Soy-isoflavone-enriched foods and markers of lipid and glucose metabolism in postmenopausal women: interactions with genotype and equol production1–3

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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); insulin; total, HDL, and LDL cholesterol; cholesterol; 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 α (XbaI and PvuII), estrogen receptor β (AluI), and estrogen receptor β(cx) (Tsp509I)] and leptin receptor (Glu223Arg)] 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 (genistin-to-daidzein 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 α. However, beneficial effects of isolated isoflavones on lipid biomarkers of cardiovascular disease risk have not yet been shown. 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 (7). However, a lack of beneficial effects on CVD, evidence of greater risk of thrombosis (8) and higher concentrations of C-reactive protein (9).

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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 (1). 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).
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(ck)], 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 completed a detailed health and lifestyle questionnaire by telephone. Persons interested in participating in the study completed a detailed health and lifestyle questionnaire by telephone. Women who met the inclusion criteria after the telephone interview were invited to take part in a physical examination and biochemical screening. A standard subject-information sheet was given to volunteers at the screening visit, and, if the volun-

teeers were willing to proceed, they were asked to sign the study’s informed consent form.

All subjects had a body mass index (BMI; in kg/m²) of 20 to 32, had not menstruated in the previous 12 mo, had not taken HRT for the previous 6 mo or antibiotics for the previous 3 mo, and had not been vaccinated in the previous 3 mo. In addition, subjects who had a history of CVD, inflammatory disease, diabetes, or other significant medical history or who were taking certain medications known to affect the outcome measures—e.g., antiinflammatories, hypertension medication, and statins—were excluded. The subjects who were included in the current study either did not smoke or smoked < 5 cigarettes/wk, consumed diets that were low in soy, did not use dietary supplements, were not following a weight-reducing diet, were not regular blood donors, and were not trained athletes or heavy exercisers. To confirm that the subjects were fit to participate, routine blood chemistry tests were carried out for liver and kidney function, hemoglobin, glucose, and plasma total cholesterol (TC), HDL cholesterol, and triacylglycerol. Women with TC > 8 mmol/L, triacylglycerol > 3 mmol/L, hemoglobin < 12 mg/dL, and diastolic and systolic blood pressures > 160 and > 90 mm Hg, respectively, were excluded and were advised to consult their general practitioners. The menopausal status of women who had last menstruated between 1 and 3 y before the start of the study was confirmed by measurements of follicle-stimulating hormone and luteinizing hormone; postmenopausal status required measurements of 41–124 IU/L and > 14 IU/L, respectively.

All volunteers gave written informed consent before beginning the study and were free to withdraw from the study at any time without obligation. Each study center obtained ethical approval from its local ethics and research committees.

Study design

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.

Soy-isoflavone-enriched foods

Subjects were asked to incorporate 2 cereal bars/d 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.
Dietary intake was evaluated at 3 timepoints during the study by the collection of 3-d food records. Diet diaries were completed at baseline (t₀) and midway (at 4 wk) during each intervention period (t₄ of the placebo intervention arm and t₈ 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 non-dominant 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 buffy coat 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 Coomassie 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 was extracted from stored buffy coats by using the QIAamp DNA blood mini-kit (Qiagen Ltd, Crawley, United Kingdom). ERα, eNOS, apolipoprotein E, and CETP polymorphisms were analyzed on 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 equol 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.
TABLE 1
Summary of primer sequences and polymerase chain reaction (PCR) conditions for analysis of estrogen receptor α (ERα), estrogen receptor β (ERβ), endothelial nitric oxide synthase (eNOS), apolipoprotein E (ApoE), and cholesteryl ester transfer protein (CETP) gene polymorphisms

<table>
<thead>
<tr>
<th>Gene and polymorphisms</th>
<th>Primers</th>
<th>PCR conditions</th>
<th>Restriction enzymes</th>
<th>Fragment separation</th>
</tr>
</thead>
<tbody>
<tr>
<td>ERα Xhol</td>
<td>5′-CTGCCACCTTATCTGTATCTTTTCTATTCTTC-3′ (F)</td>
<td>95°C/1 min 61°C/1 min 72°C/30 s</td>
<td>Xhol</td>
<td>1.2% agarose gel</td>
</tr>
<tr>
<td></td>
<td>5′-CTCTTTCCTCCCAACCCTCTCTTCTCTTA-3′ (R)</td>
<td></td>
<td></td>
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</tbody>
</table>

ERα PvuII  | 5′-CTGCCACCTTATCTGTATCTTTTCTATTCTTC-3′ (F)  | 95°C/1 min 61°C/1 min 72°C/30 s  | PvuII  | 1.2% agarose gel |
|  | 5′-CTCTTTCCTCCCAACCCTCTCTTCTCTTA-3′ (R)  |                          |

ERβ 1730G→A  | 5′-CCACGCTCTCACATACAAGTACG-3′ (F)  | 94°C/1 min 60°C/1 min 72°C/1 min  | AlaI  | 3% agarose gel |
|  | 5′-GGTTTAGGGGTGGGGTAGACTG-3′ (R)  |                          |

ERβ ERβ(cx) + 56G→A  | 5′-CTCTACTTAAAGGCAAGAAGACCTCTTC-3′ (F)  | 94°C/1 min 60°C/1 min 72°C/1 min  | Tsp509I  | 3% agarose gel |
|  | 5′-CTCTACTTAAAGGCAAGAAGACCTCTTC-3′ (R)  |                          |

Glu298Asp  | 5′-AAGCGCAAGAAGACCTCTCTCA-3′ (F)  | 94°C/30 s 57°C/1 min 72°C/1 min  | Mbol  | 2% agarose gel |
|  | 5′-CCACGCTCTCACATACAAGTACG-3′ (R)  |                          |

ApoE Apo E2, E3, and E4  | 5′-ACAGAATTCGCCCGCGCTGACAC-3′ (F)  | 95°C/1 min 61°C/1 min 72°C/30 s  | HhaI  | 10% polyacrylamide gel |
|  | 5′-AAACTCAACGACACTCTCCT-3′ (R)  |                          |

CETP<sup>1</sup>  | 5′-ACGCGCGGCGGCGGCGGCGG-3′ (F)  | 95°C/1 min 61°C/1 min 72°C/30 s  | TaqI  | 1.5% agarose gel |
|  | 5′-GATGCGCGGCGGCGGCGGCGG-3′ (R)  |                          |

LEPR Glu223Asp  | 5′-AACTTCACGACGACACTCTTTC-3′ (F)  | 94°C/30 s 50°C/45 s 72°C/1 min  | MspI  | 10% polyacrylamide gel |
|  | 5′-TGACTGACATAGTTGACTGAC-3′ (R)  |                          |

PCR conditions

Denaturation  | Annealing  | Extension  | Restriction enzymes  | Fragment separation |

95°C/1 min  | 61°C/1 min  | 72°C/30 s  | Xhol  | 1.2% agarose gel |

95°C/1 min  | 61°C/1 min  | 72°C/30 s  | PvuII  | 1.2% agarose gel |

94°C/1 min  | 60°C/1 min  | 72°C/1 min  | AlaI  | 3% agarose gel |

94°C/1 min  | 60°C/1 min  | 72°C/1 min  | Tsp509I  | 3% agarose gel |

94°C/30 s  | 57°C/1 min  | 72°C/1 min  | Mbol  | 2% agarose gel |

95°C/1 min  | 61°C/1 min  | 72°C/30 s  | HhaI  | 10% polyacrylamide gel |

95°C/1 min  | 61°C/1 min  | 72°C/30 s  | Tsp509I  | 3% agarose gel |

94°C/30 s  | 57°C/1 min  | 72°C/1 min  | Mbol  | 2% agarose gel |

95°C/1 min  | 61°C/1 min  | 72°C/30 s  | HhaI  | 10% polyacrylamide gel |

94°C/30 s  | 50°C/45 s 72°C/1 min  | MspI  | 10% polyacrylamide gel |

<sup>1</sup> F, forward; R, reverse. Forty cycles (except where indicated otherwise) of the denaturation, annealing, and extension steps were used to amplify the target DNA sequence.

<sup>2</sup> Thirty-five cycles were used.

<sup>3</sup> Twenty-eight cycles were used.

RESULTS

Eight hundred sixty-two telephone screenings were carried out, and 612 subjects were excluded on the basis of the screening
treatment arm was > 936 nmol/L (12). This amount approxi- 
mates a urinary yield of >0.45 mg/d. In one case, a urine sample 
was unavailable, so a serum concentration of >39 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 t4 of each inter-
vention arm. Macronutrient intakes at baseline were 15%, 34%, 
and 47% of energy as protein, fat, and carbohydrate, respecti-
vely, which were reported previously (20). The differences in en-
ergy 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 concen-
trations, 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 signifi-
cant reduction from baseline %sdLDL after placebo treatment.

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

The change from baseline for plasma triacylglycerol, glucose, 
and insulin concentrations did not differ according to genotype. 
However, several treatment × genotype interactions were found 
for other outcome markers: 1) eNOS (Glu298Asp) × NEFA, TC, 
LDL cholesterol, and Lp(a) interactions; 2) ERβ (AluI) × TC, 
total:HDL cholesterol, and LDL-cholesterol interactions; 3) 
ERβ(cx) (Tsp509I) × HDL cholesterol interaction; and 4) CETP 
(TaqIB) × 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 × genotype interactions shown at first-
stage analysis should be treated with caution because of prob-
lems with fit to normality before and after log transformation and 
sensitivity to outliers. In addition, slice tests showed that signifi-
cant interactions between eNOS genotype and treatment for 
NEFA, TC, LDL cholesterol, and Lp(a) concentrations were all

### Table 2

|                        | Isoflavone arm | Placebo arm | P  
|------------------------|---------------|-------------|------
| Genistein (mg/d)       | 0.37 ± 0.40[114]² | 0.37 ± 0.40 | 0.67 ± 1.21 <0.0001 |
| Daidzein (mg/d)        | 0.16 ± 0.22[114] | 0.22 ± 0.30 | 0.22 ± 0.32 <0.0001 |
| Equol (mg/d)           | 0.08 ± 0.16[114] | 0.08 ± 0.05 | 0.11 ± 0.08 <0.0001 |
| Equol producers        | 0.10 ± 0.05[31]  | 0.09 ± 0.05[33] | 0.13 ± 0.09[33] <0.0001 |
| Equol nonproducers     | 0.07 ± 0.06[82]  | 0.08 ± 0.05[84] | 0.094 ± 0.08[83] <0.0001 |

### Table 3

|                       | Isoflavone arm | Placebo arm | t  
|-----------------------|---------------|-------------|------
| Cholesterol           |               |             |      
| Total (mmol/L)        | 6.03 ± 1.32[115] | 6.13 ± 1.34 | 5.96 ± 1.19 6.10 ± 1.22[116] |
| LDL (mmol/L)          | 3.88 ± 1.11[115] | 3.84 ± 1.11 | 3.81 ± 1.02 3.83 ± 1.04[116] |
| HDL (mmol/L)          | 1.60 ± 0.39[115] | 1.74 ± 0.47 | 1.62 ± 0.35 1.71 ± 0.41[116] |
| Total:HDL             | 3.91 ± 1.08[115] | 3.71 ± 1.01 | 3.83 ± 1.04 3.73 ± 1.14[116] |
| Triacylglycerol (mmol/L) | 1.21 ± 0.50[115] | 1.22 ± 0.48 | 1.19 ± 0.46 1.22 ± 0.52[116] |
| Lp(a)                 | 226.2 ± 265.3[112] | 216.40 ± 244.4[112] | 227.38 ± 250.27[114] 233.68 ± 264.44[112] |
| NEFA (umol/L)         | 447.4 ± 193.8[113] | 411.98 ± 139.32[113] | 426.74 ± 181.35[114] 405.41 ± 185.41[113] |
| Glucose (mmol/L)      | 5.34 ± 0.61[114]  | 5.21 ± 0.60[113] | 5.32 ± 0.58[114] 5.27 ± 0.57[113] |
| Insulin (pmol/L)      | 34.8 ± 18.2[108]  | 35.48 ± 17.89[107] | 35.60 ± 18.04[108] 34.87 ± 21.02[107] |
| HOMA-IR               | 1.21 ± 0.71[105]  | 1.20 ± 0.67[105] | 1.24 ± 0.65[106] 1.21 ± 0.75[105] |

All values are ± 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).
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). Slicex tests showed that the difference in the change from baseline in HDL cholesterol between isoflavones and placebo was significant in the AA group (13%) and insufficiently robust to reach statistical significance for %sdLDL, with the difference in the equol nonproducers being mainly due to a decrease after the placebo treatment that did not occur 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 mm Hg, respectively, for isoflavones 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,
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 Lp(a) (23–25, 31). The current study, the first to examine the effect of isoflavone consumption 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-1 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 A1A1 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 Krijkkamp-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 equol 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 or a surrogate 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 isoflavone supplementation to 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(cx) 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, JP, 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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