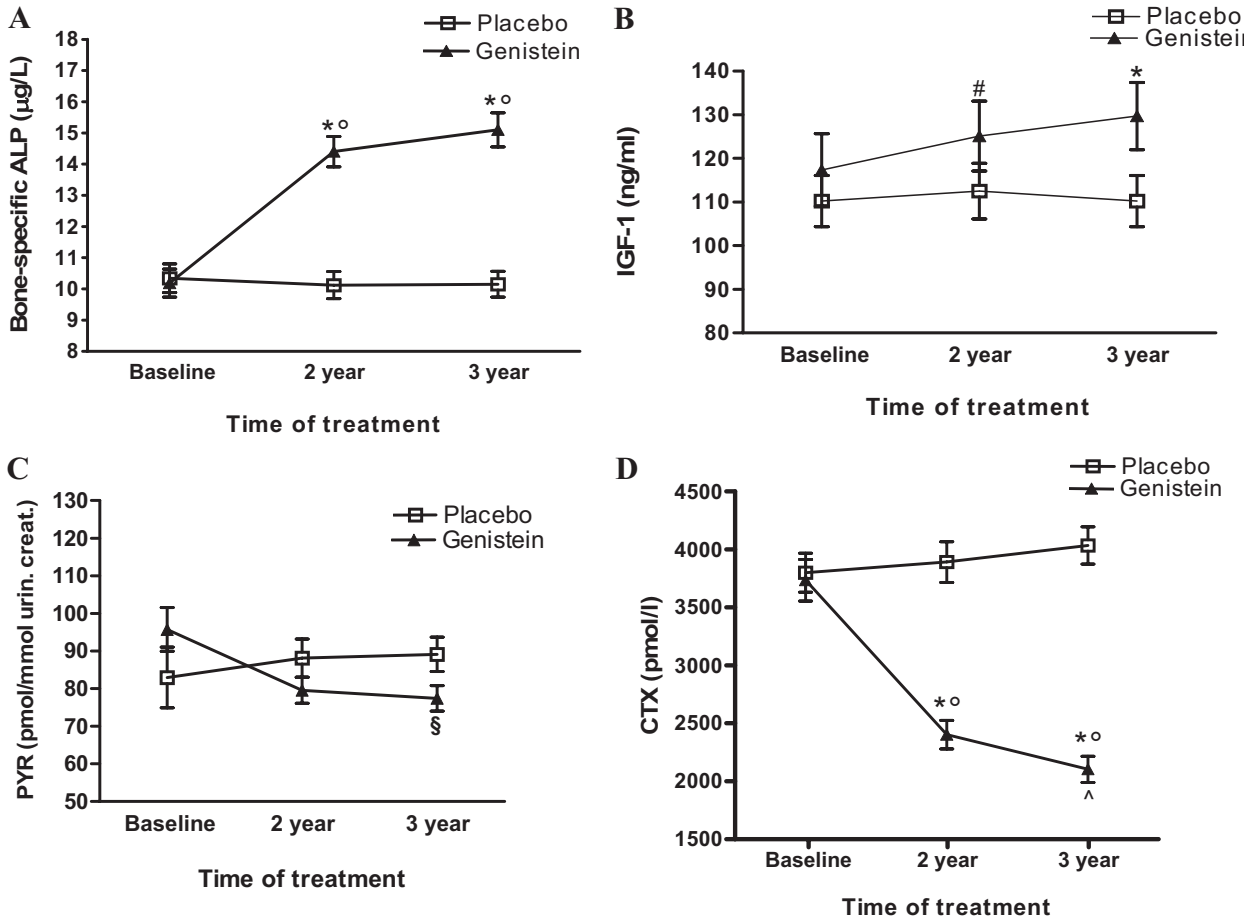


FIG. 5. A, Mean (95% CI) at different time-points in B-ALP (*, $P < 0.001$ *vs.* placebo; °, $P < 0.001$ *vs.* genistein baseline); and B, IGF-I (#, $P < 0.05$ *vs.* placebo; *, $P < 0.001$ *vs.* placebo) serum levels in the two randomized groups. C, Mean (95% CI) at different time-points in PYR (§, $P < 0.01$ *vs.* placebo); and D, mean (95% CI) at different time-points in CTX (*, $P < 0.001$ *vs.* placebo; °, $P < 0.001$ *vs.* genistein baseline; ^, $P < 0.01$ *vs.* genistein second year) in the two randomized groups.

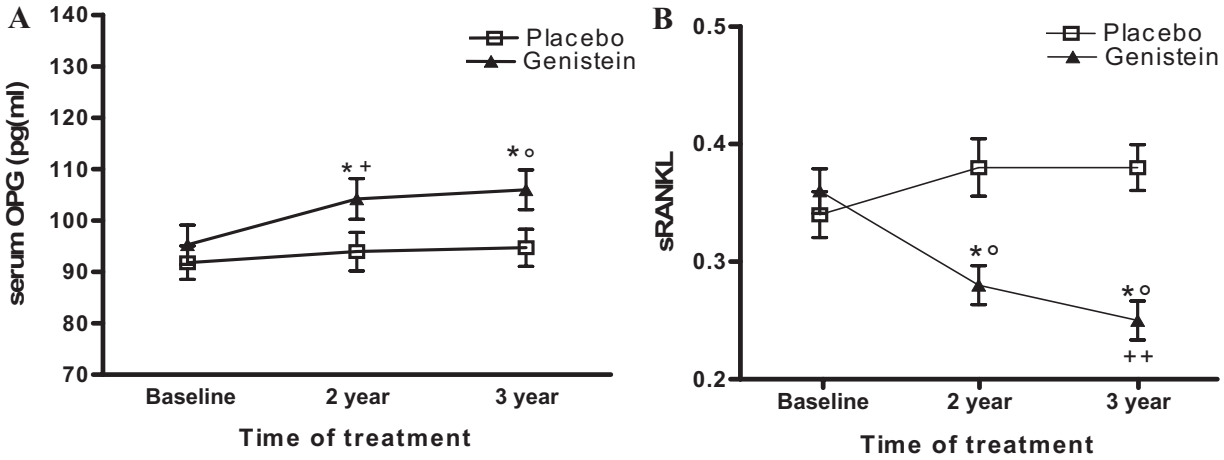


FIG. 6. A, Mean (95% CI) at different time-points in OPG serum levels (*, $P < 0.001$ *vs.* placebo; °, $P < 0.001$ *vs.* genistein baseline; +, $P < 0.01$ *vs.* genistein baseline); and B, sRANKL serum levels (*, $P < 0.001$ *vs.* placebo; °, $P < 0.001$ *vs.* genistein baseline; ++, $P < 0.05$ *vs.* genistein second year).

menopause may lead to a reduction of BRCA1 and BRCA2 mRNA levels and, hence a reduction in tumorigenesis surveillance by the these two genes. It is intriguing that the mRNA levels of BRCA1 and BRCA2 are conserved in genistein recipients but not in the placebo group. This may suggest that the genistein may help to maintain apoptotic potential and preserve DNA repair capacity in breast tissue (26–29). Accordingly, digital mammography measurements in our subcohort of patients showed that the genistein aglycone and placebo group were equivalent over the 3-yr period, both exhibiting a slight decrease in breast density. This was also confirmed by the Wolfe analysis, which showed no significant differences between treatment and placebo groups.

Recently, it has been demonstrated in a large population of menopausal women that plasma levels of genistein at baseline and after 5 and 10 yr showed a statistically significant inverse association with the risk of breast cancer (30); specifically, genistein plasma levels associated with reduction in breast cancer risk were in the same range found when subjects consumed 54 mg of genistein aglycone per day (11). Consequently, our study is the first intervention trial suggesting that the data generated by the above-mentioned prospective study might be possible through the measure of our surrogate markers. Moreover, serum concentrations of unconjugated free genistein aglycone achieved with daily intake of 54 mg are near the binding affinity of genistein seen *in vitro* for ERβ (10, 31, 32), which is abundant in bone during the mineralization phase, but are an order of magnitude lower than the *in vitro* affinity of genistein for ERα, which is associated with the development of carcinogenesis in endometrial and breast tissue (10). The selective activation of ER-β by

TABLE 3. Bone parameters by group and visit

	Genistein group (n = 71)			Placebo group (n = 67)		
Variable	**Baseline**	**2 yr**	**3 yr**	**Baseline**	**2 yr**	**3 yr**
Lumbar spine BMD (g/cm^2)	0.850 ± 0.089	0.90 ± 0.076[a]	0.928 ± 0.078[a]	0.847 ± 0.115	0.77 ± 0.08	0.748 ± 0.075
Femoral neck BMD (g/cm^2)	0.66 ± 0.054	0.7 ± 0.040[a]	0.714 ± 0.045[a]	0.675 ± 0.06	0.64 ± 0.05	0.620 ± 0.05
B-ALP (μg/liter)	10.19 ± 1.9	14.4 ± 2.05[a,d]	15.1 ± 2.3[a,d]	10.34 ± 1.88	10.12 ± 1.78	10.15 ± 1.69
IGF-I (ng/ml)	117.36 ± 35.08	125.1 ± 33.77[c]	129.71 ± 32.5[a]	107.98 ± 28.1	112.51 ± 26.11	110.22 ± 23.94
PYR (pmol/μmol creatinine)	95.74 ± 24.53	79.54 ± 14.44	77.44 ± 14.25[b]	82.96 ± 32.99	88.13 ± 20.73	89.11 ± 18.77
DPYR (pmol/ μmol creatinine)	22.01 ± 7.64	19.47 ± 2.88	18.6 ± 2.82	20.52 ± 6.3	20.13 ± 4.8	20.45 ± 4.46
CTX (pmol/liter)	3735.1 ± 757.8	2402.1 ± 520.3[a,d]	2103.2 ± 475.07[a,d,f]	3799.02 ± 690.01	3891.55 ± 717.9	4033.3 ± 656.53
OPG (pg/ml)	95.27 ± 16.12	104.18 ± 16.72[a,e]	105.98 ± 16.33[a,d]	91.81 ± 13.38	93.95 ± 15.28	94.71 ± 14.72
sRANKL (pmol/liter)	0.36 ± 0.08	0.28 ± 0.07[a,d]	0.25 ± 0.07[a,d,g]	0.34 ± 0.08	0.38 ± 0.10	0.38 ± 0.08

Values are mean ± SD.

[a] $P < 0.001$ *vs.* placebo.

[b] $P < 0.01$ *vs.* placebo.

[c] $P < 0.05$ *vs.* placebo.

[d] $P < 0.001$ *vs.* genistein baseline.

[e] $P < 0.01$ *vs.* genistein baseline.

[f] $P < 0.01$ *vs.* genistein 2nd year.

[g] $P < 0.05$ *vs.* genistein 2nd year.

genistein is likely to be mediated by a greater capacity to recruit coregulators of ER-β than those of ER-α, via *de novo* protein synthesis in osteoblasts (33). Consequently, although experimental observations indicated that the positive actions of genistein on bone could be related to a weak estrogenic effect on a subtype of ER-α (34), the accumulating evidence strongly suggests the important involvement of ER-β in bone formation and, by extension, the use of selective ER-β agonists such as genistein aglycone to treat bone loss without the harmful estrogenic activity in the breast and uterus. Previous reports have found that soy product consumption or mixed soy isoflavones were associated with a decreased risk of endometrial cancer in postmenopause (35, 36). Our findings on a lack of endometrial hyperplasia and reductions in sister chromatid exchange (37) support results from previous clinical trials demonstrating safety of genistein aglycone on endometrium (38, 39).

Our subcohort was also analyzed for continued effects on bone. Because observational studies correlated the low rate of hip fractures in postmenopausal Asians with isoflavone consumption (40), the ability of genistein and related soy isoflavones to reduce postmenopausal bone loss has been largely investigated. We previously showed that genistein increased BMD at the lumbar spine and femoral neck in postmenopausal women over 2 yr (11). The present investigation extends the effect of genistein aglycone on bone health to 3 yr of treatment in the subcohort, although additional studies of genistein aglycone effect on fractures are needed to assess its real force on bone loss during the menopause.

Specifically, genistein administration continued to decrease levels of bone resorption markers (PYR, CTX, sRANKL) and increased new bone formation markers (B-ALP, IGF-I and OPG), extending this effect to 3 yr and supporting greater bone formation in this subcohort. The positive effect of genistein aglycone on BMD in this subcohort of osteopenic, postmenopausal women is likely consistent with other studies showing positive effects on bone metabolism from food matrices and in combination with other factors (1).

Indeed, we observed a moderate number of gastrointestinal side effects in the genistein-treated women. These side effects were also observed in the placebo arm at a lower rate. It is possible that calcium carbonate may elicit a similar response and that genistein exacerbates the effect.

Overall, there are only limited data regarding the safety of highly purified soy isoflavones given to postmenopausal women for breast and endometrium. This study, in a subcohort of a larger study in postmenopausal osteopenic women, provides the first specific evidence of possible safety for the purified genistein aglycone for breast tissue. The findings for safety in the current study, however, need to be viewed in the context of a balanced diet low in saturated fats and high in fruits and vegetables that likely increased the safety and perhaps the beneficial effects of genistein aglycone in our postmenopausal women. In addition, it extends the findings of the earlier study of continued positive effects on bone tissue to 3 yr of treatment.

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