

# Soy-isoflavone-enriched foods and inflammatory biomarkers of cardiovascular disease risk in postmenopausal women: interactions with genotype and equol production<sup>1-3</sup>

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## ABSTRACT

**Background:** Dietary isoflavones are thought to be cardioprotective because of their structural similarity to estrogen. The reduction of concentrations of circulating inflammatory markers by estrogen may be one of the mechanisms by which premenopausal women are protected against cardiovascular disease.

**Objective:** Our aim was to investigate the effects of isolated soy isoflavones on inflammatory biomarkers [von Willebrand factor, intracellular adhesion molecule 1, vascular cell adhesion molecule 1 (VCAM-1), E-selectin, monocyte chemoattractant protein 1, C-reactive protein (CRP), and endothelin 1 concentrations]. Differences with respect to single-nucleotide polymorphisms in selected genes [estrogen receptor  $\alpha$  (*Xba*I and *Pvu*II), estrogen receptor  $\beta$  [*ER* $\beta$  (*Alu*I) and *ER* $\beta$ (*cx*) (*Tsp*509I), endothelial nitric oxide synthase (*Glu*298Asp), apolipoprotein E (*Apo* *E*2, *E*3, and *E*4), and cholesteryl ester transfer protein (*Taq*IB)] and equol production were investigated.

**Design:** One hundred seventeen healthy European postmenopausal women participated in this randomized, double-blind, placebo-controlled, crossover dietary intervention trial. Isoflavone-enriched (genistein-to-daidzein ratio of 2:1; 50 mg/d) or placebo cereal bars were consumed for 8 wk, with a washout period of 8 wk between the crossover. Plasma inflammatory factors were measured at 0 and 8 wk of each study arm.

**Results:** Isoflavones improved CRP concentrations [odds ratio (95% CI) for CRP values >1 mg/L for isoflavone compared with placebo: 0.43 (0.27, 0.69)]; no significant effects of isoflavone treatment on other plasma inflammatory markers were observed. No significant differences in the response to isoflavones were observed according to subgroups of equol production. Differences in the VCAM-1 response to isoflavones and to placebo were found with *ER* $\beta$  *Alu*I genotypes.

**Conclusion:** Isoflavones have beneficial effects on CRP concentrations, but not on other inflammatory biomarkers of cardiovascular disease risk in postmenopausal women, and may improve VCAM-1 in an *ER* $\beta$  gene polymorphic subgroup. *Am J Clin Nutr* 2005; 82:1260–8.

**KEY WORDS** Isoflavones, soy, cardiovascular disease, postmenopausal women, inflammatory factors, cell adhesion molecules, C-reactive protein, endothelin 1, von Willebrand factor, monocyte chemoattractant protein 1, estrogen receptor, gene-nutrient interaction

## INTRODUCTION

Cardiovascular disease is partially characterized by chronic inflammation and increased expression of cell adhesion molecules, such as intracellular adhesion molecule 1 (ICAM-1), vascular cell adhesion molecule 1 (VCAM-1), and E-selectin, on the surface of the activated endothelial cell. In addition, synthesis of chemokines, such as monocyte chemoattractant protein 1 (MCP-1), within the endothelial cell is increased. Circulating concentrations of these adhesion molecules are considered to be predictive of cardiovascular disease risk because they indicate a proinflammatory state in the vasculature (1). C-reactive protein (CRP), which is a classic downstream marker of inflammation and mediates the initiation and progression of atherosclerotic plaques by numerous molecular mechanisms (2), is considered to be a strong predictive biomarker of coronary artery disease risk (3, 4). The chronic inflammatory state is also characterized by increased plasma concentrations of von Willebrand factor (vWF), a glycoprotein that is secreted from the endothelium and that has important roles in platelet aggregation and adhesion (5). Furthermore, synthesis of the vasoconstrictor endothelin-1 is increased in atherosclerotic arteries and indicates endothelial dysfunction associated with chronic inflammation (6). Elevated concentrations of these circulating inflammatory factors are also believed to be risk factors for cardiovascular disease.

Substantial evidence indicates that the beneficial effects of estrogen on the cardiovascular system are attributable, in part, to

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a beneficial effect on the vascular endothelium (7–10). Replacement of natural hormones in the form of hormone replacement therapy (HRT) was shown to decrease concentrations of endothelin-1 (11) and cell adhesion molecules (12). Until recently, HRT was widely advocated as an effective means of delaying the progression of atherosclerosis in postmenopausal women. However, the lack of efficacy of HRT with respect to cardiovascular disease progression, evidence of an increased risk of thrombosis (13) and increased concentrations of CRP (14, 15), as well as an increased risk of hormone-dependent cancers has led to a search for alternative therapies to counteract the loss of natural estrogens at menopause.

Isoflavones are phytoestrogens (chemicals that have a structural similarity to estrogen) and can bind to estrogen receptors. The most important dietary source of isoflavones is soy. Epidemiologic evidence in humans suggests that increased soy consumption is cardioprotective. This may be due to the ability of the isoflavones found in soy (genistein, daidzein, and glycitein) to act as estrogen mimics or selective estrogen receptor modulators. In vitro experiments in cultured endothelial cells have shown that isoflavones can have antiinflammatory effects by inhibiting cell adhesion molecule expression (16–18). However, few human intervention studies have reported on the effects of isoflavone supplements on inflammatory biomarkers of cardiovascular disease risk.

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 antioxidant capacity than those of its parent compound (19). Previous studies have indicated that persons greatly vary in their ability to synthesize equol (20), and evidence suggests that responsiveness to isoflavones may vary according to a person's equol-synthesizing capacity (21). Single-nucleotide polymorphisms in cardiovascular disease risk genes or genes that are involved in estrogen action may cause the variability in response to isoflavones, as was shown for HRT therapy and estrogen receptor polymorphisms (22, 23).

The main purpose of this randomized, double-blind, placebo-controlled, crossover dietary intervention study was to examine the effects of isolated soy isoflavone consumption, which was provided within a food vehicle, on a range of circulating inflammatory molecules, including cell adhesion molecules, MCP-1, endothelin-1, CRP, and vWF. Equol and non-equol producers were identified. Genotypes of single-nucleotide polymorphisms for estrogen receptor (ER)  $\alpha$  (*Xba*I and *Pvu*II), ER $\beta$  (*Alu*I), the ER $\beta$  variant ER $\beta$ (cx) (*Tsp*509I), endothelial nitric oxide synthase (*Glu*298Asp), apolipoprotein E (*Apo* *E2*, *E3*, and *E4*), and cholesteryl ester transfer protein (*Taq*IB) were also characterized. The influence of genotype and equol production on the inflammatory response to isoflavones was then evaluated.

## SUBJECTS AND METHODS

### Subjects and study design

Each study center obtained ethical approval from their local ethics and research committees. All volunteers gave written informed consent before beginning the study and were free to withdraw from the study at any time without obligation. Healthy postmenopausal women aged between 45 and 70 y were recruited from the surrounding areas of 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 Istituto Nazionale di Ricerca per gli Alimenti e la Nutrizione (Rome, Italy). The study was advertised in the local media and via internal e-mails and posters at each study center. Women who were interested in participating in the study contacted the investigators at the 4 centers and were interviewed over the telephone with a detailed screening questionnaire. The women who met the inclusion criteria were invited to take part in a physical examination and a biochemical screening. All subjects had a body mass index (BMI; in kg/m<sup>2</sup>) between 20 and 32, had not menstruated in the previous 12 mo, had not received HRT in the previous 6 mo or antibiotics at screening in the previous 3 mo, and had not been vaccinated in the previous 3 mo. In addition, women who had a history of cardiovascular disease, inflammatory disease, diabetes, or other significant medical history, or those who were receiving certain medications known to affect the outcome measures (eg, antiinflammatories, hypertension medication, or statins) were excluded from the study. The subjects who were included in the present 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. Routine blood chemistry tests were carried out for liver and kidney function and for hemoglobin, glucose, and plasma total cholesterol, HDL cholesterol, and triacylglycerol concentrations to confirm that the subjects were fit to participate. Women with a total cholesterol concentration >8 mmol/L, triacylglycerol concentrations >3 mmol/L, hemoglobin concentrations <12 mg/dL, or a blood pressure >160/90 mm Hg were excluded and advised to consult their general practitioners. Women who had last menstruated between 1 and 3 y before the start of the study had their menopausal status confirmed by a measurement of their follicle-stimulating hormone (41–124 IU/L) and luteinising hormone (>14 IU/L) concentrations. A standard subject information sheet was given to the subjects at their screening visit, and if the subjects were willing to proceed with the study they were requested to sign the study informed consent form.

A total of 120 postmenopausal women (30 per center) were required to complete the study; 140 women were recruited to allow for dropouts. The study was a placebo-controlled, two 8 wk double-blind crossover design with an 8-wk washout period. The subjects were assigned isoflavones or placebo during arm 1 by stratified randomization according to age, BMI, and triacylglycerol concentrations.

### Soy-isoflavone-enriched foods

The subjects were requested to incorporate 2 cereal bars/d into their normal diet during the intervention periods, one with breakfast and one in the late afternoon or evening. During the test arm, the subjects consumed cereal bars that were identical to the cereal bars consumed during the placebo arm, except the products were enriched with isoflavones (50 mg/d). The isoflavone extract used was Solgen 40 (Solbar Plant Extracts Ltd, Ashdod, Israel) and had a genistein-to-daidzein ratio of 2:1. The cereal bars (40 g) were manufactured by a commercial company in the United Kingdom (Efamol Ltd, Manchester, United Kingdom) and had an average nutrient content of 652 kJ energy, 2.6 g protein, 17.3 g carbohydrate, 8.5 g fat, 1.8 g fiber, and 0.012 g sodium. The 4 different flavors of cereal bars that were offered to the subjects

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.

### Diet diaries

Dietary intake was evaluated at 3 time points during the study by the collection of 3-d food records. Diet diaries were completed at baseline (*t*<sub>0</sub>) and midway during each intervention period at 4 wk (*t*<sub>4</sub> of the placebo and isoflavone intervention arms). Estimated amounts and weights of foods consumed were recorded in the diaries, and the diaries included 2 weekdays and 1 weekend day. The dietary intake of the Danish, German, English, and Italian subjects was analyzed at each center with the use of food-composition databases that were developed in each country.

### Sample collection, assays, and blood pressure measurements

To standardize the food consumption of the subjects, a set low-fat (<15 g fat) recipe evening meal was consumed by the subjects each evening before blood samples were taken. On the morning of each visit [weeks 0, 4, and 8 (*t*<sub>0</sub>, *t*<sub>4</sub>, and *t*<sub>8</sub>)], the subjects attended the Clinical Unit after 12 h of fasting. Plasma vWF, ICAM-1, VCAM-1, E-selectin, MCP-1, and endothelin-1 concentrations were analyzed only at *t*<sub>0</sub> and *t*<sub>8</sub>, whereas CRP was analyzed at *t*<sub>0</sub>, *t*<sub>4</sub>, and *t*<sub>8</sub>. Blood was collected in 10-mL EDTA-coated tubes for the analysis of endothelin-1 and CRP and for buffy coat extractions. After centrifugation at 1600 × *g* for 10 min at 4 °C, plasma was stored at −20 °C until analyzed. Endothelin-1 concentrations were measured at the University of Reading, Reading, United Kingdom, with a Parameter human endothelin-1 immunoassay kit (R&D Systems Europe, Abingdon, United Kingdom), which had inter- and intraassay CVs of 7.0 and 9.0%, respectively. High sensitive CRP was measured at the Royal Veterinary and Agricultural University, Frederiksberg, Denmark, with a chemiluminescent immunometric assay (Diagnostic Products Corporation, Kjøge, Denmark) and an Immulite 1000 analyzer (Diagnostic Products Corporation). Inter- and intraassay CVs were 3.4 and 3.7%, respectively.

For the analysis of vWF, blood was collected in 4.5-mL Na citrate evacuated tubes and centrifuged at 3000 × *g* for 15 min at 20 °C, and the plasma was stored at −80 °C until it was analyzed at the University of Reading, Reading, United Kingdom. vWF concentrations were measured with the Von Willebrand Factor Antigen immunoassay (Quadrant Ltd, Epsom, United Kingdom), which had inter- and intraassay CVs of 10.6 and 15.0%, respectively. For E-selectin, VCAM-1, ICAM-1, and MCP-1 analyses, blood was collected in 10-mL Na heparin evacuated tubes and centrifuged at 1600 × *g* for 10 min at 4 °C, and plasma was stored at −80 °C until analyzed. E-selectin, VCAM-1, ICAM-1, and MCP-1 analyses were conducted at the University of Reading, Reading, United Kingdom; the concentrations were measured with the use of Parameter human E-selectin, VCAM-1, ICAM-1, and MCP-1 immunoassay kits (R&D Systems Europe), and the mean inter- and intraassay CVs were 6.4 and 8.1%, respectively. All plasma inflammatory factors were analyzed at *t*<sub>0</sub> and *t*<sub>8</sub>, except for CRP, which was also analyzed at *t*<sub>4</sub>.

### DNA extraction and genotyping

DNA was extracted from the stored buffy coats with the QIAamp DNA blood mini kit (Qiagen Ltd, Crawley, United

Kingdom). ERα, endothelial nitric oxide synthase, apolipoprotein E, and cholesteryl ester transfer protein polymorphisms were analyzed at the University of Reading, Reading, United Kingdom, with PCR amplification followed by enzymatic digestion with specific restriction endonucleases (24, 25, 26). ERβ polymorphisms were analyzed by the collaborating group at the Karolinska Institute, Stockholm, Sweden, with the use of restriction fragment length polymorphism analysis (27). 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 were measured at Wageningen UR, Netherlands, by HPLC (28). Samples were extracted by stirring with 20 mL of a 50% acetonitrile and 50% MilliQ water (Millipore BV, Amsterdam, Netherlands) mixture for 2 h at room temperature. The obtained solution was filtrated (Spartan 30, 0.45 μm RC filter units; Whatman International Ltd, Maidstone, United Kingdom) and diluted with extraction medium if necessary. The sample was injected into a Waters 2690 separations module (Waters Chromatography BV, Etten Leur, Netherlands) with a column oven and a Waters 960 photodiode array (Waters Chromatography BV) detector attached to it. The separation was achieved with the use of a Symmetry C18 column (250 × 4.6 mm, 5 μm; Waters Chromatography BV). The chromatographic conditions were as follows: flow, 1.5 mL/min; volume of injection, 10 μL; column temperature, 40 °C; solvent A, 10 mmol ammonium formate/L in MilliQ water (pH 2.8 with formic acid); and solvent B, gradient grade acetonitrile. The gradient consisted of 15% of B isocratic for 5 min, 15–29% of B for 31 min, and 29–35% of B for 4 min. Detection was carried out in a diode array detector. Photodiode array data between 225 and 300 nm were collected, and a signal of 260 nm was extracted for integration. The peaks were identified against previously recorded standards on the basis of their retention time and UV spectrum. Daidzein, daidzin, 6''-O-acetyldaidzin, genistein, genistin, 6''-O-acetylgenistin, glycitein, glycitin, and 6''-O-acetylglycitin were quantified, and the results were expressed as total isoflavone content and as a percentage of aglycones, glycosides, and acetyl isoflavones.

Urinary and serum genistein, daidzein, and equol concentrations were analyzed at Unilever Corporate Research, Bedfordshire, United Kingdom, with the use of time-resolved (Delfia; PerkinElmer LAS Ltd, Beaconsfield, United Kingdom) fluorescent immunoassays (29). A panel of monoclonal antibodies was prepared for genistein and equol. A monoclonal antibody to daidzein was provided by Dr Fortune Kohen from the Weizmann Institute (Rehovot, Israel). Potential monoclonal antibodies were screened, evaluated, and used to establish rapid, competitive, time resolved fluorescent immunoassays. To facilitate high throughput testing, these assays were established on an AutoDelfia (PerkinElmer) automated analyzer. The ability of the urinary daidzein, genistein, and equol assays to quantify the phytoestrogens in urine samples was assessed by measuring the recovery of known amounts in 5 urine samples with different concentrations of the phytoestrogens (ranges of phytoestrogen added; daidzein range: 15–3930 nM/L; genistein range: 289–18500 nM/L; and equol range: 39–19500 nM/L). The analytic recovery means (±SDs) of phytoestrogen from these urine samples were 90 ± 12% for daidzein (range: 72–113; *n* = 20), 109 ± 20% for genistein (range: 73–140; *n* = 15), and 110 ± 10% for equol (range: 88–124; *n* = 25). The intraassay CVs for

**TABLE 1**  
Summary of forward (F) and reverse (R) primer sequences and PCR conditions for analysis of estrogen receptor  $\alpha$  (ER $\alpha$ ), ER $\beta$ , endothelial nitric oxide synthase (eNOS), apolipoprotein E (APOE), and cholesteryl ester transfer protein (CETP) gene polymorphisms

Gene	Polymorphisms	Primers	PCR conditions				Restriction enzymes	Fragment separation
			Denaturation	Annealing	Extension			
ER $\alpha$ <sup>1</sup>	XbaI	5' -CTGCCACCCTATCTGTATCTTTTCCCTATTCTTC-3' (F) 5' -TCFTTCTCTGCCACCCTGGCGTCCGATTATCTGA-3' (R)	95°C/1 min	61°C/1 min	72°C/30 s	XbaI	1.2% agarose gel	
ER $\alpha$ <sup>1</sup>	PvuII	5' -CTGCCACCCTATCTGTATCTTTTCCCTATTCTTC-3' (F) 5' -TCFTTCTCTGCCACCCTGGCGTCCGATTATCTGA-3' (R)	95°C/1 min	61°C/1 min	72°C/30 s	PvuII	1.2% agarose gel	
ER $\beta$ <sup>1</sup>	1730G→A	5' -CCAAGCCTGCCATCACCAATGAG-3' (F) 5' -GGTTTAGGGTGGGTAGACTG-3' (R)	94°C/1 min	60°C/1 min	72°C/1 min	AclI	3% agarose gel	
ER $\beta$ <sup>1</sup>	ER $\beta$ (cx) + 56 G→A	5' -CTTACTTAAAGGGCAGAAAAGGCCCTCTC-3' (F) 5' -GTTGGATTGATAATAGAAAAGGAAGGTG-3' (R)	94°C/1 min	60°C/1 min	72°C/1 min	Tsp509I	3% agarose gel	
eNOS <sup>2</sup>	Glu298Asp	5' -AAGCAGGAGACAGTGGATGGA-3' (F) 5' -CCCAGTCAATCCCTTTGGTCTCA-3' (R)	94°C/30 s	57°C/1 min	72°C/1 min	MboI	2% agarose gel	
APOE <sup>1</sup>	Apo E2, E3, and E4	5' -ACAGAAATCGCCCCCGCCCTGGTACAC-3' (F) 5' -TAAGCTTGGCACGGCTGTCCAAGGA-3' (R)	95°C/1 min	61°C/1 min	72°C/30 s	HhaI	10% polyacrylamide gel	
CETP <sup>3</sup>	TaqIB	5' -CACTAGCCCAGAGAGAGGTGCC-3' (F) 5' -CTGAGCCCAGCCGACACTAAC-3' (R)	95°C/30 s	60°C/30 s	72°C/45 s	TaqI	1.5% agarose gel	

<sup>1-3</sup>Cycles of denaturation, annealing, and extension steps that were used to amplify the target DNA sequence: <sup>1</sup> 40, <sup>2</sup> 35, and <sup>3</sup> 28.

TABLE 2

Urinary isoflavone yields at baseline (*t*0) and week 8 (*t*8) of the isoflavone and placebo intervention arms<sup>1</sup>

	Isoflavones		Placebo		<i>P</i> <sup>2</sup>
	<i>t</i> 0	<i>t</i> 8	<i>t</i> 0	<i>t</i> 8	
Genistein (mg/d)	0.37 ± 0.40 [114]	7.27 ± 3.58 [117]	0.37 ± 0.40 [117]	0.42 ± 0.32 [117]	< 0.0001
Daidzein (mg/d)	0.16 ± 0.22 [114]	5.76 ± 2.70 [117]	0.22 ± 0.30 [117]	0.22 ± 0.32 [117]	< 0.0001
Equol (mg/d)	0.08 ± 0.06 [114]	0.85 ± 1.43 [117]	0.08 ± 0.05 [117]	0.11 ± 0.08 [117]	< 0.0001
Equol producers <sup>3</sup>	0.10 ± 0.05 [31]	2.61 ± 1.73 [33]	0.09 ± 0.05 [33]	0.13 ± 0.09 [33]	< 0.0001
Equol nonproducers <sup>4</sup>	0.07 ± 0.06 [82]	0.15 ± 0.08 [83]	0.08 ± 0.05 [84]	0.094 ± 0.08 [83]	< 0.0001

<sup>1</sup> All values are  $\bar{x} \pm SD$ , *n* in brackets.<sup>2</sup> *P* values are shown for the treatment effect within a stepwise-generated general linear mixed model. Differences from baseline were used as the response variable. Only subjects with no missing data points were included in the analysis.<sup>3</sup> Urinary equol concentration >0.45 mg/d.<sup>4</sup> Urinary equol concentration <0.45 mg/d.

urinary daidzein concentrations of 31.4 and 786 nmol/L were 4.9% and 2.2%, respectively, and the interassay CVs were 2.4% and 1.4%, respectively. The intraassay CVs for urinary genistein concentrations of 231 and 3700 nmol/L were 13.4% and 3.9%, respectively, and the interassay CVs were 9.7% and 2.2%, respectively. The intraassay CVs for urinary equol concentrations of 19.5, 390, and 1950 nmol/L were 6.7%, 5%, and 4.6%, respectively, and the interassay CVs were 6.7%, 1.5%, and 2.7%, respectively.

### Statistical analysis

SAS 9.1 (SAS Institute Inc, Cary, NC) was used for all statistical analyses (PROC MIXED procedure), except where stated otherwise. Data are given as means ± SDs or means ± SEMs. Changes from baseline [ie, week 8 – week 0 (*t*8 – *t*0)] were used as the dependent variables. If the original data were approximately normally distributed, then changes from baseline were calculated on the original scale; if a log transform was deemed appropriate, then changes from baseline were calculated on the log scale, and these changes then corresponded to a multiplicative change from the baseline original scale. The 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. The fixed effects that were always included in the final model were baseline values, treatment, center, time, and treatment order. The fixed effects that were included in the final model if the effect was significant were treatment × time interaction, treatment order × treatment interaction, baseline × treatment interaction, center × treatment interaction, and baseline values of BMI, age, and triacylglycerols as fixed covariates (these values were used in the stratified randomization procedure). Additional exploratory investigations of equol group and genotype were included in the model. Slice tests were used when statistically significant interactions were found to test for any effects within levels of an interaction. In the case of plasma CRP concentrations, an additional analysis was required because of the instability and lack-of-fit of the linear regression models that were used. A logistic regression (SAS PROC LOGISTIC) was used to model the CRP values at *t*4 and *t*8, with time and the treatment × time interaction included as potentially significant effects. The median (0.995 mg/L) was used to separate the data into a binary variable corresponding to

VAR = 1 if CRP >1 and VAR = 0 if CRP ≤1. A stepwise variable selection procedure was then followed, with the fixed and random effects as previously described and in the same manner as previously described.

SPSS for WINDOWS (version 12.0.1; SPSS Inc, Chicago, IL) was used to calculate the difference between dietary intakes at baseline and midisoflavone intervention arm (*t*4) or midplacebo intervention arm (*t*4) with the use of repeated-measures analysis of variance (ANOVA). The observed genotype frequencies of the ERα and ERβ genes were compared with those expected under the Hardy-Weinberg equilibrium with standard chi-square tests.

### RESULTS

No significant changes in body weight were evident after the dietary intervention. A total of 133 subjects were recruited and 117 subjects completed the study (a dropout rate of 12% with a target of 120 subjects). The mean (±SD) age, BMI, blood pressure, and fasting plasma lipids and glucose concentrations of the subjects at baseline were as follows: age, 57.7 ± 5.4 y; BMI, 25.0 ± 2.9; systolic blood pressure, 120.6 ± 15.4 mm Hg; diastolic blood pressure, 76.1 ± 8.3 mm Hg; total cholesterol, 5.88 ± 0.93 mmol/L; LDL cholesterol, 3.59 ± 0.80 mmol/L; HDL cholesterol, 1.79 ± 0.38 mmol/L; triacylglycerols, 1.10 ± 0.47 mmol/L; and fasting glucose, 5.17 ± 0.47 mmol/L. Compliance was assessed from the study diaries, number of empty cereal bar packets, and serum and urinary isoflavone analyses. Urinary isoflavone concentrations after the isoflavone supplementation and placebo are shown in **Table 2**. After the isoflavone treatment, genistein and daidzein serum concentrations increased 20-fold and 36-fold, respectively, with no significant increase in concentrations evident after the placebo treatment.

The subjects were defined as equol producers if their urinary equol concentration in a 24-h urine sample during the isoflavone treatment arm was >936 nmol/L (20). This approximates to 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 decide the equol producer status. With these cutoffs, 33 of 117 subjects (28.2%) were classified as equol producers.

Dietary intake was assessed at baseline and at *t*4 of each intervention arm. Macronutrient intakes at baseline were 15% of energy as protein, 34% of energy as fat, and 47% of energy as carbohydrate (**Table 3**). No significant differences in energy

TABLE 3

Calculated daily nutrient intakes for baseline (*t*0) and week 4 (*t*4) of the placebo and isoflavone intervention arms<sup>1</sup>

	Baseline <i>t</i> 0	Isoflavones <i>t</i> 4	Placebo <i>t</i> 4	<i>P</i> <sup>2</sup>
Energy (kJ/d)	8450 ± 1953 <sup>3</sup>	8466 ± 1676	8638 ± 1627	0.427
Protein				
(g/d)	75 ± 19	74 ± 15	76 ± 14	0.506
(% of energy)	15	15	15	
Fat				
(g/d)	76 ± 23	76 ± 19	81 ± 38	0.116
(% of energy)	34	33	35	
SFA				
(g/d)	27 ± 14	27 ± 16	26 ± 11	0.749
(% of energy)	12	12	11	
PUFA				
(g/d)	12 ± 4	12 ± 4	14 ± 19	0.251
(% of energy)	5	5	5	
MUFA				
(g/d)	28 ± 13	28 ± 10	29 ± 12	0.368
(% of energy)	12	12	13	
Carbohydrate				
(g/d)	240 ± 66	242 ± 58	246 ± 60	0.608
(% of energy)	47	47	47	
Total dietary fiber (g/d)	23 ± 9	23 ± 8	24 ± 9	0.220
Dietary cholesterol (mg/d)	277 ± 151	249 ± 108	267 ± 112	0.115
Alcohol				
(g/d)	12 ± 15	12 ± 14	11 ± 14	0.313
(% of energy)	4	4	4	

<sup>1</sup> *n* = 113. SFA, saturated fatty acids; PUFA, polyunsaturated fatty acids; MUFA, monounsaturated fatty acids.<sup>2</sup> Repeated-measures ANOVA with treatment as a within-subject factor for the difference between baseline, midplacebo arm (*t*4), and midisoflavone arm (*t*4). No significant differences in the dietary intake of energy or in macronutrient intakes were seen between baseline, midplacebo arm, and midintervention arm.<sup>3</sup>  $\bar{x} \pm$  SD (all such values).

intake or macronutrient intake were observed either across the treatments or compared with baseline.

No significant differences in plasma VCAM-1, ICAM-1, E-selectin, MCP-1, endothelin-1, and vWF concentrations were seen after the 2 intervention periods (Table 4). CRP concentrations, which were measured at *t*4 in addition to *t*8, showed an overall treatment effect ( $P < 0.05$ ), but not when the analysis was repeated without *t*4 ( $P > 0.05$ ). A high degree of skewness

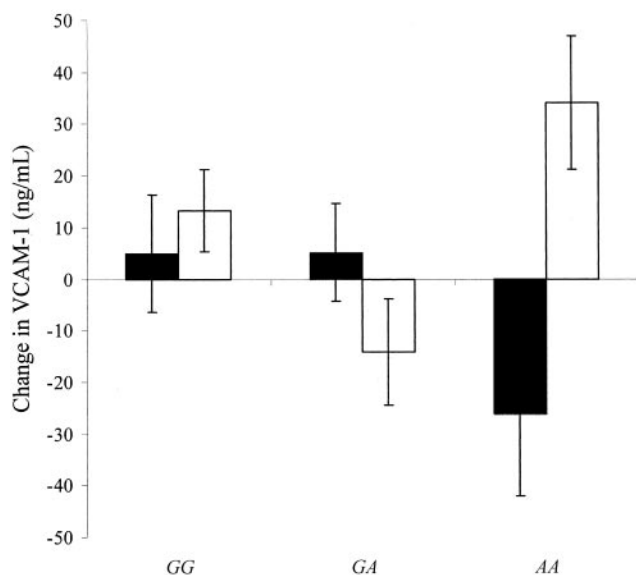
remained in the data after log transformation, particularly at *t*4. Therefore, a logistic regression procedure was performed. The model fitted well to the data ( $P > 0.20$ , Hosmer and Lemeshow goodness-of-fit test) and the results were robust to the removal of influential points. An overall treatment effect including *t*4 and *t*8 was shown ( $P < 0.001$ ). The odds ratio (and 95% Wald CI) estimate for the treatment effect was 0.43 (0.27, 0.69). This indicated that CRP concentrations at *t*4 and *t*8 were significantly

TABLE 4

Plasma inflammatory factor concentrations at baseline (*t*0) and week 8 (*t*8) of the isoflavone and placebo intervention arms<sup>1</sup>

	Isoflavones		Placebo		<i>P</i> <sup>2</sup>
	<i>t</i> 0	<i>t</i> 8	<i>t</i> 0	<i>t</i> 8	
vWF (IU/dL)	104.96 ± 53.77 [116]	105.46 ± 53.07 [116]	103.27 ± 49.46 [115]	99.99 ± 39.92 [116]	0.883
sICAM-1 (ng/mL)	215.04 ± 51.60 [116]	220.40 ± 52.77 [117]	217.45 ± 52.21 [116]	217.78 ± 48.28 [115]	0.147
sVCAM-1 (ng/mL)	504.79 ± 134.39 [114]	503.48 ± 146.66 [113]	498.14 ± 129.00 [114]	499.76 ± 135.88 [111]	0.475
E-selectin (ng/mL)	42.14 ± 15.41 [117]	42.17 ± 15.82 [117]	40.67 ± 15.05 [117]	41.26 ± 15.17 [117]	0.307
MCP-1 (ng/mL)	259.36 ± 95.93 [117]	260.43 ± 101.23 [117]	262.40 ± 85.74 [117]	260.49 ± 106.17 [117]	0.928
Endothelin-1 (pg/mL)	1.15 ± 0.39 [107]	1.20 ± 0.43 [107]	1.15 ± 0.39 [106]	1.21 ± 0.40 [107]	0.800
hs-CRP (mg/L) <sup>3</sup>	1.71 ± 1.89 [114]	1.70 ± 1.89 [113]	1.64 ± 1.73 [116]	1.76 ± 1.83 [113]	0.086

<sup>1</sup> All values are  $\bar{x} \pm$  SD, *n* in brackets. vWF, von Willebrand Factor; sICAM-1, soluble intracellular adhesion molecule 1; sVCAM-1, soluble vascular cell adhesion molecule 1; MCP-1, monocyte chemoattractant protein 1; hs-CRP, highly sensitive C-reactive protein.<sup>2</sup> *P* values are shown for the treatment effect within a stepwise-generated general linear mixed model. Differences from baseline were used as the response variable. Only subjects with no missing data points were included in the analysis. No significant differences were observed between the isoflavone and placebo intervention arms.<sup>3</sup> Hs-CRP was analyzed at *t*4 in addition to *t*0 and *t*8. The mean results at *t*4 were 1.54 ± 1.67 mg/L (isoflavones) and 1.89 ± 3.24 mg/L (placebo). A significant effect of treatment on CRP concentrations was seen when change from baseline at *t*4 and *t*8 was considered ( $P = 0.015$ ); however, *t*4 data were highly skewed despite log transformation, and the statistical data at *t*4 were not robust.



**FIGURE 1.** Mean ( $\pm$ SEM) change in plasma vascular cell adhesion molecule 1 (VCAM-1) concentrations according to estrogen receptor  $\beta$  *AluI* genotype, after treatment with isoflavones (■) and placebo (□). A significant genotype  $\times$  treatment interaction ( $P = 0.023$ ) was observed when the data were analyzed in a stepwise-generated general linear mixed model. Differences from baseline were used as the response variable (only subjects with no missing data points were included in the analysis). Slice tests showed no significant difference in the *GG* genotype group ( $n = 46$ ) or the *GA* genotype group ( $n = 47$ ), but a significant treatment effect was observed in the *AA* genotype group ( $n = 18$ ;  $P = 0.016$ ).

less likely to be  $>1$  mg/L after the isoflavone treatment than after the placebo treatment.

The change in plasma VCAM-1 concentrations ( $t8 - t0$ ) was significantly different according to ER $\beta$  *AluI* genotype ( $P < 0.05$ ) (Figure 1). Slice tests uncovered a significant difference in VCAM-1 response after both isoflavone and placebo treatments in the subjects with the *AA* genotype ( $P < 0.05$ ), but not in the subjects with the *GG* or *GA* genotypes, with a decrease after the isoflavone treatment and an increase after the placebo treatment. The genotype distribution of ER $\beta$  *AluI* did not deviate from Hardy-Weinberg equilibrium (data not shown). The change in plasma ICAM-1, E-selectin, MCP-1, endothelin-1, CRP, and vWF concentrations from baseline did not significantly differ according to genotype. No significant differences in the inflammatory response to isoflavones and placebo were observed between the subjects who were equol producers ( $n = 33$ ) and the subjects who were non-equol producers ( $n = 84$ ).

## DISCUSSION

The aim of the current study was to determine whether the attenuation of endothelial inflammation by isoflavones that was observed in vitro (16–18) was also seen when isoflavones are administered to postmenopausal women in vivo, as measured by circulating concentrations of adhesion molecules and biomarkers of endothelial function. The release of inflammatory factors by the endothelium is integral to the development of atherosclerosis. The present study found no beneficial effects of isoflavone supplementation on circulating concentrations of ICAM-1, VCAM-1, E-selectin, or MCP-1. Previously, soy protein had been found to have no effects on adhesion molecule expression

in vivo (30, 31), and 114 mg encapsulated isoflavones/d was shown to have no effect on plasma E-selectin concentrations (32). However, Teede et al (33) observed a decrease in VCAM-1, together with a decrease in arterial stiffness, after 80 mg formononetin/d in Australian men and women ( $n = 80$ ). Formononetin is a precursor of daidzein that is found in red clover, and, therefore, significant conversion of formononetin to daidzein may have occurred. The dose of daidzein that was used in the present study was much lower than the dose used by Teede et al (17 mg daidzein/d compared with 33 mg genistein/d), which may be a significant factor in the different outcomes. vWF, which is considered to be an important indicator of endothelial dysfunction, did not respond to isoflavone supplementation in the present study, a finding that agrees with a previous soy protein dietary intervention study that was conducted in type 2 diabetics (34). Squadrito et al (35, 36) showed that endothelin-1 concentrations decreased in postmenopausal women after 6 and 12 mo of supplementation with 54 mg genistein/d, although it is not clear whether these 2 studies represent a single sample population or 2 separate study groups. The present results do not replicate those findings, perhaps because the present study had a shorter duration of intervention or used a lower dose of genistein. The use of endothelin-1 as a biomarker of endothelial function has been questioned because endothelin-1 is not secreted into the lumen of the blood vessel but released directly from the endothelial cells into the underlying smooth muscle cells (37). However, some endothelin-1 is discharged into the circulation, and evidence exists that high plasma concentrations of endothelin-1 are associated with cardiovascular diseases (38, 39).

High plasma concentrations of the acute phase protein CRP are indicative of systemic inflammatory stress, and, as such, this biomarker is extremely sensitive to any source of inflammation, including infections. This may limit CRP as a useful biomarker of vascular inflammation in smaller sample populations, although it was previously shown to be a powerful prognostic indicator of cardiovascular disease risk in large populations (eg, in postmenopausal women from the Women's Health Study;  $n = 28\ 263$ ) (3). Plasma CRP concentrations have been shown to increase after HRT, and, because this was an adverse effect, the potential response of CRP to isoflavones was of interest. The findings of the present study show no significant increase in plasma CRP concentrations after supplementation with genistein and daidzein, which suggests that isoflavones, at this dose, do not act as estrogen mimics in this respect. Problems with statistical processing of the highly skewed data suggest that CRP may not be a good biomarker of cardiovascular disease risk in a healthy population sample of this size. However, thorough statistical analysis of the data by logistic regression to correct the poor fit to the linear regression model showed that isoflavone consumption did have a beneficial effect on CRP concentrations compared with the placebo treatment. During isoflavone treatment, the subjects were less likely to show elevated concentrations of CRP ( $>1$  mg/L) than during the placebo treatment. This result is in contrast to the findings of another isoflavone supplementation intervention study in which CRP was measured (32). The latter study used an isoflavone supplement with a different aglycone profile to that used here, which provided 66 mg glycitein/d, 42 mg daidzein/d, and only 6 mg genistein/d in the form of capsules, whereas 50 mg isoflavones/d (genistein-to-daidzein ratio of 2:1) was consumed in the present study by the subjects as part of a


food vehicle. This difference may account for the conflicting results.

Isoflavones may have the potential to attenuate the inflammatory response in cultured endothelial cells, but the results of the present human intervention study showed that this did not occur in vivo for most of the inflammatory biomarkers. Several factors should be considered. First, circulating inflammatory factors are thought to be an early indicator of atherogenesis and endothelial dysfunction, and the present study was designed to show an inhibitory effect on vascular inflammation. It is possible that the vascular health of this group of postmenopausal women was not impaired to any clinically significant extent at baseline, and therefore any detectable improvement on inflammatory status by isoflavone treatment would not be sufficiently uncovered. Second, the hypothesis that isoflavones may improve cardiovascular health was based on epidemiologic evidence that showed lower rates of heart disease in countries that consume relatively large amounts of soy protein (and consequently more isoflavones). The subjects in the present intervention study consumed isoflavone-enriched cereal bars for 8 wk. Although this intervention period is a biologically acceptable length of time for a clinical intervention study, it may be an unrealistically short period to observe the benefits from weak estrogenic plant compounds that were observed after a lifetime exposure in epidemiologic studies. Third, the effects of isoflavones may depend on the type and the dose. The present study chose a dose of 50 mg/d with a genistein-to-daidzein ratio of 2:1 because this was thought to represent a typical dietary isoflavone intake in Asian countries, such as Japan. However, because of the relatively short exposure time, this dose might not have been sufficient to exert significant protective effects. Serum and urinary isoflavone analyses confirmed that the supplements were biologically available, but the possibility remains that isoflavones may only be cardioprotective when contained in the soy protein matrix. Previous studies have shown that isoflavone-rich soy protein reduces cholesterol to a greater extent than does isoflavone-depleted soy protein (40). However, it has also been shown that plasma ICAM-1, VCAM-1, E-selectin, and endothelin-1 do not change with either isoflavone-rich or isoflavone-poor soy protein (30).

Some inconsistencies exist between the present results and the few previous studies that have investigated the effect of isoflavones on circulating inflammatory factors (33, 34). These contradictory results may be due to genotypic variation in candidate genes for cardiovascular disease risk and estrogen receptor genes, although it is unlikely that this would entirely account for the different results. Analysis of key selected genotypes in this population of European postmenopausal women uncovered a significant diet-gene interaction for isoflavones between the *ERβ AluI* genotypes, with isoflavones reducing plasma VCAM-1 in the variant *AA* genotype ( $n = 18$ ) but not the homozygous wildtype (*GG*) or heterozygous (*GA*) genotypes ( $n = 46$  and  $47$ , respectively). This is of particular interest because estrogen was shown to inhibit VCAM-1 expression in endothelial cells via estrogen receptor-mediated mechanisms (41). In addition, low concentrations of genistein (1 and 10 nM) attenuated tumor necrosis  $\alpha$ -induced VCAM-1 mRNA expression in endothelial cells, an effect that was reduced by an estrogen receptor antagonist (17). Therefore, the notion that variation in the function of the estrogen receptor may influence the expression of VCAM-1 in response to estrogen or phytoestrogens is highly plausible. However, the *ERβ AluI* polymorphism is positioned in

the 3'-untranslated region in exon 8 of the *ERβ* gene, a noncoding region, which may suggest that it has no functional implications (42). Nevertheless, the *ERβ AluI* polymorphism may be in linkage disequilibrium with another unidentified variation in the *ERβ* gene that could possibly influence the interaction between *ERβ* and circulating ligands and thereby modulate VCAM-1 expression. Another explanation would be that the *ERβ AluI* polymorphism may be linked with polymorphisms of other as yet unidentified genes that flank *ERβ* and modulate VCAM-1 expression either directly or indirectly. This is a novel and exciting area and clearly additional studies are needed to confirm this observation of the present study.

It was previously suggested that the status of a person as an equol producer or non-equol producer may influence their response to dietary isoflavone supplementation (21). A recent study by Kreijkamp-Kaspers et al (43) showed that blood pressure response to soy protein differed when 202 postmenopausal women aged 60–75 y were subdivided into equol and non-equol producers. However, we observed no significant differences in the concentrations of circulating inflammatory factors or in blood pressure (data not shown) between equol producers and non-equol producers. This may be because a younger age group of postmenopausal women with lower baseline blood pressures was investigated in the present study (aged 46–70 y) than in the study by Kreijkamp-Kaspers et al (43). The subjects were therefore more likely to have a lower risk of cardiovascular disease at the outset, and, consequently, differences with respect to equol status may not be detected.

In conclusion, we showed that isoflavone-enriched foods resulted in no significant improvement in the concentrations of circulating inflammatory factors, including cell adhesion molecules, vWF, MCP-1, and endothelin-1, in our group of postmenopausal women. However, isoflavones had a beneficial effect on plasma CRP concentrations. The findings of the present study are important because it was previously unclear whether isoflavones alone (not contained within soy protein) had any antiinflammatory benefits for the vascular system of healthy postmenopausal women. The findings for a beneficial effect of isoflavones on CRP concentrations suggest that there may be some basis for the recommendation of isoflavone supplements for healthy postmenopausal women for the reduction of inflammatory cardiovascular disease risk factors, although the majority of inflammatory biomarkers were unaffected. However, certain subpopulations may respond more beneficially to isoflavone supplementation, as shown here by the decrease in plasma VCAM-1 concentrations in one of the genotypes of the *ERβ AluI* polymorphism. 

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WLH, KV, JH, SB, CK, MR, MF, FB, DT, TD, A-MM, and CMW contributed to the design of the study and the collection and analysis of the data. WLH, KV, A-MM, and CMW prepared the manuscript. MN, KD-W, and J-AG analyzed the estrogen receptor- $\beta$  genotypes. None of the authors had conflicts of interest.

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