**Soy-isoflavone-enriched foods and markers of lipid and glucose metabolism in postmenopausal women: interactions with genotype and equal production**

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**ABSTRACT**

**Background:** The hypocholesterolemic effects of soy foods are well established, and it has been suggested that isoflavones are responsible for this effect. However, beneficial effects of isolated isoflavones on lipid biomarkers of cardiovascular disease risk have not yet been shown.

**Objective:** The objective was to investigate the effects of isolated soy isoflavones on metabolic biomarkers of cardiovascular disease risk, including plasma total, HDL, and LDL cholesterol; triacylglycerols; lipoprotein(a); the percentage of small dense LDL; glucose; nonesterified fatty acids; insulin; and the homeostasis model assessment of insulin resistance. Differences with respect to single nucleotide polymorphisms in selected genes [ie, estrogen receptor (XbaI and PvuII), estrogen receptor β (AluI), and estrogen receptor β (Tsp509I), endothelial nitric oxide synthase (Glu298Asp), apolipoprotein E (Apo E2, E3, and E4), cholesteryl ester transfer protein (TagIB), and leptin receptor (Gln223Arg)] and with respect to equol production were investigated.

**Design:** Healthy postmenopausal women (n = 117) participated in a randomized, double-blind, placebo-controlled, crossover dietary intervention trial. Isoflavone-enriched (genisteano-daizein ratio of 2:1; 50 mg/d) or placebo cereal bars were consumed for 8 wk, with a wash-out period of 8 wk before the crossover.

**Results:** Isoflavones did not have a significant beneficial effect on plasma concentrations of lipids, glucose, or insulin. A significant difference between the responses of HDL cholesterol to isoflavones and to placebo was found with estrogen receptor genotypes AA and TG (p = 0.05). Isoflavone supplementation, when provided in the form of hormone replacement therapy (HRT), have been shown to reduce plasma concentrations of LDL cholesterol and increase concentrations of HDL cholesterol. However, a lack of beneficial effects on CVD, evidence of greater risk of thrombosis (8) and higher concentrations of C-reactive protein (9), makes the possibility of a lack of benefit unclear.

**Conclusions:** Isoflavone supplementation, when provided in the form and dose used in this study, had no effect on lipid or other metabolic biomarkers of cardiovascular disease risk in postmenopausal women but may increase HDL cholesterol in an estrogen receptor β gene–polymorphic subgroup.

**KEY WORDS** Isoflavones, soy, cardiovascular disease, postmenopausal women, plasma lipids, cholesterol, triacylglycerols, glucose, insulin, estrogen receptor, gene-nutrient interaction

**INTRODUCTION**

Isoflavones are phytoestrogens (chemicals with structural similarity to estrogen). They can bind to estrogen receptors to modulate gene transcription and cell-signaling pathways, and they also may act independently of the estrogen receptor. Epidemiologic evidence in humans suggests that increased consumption of soy, the main dietary source of isoflavones, is cardioprotective (2), which may be due to estrogenic properties of soy isoflavones (ie, genistein, daidzein, and glycitein). Evidence of the ability of soy products to bring about a beneficial change in the blood lipoprotein profile led the Food and Drug Administration to approve a claim that “25 g of soy protein a day, as part of a diet low in saturated fat and cholesterol, may reduce the risk of heart disease” (3). It is currently uncertain whether soy isoflavones contribute to the reported cholesterol lowering effects of soy, although a meta-analysis suggests that isoflavone dose is not related to changes in LDL or HDL cholesterol after soy consumption (4).

The increase in cardiovascular disease (CVD) risk that is associated with decreased ovarian function at the menopause (5, 6) is in part attributable to arterial dysfunction and a less favorable blood lipid profile. Exogenous estrogen and progesterone, in the form of hormone replacement therapy (HRT), have been shown to reduce plasma concentrations of LDL cholesterol and increase concentrations of HDL cholesterol (7). However, a lack of beneficial effects on CVD, evidence of greater risk of thrombosis (8) and higher concentrations of C-reactive protein (9), makes the possibility of a lack of benefit unclear.
protein and triacylglycerols (9, 10), and greater risk of hormone-dependent cancers have led to a search for alternative to HRT, such as isoflavones.

Equol, a gut bacterial metabolite of daidzein, may prove to be an important bioactive metabolite of isoflavones because of its greater binding affinity to estrogen receptors and a greater antioxidant capacity than are seen with the parent compound (11). Previous studies indicated that persons vary greatly in their ability to synthesize equol (12), and evidence suggests that responsiveness to isoflavones may vary according to a person’s equal-synthesizing capacity (13). Variability in response to isoflavones may also be observed with respect to variability in CVD risk genes or genes involved in estrogen action, as has been shown for HRT therapy with respect to estrogen receptor polymorphisms (14, 15).

Relatively few studies have reported on the effects of isolated isoflavones on biomarkers of CVD risk, and most of these studies have been hampered by inadequate statistical power. The main hypothesis of the current study was that the consumption of isolated soy isoflavones, provided within a food vehicle, would reduce the circulating lipid biomarkers of CVD risk (except HDL cholesterol, which would be increased). A second hypothesis was that the response to isoflavones is not uniform across the study population—that it will differ according to estrogen receptor− or CVD risk−related genotype and according to whether a person is an “equol producer” or “equol nonproducer.” Genotypes for single-nucleotide polymorphisms in estrogen receptor α (ERα), estrogen receptor β (ERβ) and an ERβ variant [ERβ(Δ57)] , endothelial nitric oxide synthase (eNOS), apolipoprotein E, cholesteryl ester transfer protein (CETP), and the leptin receptor were characterized, and equol-producing status was defined.

SUBJECTS AND METHODS

Subjects

Healthy postmenopausal women aged 45–70 y were recruited from the areas around the University of Reading (Reading, United Kingdom), the German Institute of Human Nutrition (Nuthetal, Germany), The Royal Veterinary and Agricultural University (Copenhagen, Denmark), and the National Institute for Food and Nutrition Research (Rome, Italy). Each study center advertised the study in its local media and via internal e-mails and posters. Persons interested in participating in the study com-

All volunteers gave written informed consent before begin-

The study was a randomized, placebo-controlled, 2 × 8−wk double-blind crossover design with an 8−wk washout period. The number of subjects needed to enable a statistically significant effect on risk biomarkers was calculated by using the method of least standardized difference, a power of 90% with a significance level of 5%, and a predicted plasma cholesterol reduction of 10% as the marker of response to isoflavone supplementation. Use of this outcome (reduction in plasma cholesterol) would require a total sample size of 50 women, but additional subjects were included to account for the unknown or greater variability in some outcome markers that are not often investigated. Therefore, a total of 120 postmenopausal women (30 center) was required to complete the study, and 140 women were recruited to allow for dropouts. All participating women were stratified at the University of Reading according to age, BMI, and triacylglycerol before study entry and then randomly assigned to 1 of 2 groups. One group began the study with the treatment arm, and, after the washout period, they went into the placebo arm; the other group followed the reverse order.

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Soy isoflavone−enriched foods

Subjects were asked to incorporate 2 cereal bars/day into their normal diet during the intervention periods—one with breakfast and one in the late afternoon or early evening. During the isoflavone treatment arm, subjects consumed cereal bars identical to those consumed during the placebo phase, except that the treatment arm products were enriched with isoflavones (50 mg/d). The isoflavone extract (Solbar Plant Extracts Ltd, Ashdod, Israel) had a genistein−to−daidzein ratio of 2:1. The cereal bars (40 g; Efamol, Ltd, Manchester, United Kingdom) had an average nutrient content of 652 kJ of energy, 2.6 g protein, 17.3 g carbohydrate, 8.5 g fat, 1.8 g fat, and 0.012 g sodium. The 4 flavors offered to the volunteers were apricot and almond, apple and cardamon, hazelnut, and raspberry. The subjects perceived the isoflavone−enriched and placebo foods as identical in appearance and taste.
Analyses were carried out at the University of Reading. All plasma lipids at baseline (t0) and midway (at 4 wk) during each intervention period (4 of the placebo intervention arm and 4 of the isoflavone intervention arm). The diaries used estimated amounts and weights of foods consumed and included 2 weekdays and 1 weekend day. The dietary intake of the Danish, German, English, and Italian subjects was analyzed at each center by using food-composition databases developed in each country.

Sample collection, assays, and blood pressure measurements

Once recruited into the study, subjects were required to attend the clinical unit on 6 occasions (t0, t4, and t8 on each arm of the study). To standardize the food consumption of subjects the evening before blood samples, a low-fat evening meal (<15 g fat) was consumed the evening before each of the 6 clinical visits. Three standard menus were provided to subjects, with recipes and preparation instructions; these menus were low in fat and provided no more than 15 g fat for the entire evening meal. Each subject consumed the same meal from the 3 menu selections on each evening before blood sampling.

On the morning of each visit, subjects attended the clinical unit in a 12-h fasted state. Blood pressure was measured on the nondominant arm while the subject was in a sitting position after a 10-min rest. Blood was collected in 10-mL EDTA-coated tubes for the analysis of TC, HDL cholesterol, triacylglycerol, lipoprotein(a) [Lp(a)], LDL subclasses, glucose, nonesterified fatty acids (NEFAs), and buoyant cloud extraction. After centrifugation at 1600×g for 10 min at 4 °C, plasma was stored at −20 °C until analysis. Triacylglycerol, TC, and HDL cholesterol were analyzed by using an ILab 600 biochemical analyzer and enzymatic colorimetric kits (both: Instrumentation Laboratories Ltd, Warrington, United Kingdom). LDL cholesterol was calculated by using the formula of Friedewald et al (16). Lp(a) (Biokit, New Ash Green Longfield, United Kingdom), glucose (Instrumentation Laboratories Ltd) and NEFA (Alpha Laboratories, Eastleigh, United Kingdom) concentrations were also analyzed by using the ILab 600 biochemical analyzer. LDL subclasses were separated by ultracentrifugation with the use of Cooamassie Blue–stained plasma on a self-forming density gradient of iodixanol, and the subclasses were analyzed by using digital photography and gel-scan software (17). For insulin measurements, blood was collected in 10-mL sodium heparin–evacuated tubes and centrifuged at 1600×g for 10 min at 4 °C, and plasma was stored at −80 °C until it was analyzed. Insulin was measured with an enzyme-linked immunosorbent assay by using a commercially available kit (Dako Cytomation, Ely, United Kingdom). A formula was used to describe insulin resistance, ie, the homeostasis model assessment (HOMA): insulin resistance = fasting glucose (mmol/L) × fasting insulin (µU/mL)/22.5 (18). Analyses of TC, HDL cholesterol, triacylglycerol, Lp(a), LDL subclasses, glucose, insulin, and NEFAs were conducted at the University of Reading. All plasma lipids were analyzed at t0, t4, and t8, except for LDL subclasses, which were analyzed at t0 and t8. Glucose, insulin, and NEFA analyses were carried out at t0 and t8.

DNA extraction and genotyping

DNA was extracted from stored buffy coats by using the QIAamp DNA blood mini-kit (Qiagen Ltd, Cheadle, United Kingdom). ERα, eNOS, apolipoprotein E, and CETP polymorphisms were analyzed at the University of Reading by using PCR amplification and then enzymatic digestion with specific restriction endonucleases. The collaborating group at the Karolinska Institute (Stockholm, Sweden) used RFLP analysis to analyze ERβ polymorphisms. The details of the primer sequences and PCR conditions are shown in Table 1.

Isoflavone analysis

The isoflavone content of the enriched and unenriched cereal bars was measured at Wageningen University and Research Centre (Wageningen, Netherlands) with the use of HPLC (19), as described previously (20). Urinary and serum genistein, daidzein, and equol were analyzed at Unilever Corporate Research (Sharnbrook, United Kingdom) by using time-resolved fluorescence immunoassays (Delfia; Perkin-Elmer Life Sciences, Cambridge, United Kingdom) (D Talbot R Ogborne, T Dodd, et al, unpublished observations, 2004), also described previously (20).

Statistical analysis

We used SAS software (version 9.1; SAS Institute Inc, Cary, NC) for all statistical analyses (PROC MIXED procedure) except when stated otherwise. Data are given as the mean ± SD, except in Figure 1, where mean ± SEM values are given. Changes from baseline to week 8 (ie, t8 − t0) were used as the dependent variables. Observations with missing data were excluded from the analysis. If the original data were approximately normally distributed, then changes from baseline on the original scale were calculated; if a log transform was deemed appropriate, then changes from baseline on the log scale were calculated, and these changes corresponded to a multiplicative change from baseline on the original scale. Subjects were included as a random factor within a linear mixed model. Residual analyses were conducted to check that the assumptions of the modeling process were justifiable and to identify any potential outliers. Sensitivity analyses were then carried out to check the degree of influence of any such outliers. Fixed effects always included in the final model were baseline values, treatment, center, time, and treatment order. Fixed effects included in the final model, if the effect was significant, were treatment order × treatment interaction; baseline × treatment interaction; baseline values of BMI, age, and triacylglycerol as fixed covariates (these baseline values were used in the stratified randomization procedure); and center × treatment interaction. Further exploratory investigation of the equal group and of genotype was included in the model. When statistically significant interactions were found, slice tests were used to test for effects within levels of an interaction. SPSS for WINDOWS software (version 12.0.1; SPSS Inc, Chicago, IL) was used to calculate the difference between dietary intakes at baseline and at the midpoints (t4 of the isoflavone and placebo intervention arms by using repeated-measures analysis of variance. Observed genotype frequencies were compared with those expected under the Hardy-Weinberg equilibrium by using standard chi-square tests.
RESULTS

Eight hundred sixty-two telephone screenings were carried out, and 612 subjects were excluded on the basis of the screening questionnaire. The remaining 250 volunteers attended a screening session at which a blood sample was taken and a physical examination was carried out to assess the volunteers’ suitability for the study. After the screening session, 133 volunteers were eligible for inclusion and chose to take part in the study; they were allocated by stratified randomization to the isoflavone-placebo or placebo-isoflavone groups. Sixteen subjects withdrew from the study after the start because of a new regimen of excluded medication, dislike of the study foods, or personal reasons; 117 subjects completed the study (a drop-out rate of 12% with a target of 120 subjects). Randomized groups remained comparable for the stratification variables, BMI, age and triacylglycerol after 16 subjects dropped out. Mean (±SD) values at baseline were 57.7 ± 5.4 y for age, 25.0 ± 2.9 for BMI, 120.6 ± 15.4 mm Hg for systolic blood pressure, 76.1 ± 8.3 mm Hg for diastolic blood pressure, 5.88 ± 0.93 mmol/L for TC, 3.59 ± 0.80 mmol/L for LDL cholesterol, 1.79 ± 0.38 mmol/L for HDL cholesterol, 1.10 ± 0.47 mmol/L for triacylglycerol, and 5.17 ± 0.47 mmol/L for fasting glucose. No changes in body weight were evident after the dietary intervention. Compliance was monitored by using study diaries, by counting the number of empty cereal bar packets returned, and by analyzing serum and urinary isoflavones. Urinary isoflavone concentrations after consumption of the isoflavone supplementation and placebo are shown in Table 2. After the isoflavone treatment, genistein and daidzein urinary concentrations were 17- and 26-fold those of placebo, respectively.

Volunteers were defined as “equol producers” if their urinary equol concentration in a 24-h urine sample during the isoflavone
treatment arm was $>936\ \text{nmol/L}$ (12). This amount approximates a urinary yield of $>0.45\ \text{mg/d}$. In one case, a urine sample was unavailable, so a serum concentration of $>39\ \text{nmol/L}$ was used to identify equol producer status (13). With the use of these cutoffs, 33 (28.2%) of 117 subjects were classified as equol producers.

Dietary intake was assessed at baseline and 4 of each intervention arm. Macronutrient intakes at baseline were 15%, 34%, and 47% of energy as protein, fat, and carbohydrate, respectively, which were reported previously (20). The differences in energy or macronutrient intake across the treatments and compared with baseline were not significant.

The differences in plasma TC, LDL or HDL cholesterol, total: HDL, triacylglycerol, Lp(a), NEFA, glucose and insulin concentrations, and HOMA after the 2 intervention periods were not significant (Table 3). For the percentage of small dense LDL (%sdLDL), a weak treatment effect was found ($P < 0.05$), which least-squares mean estimates showed to be a result of a significant reduction from baseline %sdLDL after placebo treatment.

The significant differences in mean systolic or diastolic blood pressure after isoflavone or placebo treatment were not significant (data not shown).

The change from baseline for plasma triacylglycerol, glucose, and insulin concentrations did not differ according to genotype. However, several treatment $\times$ genotype interactions were found for other outcome markers: 1) eNOS (Glu298Asp) $\times$ NEFA, TC, LDL cholesterol, and Lp(a) interactions; 2) ER$\beta$ (AluI) $\times$ TC, total: HDL cholesterol, and LDL-cholesterol interactions; 3) ER$\beta$(cc) (Tsp509I) $\times$ HDL cholesterol interaction; and 4) CETP (TaqI) $\times$ total: HDL cholesterol interaction. These interactions are summarized in Table 4. The detailed statistical treatments applied to these data (see Methods) suggest that many of the significant treatment $\times$ genotype interactions shown at first-stage analysis should be treated with caution because of problems with fit to normality before and after log transformation and sensitivity to outliers. In addition, slice tests showed that significant interactions between eNOS genotype and treatment for NEFA, TC, LDL cholesterol, and Lp(a) concentrations were all

### Table 2

<table>
<thead>
<tr>
<th></th>
<th>Isoflavone arm</th>
<th>Placebo arm</th>
<th>$P^{1}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Genistein (mg/d)</td>
<td>0.37 ± 0.40 [114]$^2$</td>
<td>0.37 ± 0.40</td>
<td>0.42 ± 0.32</td>
</tr>
<tr>
<td>Daidzein (mg/d)</td>
<td>0.16 ± 0.22 [114]</td>
<td>0.22 ± 0.30</td>
<td>0.22 ± 0.32</td>
</tr>
<tr>
<td>Equol (mg/d)</td>
<td>0.08 ± 0.06 [114]</td>
<td>0.08 ± 0.05</td>
<td>0.11 ± 0.08</td>
</tr>
<tr>
<td>Equol producers$^3$</td>
<td>0.10 ± 0.05 [31]</td>
<td>0.09 ± 0.05 [33]</td>
<td>0.13 ± 0.09 [33]</td>
</tr>
<tr>
<td>Equol nonproducers$^3$</td>
<td>0.07 ± 0.06 [82]</td>
<td>0.08 ± 0.05 [84]</td>
<td>0.094 ± 0.08 [83]</td>
</tr>
</tbody>
</table>

1 $P$ values are shown for the treatment effect within a stepwise-generated general linear mixed model implemented in SAS PROC MIXED (see Methods). Differences from baseline were used as the response variable. The differences in the change in urinary isoflavone concentrations between the placebo and treatment arms were significant for genistein, daidzein, and equol ($P < 0.0001$). The differences in the change in urinary equol concentrations between the placebo and treatment arms in both equol producers and equol nonproducers were also significant ($P < 0.0001$).

2 $\overline{x} \pm SD$; n in brackets (all such values).

3 Equol producers: $>0.45\ \text{mg/d}$; equol nonproducers: $<0.45\ \text{mg/d}$

### Table 3

<table>
<thead>
<tr>
<th></th>
<th>Isoflavone arm</th>
<th>Placebo arm</th>
<th>$t$</th>
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</thead>
<tbody>
<tr>
<td>Cholesterol</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total (mmol/L)</td>
<td>6.03 ± 1.32 [115]</td>
<td>6.13 ± 1.34</td>
<td>5.96 ± 1.19</td>
</tr>
<tr>
<td>LDL (mmol/L)</td>
<td>3.88 ± 1.11 [115]</td>
<td>3.84 ± 1.11</td>
<td>3.81 ± 1.02</td>
</tr>
<tr>
<td>HDL (mmol/L)</td>
<td>1.60 ± 0.39 [115]</td>
<td>1.74 ± 0.47</td>
<td>1.62 ± 0.35</td>
</tr>
<tr>
<td>Total: HDL</td>
<td>3.91 ± 1.08 [115]</td>
<td>3.71 ± 1.01</td>
<td>3.83 ± 1.04</td>
</tr>
<tr>
<td>Triacylglycerol (mmol/L)</td>
<td>1.21 ± 0.50 [115]</td>
<td>1.22 ± 0.48</td>
<td>1.19 ± 0.46</td>
</tr>
<tr>
<td>Lp(a)</td>
<td>226.2 ± 265.3 [112]</td>
<td>216.40 ± 244.45 [112]</td>
<td>227.38 ± 250.27 [114]</td>
</tr>
<tr>
<td>NEFA (umol/L)</td>
<td>447.4 ± 193.8 [113]</td>
<td>411.98 ± 139.32 [113]</td>
<td>426.74 ± 181.35 [114]</td>
</tr>
<tr>
<td>Glucose (mmol/L)</td>
<td>5.34 ± 0.61 [114]</td>
<td>5.21 ± 0.60 [113]</td>
<td>5.32 ± 0.58 [114]</td>
</tr>
<tr>
<td>Insulin (pmol/L)</td>
<td>34.8 ± 18.2 [108]</td>
<td>35.48 ± 17.89 [107]</td>
<td>35.60 ± 18.04 [108]</td>
</tr>
<tr>
<td>HOMA-IR</td>
<td>1.21 ± 0.71 [105]</td>
<td>1.20 ± 0.67 [105]</td>
<td>1.24 ± 0.65 [106]</td>
</tr>
</tbody>
</table>

1 All values are $\overline{x} \pm SD$; n = 117 unless otherwise stated (in brackets). Lp(a), lipoprotein (a); percentage sdLDL, percentage of total LDL that is small dense LDL; NEFA, nonesterified fatty acids; HOMA-IR, homeostasis model of assessment of insulin resistance. Differences from baseline were used as the response variable (SAS PROC MIXED). The differences between the change from baseline in the isoflavone and placebo arms for plasma total, HDL, and LDL cholesterol; triacylglycerol; Lp(a); glucose; insulin; HOMA-IR; and NEFA were not significant ($P < 0.05$). The difference between isoflavones and placebo in percentage sdLDL was small but significant ($P = 0.044$).
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TABLE 4
Summary of significant treatment × genotype interactions after analysis of the change from baseline of plasma lipid, glucose, and insulin concentrations during the intervention (isoflavone-enriched cereal bars) and placebo (cereal bars) arms

<table>
<thead>
<tr>
<th>Genotype and outcome</th>
<th>Treatment × genotype interaction (P²)</th>
<th>Significant differences between the treatments</th>
<th>Genotype[^a]</th>
<th>P[^a]</th>
<th>Comments</th>
</tr>
</thead>
<tbody>
<tr>
<td>eNOS: Gln298Asp</td>
<td></td>
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<tr>
<td>NEFA</td>
<td>0.009</td>
<td>AA (n = 7)</td>
<td>0.007</td>
<td>Small subject numbers in genotype of interest</td>
<td></td>
</tr>
<tr>
<td>Total cholesterol</td>
<td>0.021</td>
<td>AA (n = 8)</td>
<td>0.048</td>
<td>Small subject numbers in genotype of interest</td>
<td></td>
</tr>
<tr>
<td>LDL cholesterol</td>
<td>0.021</td>
<td>AA (n = 8)</td>
<td>0.046</td>
<td>Small subject numbers in genotype of interest</td>
<td></td>
</tr>
<tr>
<td>Lp(a)</td>
<td>0.005</td>
<td>AA (n = 8)</td>
<td>0.017</td>
<td>Small subject numbers in genotype of interest; poor fit to normality</td>
<td></td>
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<tr>
<td>ERβ AluI</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total cholesterol</td>
<td>0.111</td>
<td>GA (n = 48)</td>
<td>0.046</td>
<td>Clinically relevant change?</td>
<td></td>
</tr>
<tr>
<td>Total:HDL cholesterol</td>
<td>0.007</td>
<td>GA (n = 48)</td>
<td>0.039</td>
<td>Poor fit to normality</td>
<td></td>
</tr>
<tr>
<td>LDL cholesterol</td>
<td>0.011</td>
<td>GA (n = 48)</td>
<td>0.036</td>
<td>Clinically relevant change?</td>
<td></td>
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<tr>
<td>ERβ(cx) Tsp509I</td>
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<td></td>
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<tr>
<td>HDL cholesterol</td>
<td>0.009</td>
<td>AA (n = 31)</td>
<td>0.002</td>
<td></td>
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<tr>
<td>CETP Tsp509I</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total:HDL cholesterol</td>
<td>0.004</td>
<td>B1B2 (n = 55)</td>
<td>0.044</td>
<td>Poor fit to normality</td>
<td></td>
</tr>
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</table>

[^a] P values are shown for all significant (P < 0.05) treatment × genotype interactions (SAS PROC MIXED). Differences from baseline were used as the response variable. Subjects with the ERβ(cx) Tsp509I AA genotype, but not in the total:HDL cholesterol, showed a significant mean increase in HDL of 0.21 mmol/L after isoflavone treatment compared with 0.04 mmol/L in the placebo period (P < 0.005), a clinically relevant and statistically robust change.

[^b] After slice tests.

a result of differences in the AA homozygote. The number of subjects who were AA homozygotes for the eNOS polymorphism studied here was low (n = 8) and insufficiently robust to allow clear conclusions as to a true treatment × genotype interaction. However, 3 treatment × genotype interactions remained: ERβ(cx) Tsp509I × HDL cholesterol, ERβ (AluI) × LDL cholesterol, and ERβ (AluI) × TC. The change in plasma HDL-cholesterol concentrations was significantly (P < 0.01) different for the ERβ(cx) Tsp509I genotype (Figure 1). Slice tests showed that the difference in the change from baseline in HDL cholesterol between isoflavones and placebo was significant in the AA genotype (P < 0.005) but not in GG (n = 26) or GA (n = 54); in addition, the tests showed that the significant difference in HDL-cholesterol change in the AA group was due to a 13% increase after isoflavone treatment, as compared with a 2% increase after placebo treatment. This effect was robust to the removal of outliers.

Changes in plasma LDL-cholesterol and TC concentrations were significantly different according to ERβ AluI genotype (P < 0.05 for both interactions). Slice tests showed that the difference in the response of LDL cholesterol and TC between isoflavones and placebo was significant (P < 0.05 for both) in the GA genotype but not in GG or AA. The change in TC concentrations was an increase of ≈3% after isoflavone treatment and a decrease of 1% after placebo treatment. For LDL-cholesterol concentrations, the changes were an increase of ≈2% after isoflavone treatment and a decrease of 2% after placebo treatment. These effects were robust to the removal of outliers, but the magnitude of these changes is unlikely to be of any clinical relevance.

There were no differences between equol producers (n = 33) and equol nonproducers (n = 84) in plasma TC, LDL or HDL cholesterol, total:HDL, triacylglycerol, NEFA, glucose, insulin, or HOMA response to isoflavones and placebo. The interaction between equol group and treatment was significant (P < 0.05) for Lp(a), with a difference in Lp(a) concentrations in the equol producers. However, these results should be treated with caution, because the data were highly skewed and the fit to normality was poor, even after log transformation of the data. The interaction between equol group and treatment also was significant (P < 0.05) for %sdLDL, with the difference in the equol nonproducers not occurring after treatment with the isoflavone-enriched foods. The magnitude (%) of these changes, however, is likely to be clinically insignificant. In equol producers, the %sdLDL values at 0 and 8 were 24.51 ± 8.49% and 26.28 ± 11.82%, respectively, for isoflavones and 23.55 ± 7.85% and 26.14 ± 9.99%, respectively, for placebo. In equol nonproducers, the values at 0 and 8 were 22.83 ± 15.24% and 23.54 ± 15.77% for isoflavones and 22.79 ± 12.75% and 22.29 ± 17.12% for placebo. Mean systolic and diastolic blood pressures also did not differ significantly according to equol status. In equol producers, the systolic values at 0 and 8 were 113 ± 14 and 117 ± 13 mm Hg, respectively, for isoflavones and 117 ± 14 and 117 ± 14 mm Hg, respectively, for placebo. In equol nonproducers, the systolic values at 0 and 8 were 119 ± 17 and 121 ± 16 mm Hg, respectively, for isoflavones and 119 ± 17 and 119 ± 17 mm Hg, respectively, for placebo. In equol producers, the diastolic values at 0 and 8 were 71 ± 9 and 72 ± 10 mm Hg, respectively, for isoflavones and 72 ± 10 and 72 ± 8 mm Hg, respectively, for
placebo. In equol nonproducers, the diastolic values at t0 and t8 were 74 ± 10 and 75 ± 9 mm Hg, respectively, for isoflavones and 74 ± 11 and 74 ± 10 mm Hg, respectively, for placebo.

**DISCUSSION**

It has been recognized for many years that reductions in circulating TC, LDL cholesterol, and triacylglycerols and increases in HDL cholesterol can result from consumption of soy protein. Most soy protein studies have shown lipid-lowering effects (21), but the few studies that have included supplementation with isoflavones alone have, to date, produced negative results (22–26). Many of these soy protein studies had low statistical power, and only a few of those included a randomized, double-blind, placebo-controlled crossover design. Therefore, the question of a possible hypocholesterolemic effect of isoflavones is still open. Furthermore, in most isoflavone intervention studies to date, the treatment and placebo were administered in a capsule, which increases the possibility of poor intestinal absorption in the absence of a food vehicle. In the current study, isoflavones were administered as a food, and serum isoflavone analysis confirmed that genistein and daidzein concentrations after isoflavone treatment were 17 and 26 times as high as after placebo treatment.

The current study confirms previous findings that the consumption of genistein and daidzein has no effect on plasma TC, HDL or LDL cholesterol, or triacylglycerol (25–27). These findings agree with those of previous studies—apart from a minority of studies that found adverse effects (28–30)—of no effect of isolated isoflavones on LDL subclass distribution, found a weak but significant treatment effect on %sdLDL, which occurred after placebo treatment but not after isoflavone treatment. The changes were small, and therefore their biological significance is questionable. The effect of isolated isoflavones on plasma insulin concentrations is not yet clear. Although Cheng et al (32) found beneficial effects of isolated isoflavones on plasma insulin and glucose, the current study agrees with the findings of Blakesmith et al (31) and Nikander et al (25) that isoflavone-enriched foods have no significant effects on insulin or glucose concentrations in healthy postmenopausal women.

The observed lack of efficacy of isoflavones in the current study could be due to a number of factors. First, although mean baseline TC and LDL-cholesterol concentrations in this postmenopausal group were slightly higher than those in the overall adult population, it is possible that baseline lipoprotein metabolism and insulin sensitivity were not impaired to any clinically significant extent in the group as a whole, and therefore there was insufficient scope for a detectable improvement by isoflavone treatment. Second, the subjects in the current intervention study consumed isoflavone-enriched cereal bars for 8 wk; although seen as a biologically acceptable length of time for a clinical intervention study, that span may be an unrealistically short period in which to observe, in epidemiologic studies, the benefits from weak estrogenic plant compounds that have been observed after a lifetime exposure to soy foods. Third, the effects of isoflavones may depend on the type and the dose. The current study used a dose of 50 mg/d with genistein:daidzein at 2:1 because this was thought to represent a typical dietary intake in Asian countries such as Japan (33, 34). However, because of the relatively short exposure time, this dose may not have been sufficient to exert significant protective effects. Serum and urinary isoflavone analyses confirmed that the supplements were biologically available, but it is possible that isoflavones may be cardioprotective only when contained in the soy protein matrix, especially in light of previous studies showing that isoflavone-rich soy protein reduces cholesterol to a greater extent than does isoflavone-depleted soy protein (35).

To our knowledge, apart from one study that examined the effect of apolipoprotein E genotype (36), this is the first study to investigate the effect of various polymorphisms in genes relevant to estrogen action and lipoprotein metabolism on the lipid response to isoflavone supplementation. In the current study, postmenopausal women with the ERβ(cx) Tsp509I AA genotype (but not the GA or GG genotypes) showed a significant mean increase in HDL cholesterol (0.21 mmol/L) after isoflavone treatment but an increase of only 0.04 mmol/L after placebo treatment. The molecular mechanism of this genotype × isoflavone interaction is not yet known. ERβ(cx) Tsp509I polymorphism is located in exon 9 of ERβ(cx), a splice variant of the ERβ gene that uses an alternative exon of the gene (37). ERβ(cx) can act as a dominant-negative inhibitor of ERα, and it influences the response to endocrine therapy in breast cancer and thus is a potential predictive molecule on response to estrogen-like compounds (39). However, the effect of this isoform on risk markers for the cardiovascular system is currently unknown. The current results are difficult to interpret because the ERβ(cx) isoform lacks the amino acid residues of the ERβ that are required for estrogen binding, and therefore it is incapable of triggering an estrogen-induced transcriptional activation (39). Isoflavones may be acting via ERβ(cx) through nongenomic pathways, such as activation of cell-signaling pathways. It should also be taken into account that this polymorphism in ERβ(cx) isoform is positioned in the 3’UTR, a noncoding region, which may suggest that it affects mRNA stability (37). It is possible that the ERβ(cx) Tsp509I polymorphism is in linkage disequilibrium with another gene variant that may be involved in metabolic pathways of HDL synthesis such as apolipoprotein A-I synthesis and hepatic lipase activity (40). The molecular mechanisms that would explain the interplay between polymorphisms in the ERβ(cx) isoform and the HDL response to isoflavone intake are a novel area of potential research and warrant further investigation.

The difference in the LDL-cholesterol and TC response between isoflavones and placebo was significant in the GA genotype of the ERβ A114l polymorphism but not in GG or AA. Although statistically significant, the effect of a difference of 1% to 3% in plasma TC or LDL-cholesterol concentrations according to treatment is biologically small and unlikely to affect overall risk of CVD. In addition, it is difficult to suggest a possible mechanism whereby a heterozygous genotype would affect phenotype, at the same time that the 2 homozygous genotypes do not.

It was previously suggested that the status of a person as an equol producer or equol nonproducer may influence the person’s response to dietary isoflavone supplementation (13). A recent study by Kreijkamp-Kaspers et al (41) found that the response of the blood pressure to soy protein differed when 202 postmenopausal women aged 60–75 y were subdivided into equol producers and nonproducers. However, the current study shows that the differences between the groups in concentrations of plasma lipids, glucose or insulin or in blood pressure according to equol-producing status were not significant. The postmenopausal women in the current study were younger (46–70 y old) than
were those in the study of Krijkamp-Kaspers et al (41). The subjects in the current study, therefore, were more likely to have a low risk of CVD at the outset, and, consequently, differences with respect to equal status may not be detected.

In summary, this randomized, controlled crossover study in 117 postmenopausal women showed that isoflavones, when provided in the form and the dose used in this study, have no effects on a range of blood lipids and lipoproteins, glucose, or insulin in a surrogates marker of insulin resistance, HOMA-IR. On the basis of this substantial evidence, and considering the fact that the effects on a range of circulating inflammatory factors were few (20), little scientific basis exists for the recommendation of soy isoflavones in all healthy postmenopausal women for the improvement of cardiovascular health. These results are of considerable importance because it was previously unclear whether isolated isoflavones (ie, those not contained within soy protein) had any cardioprotective benefits. However, the variation in plasma HDL-cholesterol response to isoflavones according to ERβ(ox) Tsp509I genotype suggests that a subgroup of the population may benefit from a high isoflavone intake, and further research into variations in the ERβ gene and the interplay with dietary isoflavones would be of the utmost interest.

WLH, KV, JH, SB, CK, H-JFZ, MF, FB, DT, J-P, A-MM, and CMW contributed to the design and conduct of the study and to the collection of samples and data. WLH, KV, AMM, and CMW were responsible for laboratory analysis and manuscript preparation. MN, KD-W, and J-ÅG analyzed the estrogen receptor β genotypes. TD carried out part of the statistical analysis by using SAS. None of the authors had any financial or personal conflict of interest.

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