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Estrogen Receptor–Mediated Effects of Isoflavone Supplementation Were Not Observed in Whole-Genome Gene Expression Profiles of Peripheral Blood Mononuclear Cells in Postmenopausal, Equol-Producing Women[1–4]

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Abstract

Isoflavones (genistein, daidzein, and glycitein) are suggested to have benefits as well as risks for human health. Approximately one-third of the Western population is able to metabolize daidzein into the more potent metabolite equol. Having little endogenous estradiol, equol-producing postmenopausal women who use isoflavone supplements to relieve their menopausal symptoms could potentially be at high risk of adverse effects of isoflavone supplementation. The current trial aimed to study the effects of intake of an isoflavone supplement rich in daidzein compared with placebo on whole-genome gene expression profiles of peripheral blood mononuclear cells (PBMCs) in equol-producing, postmenopausal women. Thirty participants received an isoflavone supplement or a placebo for 8 wk each in a double-blind, randomized cross-over design. The isoflavone supplement was rich in daidzein (60%) and provided 94 mg isoflavones (aglycone equivalents) daily. Gene expression in PBMCs was significantly changed ($P < 0.05$) in 357 genes after the isoflavone intervention compared with placebo. Gene set enrichment analysis revealed downregulated clusters of gene sets involved in inflammation, oxidative phosphorylation, and cell cycle. The expression of estrogen receptor (ER) target genes and gene sets related to ER signaling were not significantly altered, which may be explained by the low ERα and ERβ expression in PBMCs. The observed downregulated gene sets point toward potential beneficial effects of isoflavone supplementation with respect to prevention of cancer and cardiovascular disease. However, whether ER-related effects of isoflavones are beneficial or harmful should be studied in tissues that express ERs. J. Nutr. 143: 774–780, 2013.

Introduction

Isoflavones are phytoestrogens present in soy and red clover. The main isoflavones in these plants are the glucosides genistin, daidzin, and glycitin. Their aglycones are known as genistein, daidzein, and glycitein. A potent metabolite of daidzein, equol, can be produced by certain intestinal bacteria that are present in 20–30% of the Western population (1). Positive health effects of isoflavones include a reduced risk of cancer and cardiovascular disease, as suggested by epidemiologic research in Asian populations who daily consume soy products (2). Other beneficial effects of isoflavones, which are relevant for peri- and postmenopausal women, relate to diminishing menopausal symptoms and osteoporosis. Due to these advocated effects of isoflavones, isoflavone supplementation is becoming more common in Western countries (3). However, the daily doses recommended by the suppliers of these supplements are relatively high compared with the traditional Asian soy diet; therefore, the safety of these supplements requires investigation. In this regard, recent reviews state that human, animal, and cell experimental studies remain inconclusive on whether isoflavones, especially as food supplements, are beneficial or potentially harmful (4,5). Suggested adverse effects relate to cancer promotion in hormone-sensitive cancers, such as breast and uterus cancer, and effects on the thyroid gland when iodine intake is insufficient (4).

With the positive and negative effects of isoflavone supplementation still under debate, we hypothesized that postmenopausal, equol-producing women are particularly susceptible to the possible effects of isoflavone supplementation. Postmenopausal

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[3] This trial was registered at clinicaltrials.gov as NCT01232751.
[4] Supplemental Figures 1 and 2 and Supplemental Tables 1 and 2 are available from the "Online Supporting Material" link in the online posting of the article and from the same link in the online table of contents at http://jn.nutrition.org.
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women produce little endogenous estradiol to compete with isoflavones for the estrogen receptor (ER)[7] (6); therefore, isoflavones could theoretically induce estrogen-responsive effects. Moreover, equol is a more potent activator of ERs than its precursor, daidzein (7), and could therefore potentially be responsible for adverse effects of isoflavone supplementation. Effects of isoflavones are induced via ERα and ERβ, which are differentially distributed in different tissues (5,8). Isoflavones may regulate transcription of ER target genes via the estrogen-responsive elements (EREs) present within gene promoter regions but may also have ER- and ERE-independent effects.

A good tool to study these isoflavone supplementation–induced pathways and signaling routes is whole-genome gene expression. Peripheral blood mononuclear cells (PBMCs) are easily accessible, and previous studies have shown that those cells can be used to study nutrition-induced effects on gene expression (9). A previous 84-d parallel intervention study demonstrated differential gene expression changes in PBMCs upon isoflavone supplementation (approximately 900 mg daily) in equol producers and nonproducers, also on estrogen-responsive genes (10). These effects were observed with a 16K oligo array upon consumption of a genistein-rich isoflavone supplement in 10 postmenopausal women receiving placebo treatment compared with 11 postmenopausal women receiving isoflavone treatment, 2 and 5 of whom, respectively, were equol producers.

The current study aimed to determine effects on PBMC whole-genome gene expression of an isoflavone supplement rich in daidzein in a common dose for supplement users. For this study, 30 equol-producing, postmenopausal women were selected (i.e., those who convert the daidzein in this supplement to the more potent equol).

Participants and Methods

Participants. A total of 30 postmenopausal, equol-producing women were recruited for the trial. Equol producer status was tested following the protocol of Setchell and Cole (11). In short, the potential participants took 75 mg (aglycone equivalents) isoflavones as supplements daily for 3 d and delivered morning urine on the fourth day. Participants were designated as equol producers when the $\log_{10}$-transformed urinary S-equol:daidzein ratio was higher than -1.75. The participants were defined as postmenopausal when they had had their last menses >1 y before the start of the study. When the participants' last menses was between 3 mo and 1 y ago, or when the potential participant had undergone a hysterectomy, the inclusion criterion was a follicle-stimulating hormone (FSH) concentration >40 IU/L. Exclusion criteria were as follows: regular use of soy products or regular intake of isoflavone supplements (more than once a week), current use of contraceptives containing hormones or hormone replacement therapy, current use of medication containing sex hormones or sex hormone–triggering compounds, current use of antiinflammatory medicines, and use of antibiotics in the past 3 mo. Furthermore, women with severe heart disease, thyroid disorders, a removed thyroid gland, removed ovaries or prior diagnosis of cancer could not participate. Lifestyle-related exclusion criteria included alcohol and drug abuse, smoking, a BMI >35 kg/m^2, and allergy to soy. All participants consented to participate in the study. The study was approved by the medical ethical committee of Wageningen University.

Study design. This randomized, double-blind, cross-over, placebo-controlled study included two 8-wk intervention periods with one 8-wk washout period in between. A sample size of 30 participants was postulated to be sufficient on the basis of previous nutritional intervention studies using whole-genome transcriptomics that found significant effects on gene expression profiles (9,12). The participants were randomly allocated to receive either the isoflavone supplement or the placebo supplement in the first intervention period of 8 wk and the other treatment in the second intervention period. Stratified randomization was performed by an independent research assistant by using a computer-generated table of random numbers. Researchers as well as participants were blinded to randomization until after data analysis. The participants were asked to consume 4 capsules/d, 2 in the morning and 2 in the evening. The participants consumed 94 mg isoflavones (aglycone equivalents) daily (56 mg daidzein, 26 mg glycitein, and 12 mg genistein as analyzed in our laboratory). There was a run-in period of 4 wk before the start of the experimental treatments. The participants were asked not to eat soy foods during this period as well as during the trial. To aid in this, a detailed list of isoflavone-containing foods was provided to direct the participants. At the end of each 8-wk intervention period, the participants were asked to fill out an FFQ in order to monitor their eating habits during these periods (13).

Isoflavone supplement and placebo. The isoflavone supplement in capsules was commercially available (Phytosoya Forte, 35 mg) and manufactured and purchased from Arkopharma. For the placebo supplement, identical empty capsules and capsule bottles were purchased from Arkopharma and filled by Fagron. The placebo capsules were filled with microcrystalline cellulose; this was also the filler component of the isoflavone supplement. The capsules were made of hydroxypropylmethyl cellulose and dyed with iron oxide and titanium oxide.

Blood sampling and PBMC isolation. During each intervention period, the participants arrived in a fasted state at the research venue, at Wageningen University, at the start of the study, after 4 wk, and after 8 wk (6 visits in total). At each visit, fasting venous blood samples were obtained. Plasma was collected into 6-mL EDTA Vacutainers [Becton Dickinson (BD)], centrifuged for 10 min at 1190 $\times$ *g* at a temperature of 4°C. Serum was collected in 8-mL BD SST tubes, centrifuged for 10 min at 1580 $\times$ *g* at 20°C. For PBMC isolation, 8 mL of blood was collected in BD Vacutainer cell preparation tubes. PBMCs were isolated immediately after blood collection according to the manufacturer's instructions, dissolved in RLT buffer (Qiagen). All samples were stored until further analysis at -80°C.

RNA extraction and microarray processing. RNA was isolated from all PBMC samples by using the Qiagen RNeasy Micro Kit (Qiagen). RNA yield was quantified on a Nanodrop ND 1000 spectophotometer (Nanodrop Technologies), and integrity was measured on an Agilent 2100 Bioanalyzer with RNA 6000 Nanochips (Agilent Technologies). PBMC samples from all 30 participants, after both placebo and isoflavone supplementation, yielded enough RNA of sufficient quality to perform microarray analysis. Microarray analysis was performed on samples from each individual obtained after 8 wk of isoflavone supplementation and 8 wk of placebo supplementation, resulting in a total of 60 microarrays. Total RNA was labeled by using a 1-cycle cDNA labeling kit (MessageAmp II-Biotin Enhanced Kit; Ambion) and hybridized to GeneChip Human Gene 1.1 ST Array targeting 19,738 unique gene identifiers (Affymetrix). Sample labeling, hybridization to chips, and image scanning were performed according to the manufacturers' instructions.

cDNA synthesis and qPCR were performed to confirm the microarray data for ERα and ERβ expression. First, 500 ng of total RNA was reverse-transcribed with a Promega cDNA synthesis kit (Promega

[7] Abbreviations used: AhR, aryl hydrocarbon receptor; *BCL2*, B-cell CLL/lymphoma; BD, Becton Dickinson; *CCND1*, cyclin D1; ER, estrogen receptor; ERE, estrogen-responsive element; *FOXM1*, forkhead box protein M1; FSH, follicle-stimulating hormone; GPER, G protein–coupled estrogen receptor; GSEA, gene set enrichment analysis; *IL1β*, interleukin 1β; *LXR*, liver X receptor; *NDUF*, NADH dehydrogenase ubiquinone flavoprotein; OXPHOS, oxidative phosphorylation; PBMC, peripheral blood mononuclear cell; *PLAUR*, plasminogen activator, urokinase receptor; *PPARα*, peroxisome proliferator receptor α; *PR*, progesterone receptor; *pS2*, presenelin-2; ROS, reactive oxygen species; *SOD1*, superoxide dismutase 1; *TIRAP*, Toll–interleukin 1 receptor domain containing adaptor protein; *TLR*, Toll-like receptor; *TLR4*, Toll-like receptor 4; TNF-α, tumor necrosis factor α; *ZNF*, zinc finger.

Benelux BV). cDNA was PCR-amplified with Platinum Taq DNA polymerase (Invitrogen) on a Biorad I-cycler PCR machine (Bio-Rad Laboratories BV). Primer sequences of *ERα* and *ERβ* were chosen on the basis of the sequences available in the National Center for Biotechnology Information's BLAST (Basic Local Alignment Search Tool) (14). The mRNA expression of all genes was normalized to human ribosomal protein LP0 expression and expressed as 2-log ratios.

HPLC analysis of isoflavones in supplements and plasma. HPLC analyses were carried out with a CoulArray electrochemical detector equipped with a high-sensitivity analytical cell (model 6210, 4-sensor cell; ESA). CoulArray for Windows (ESA) was used for controlling the instrument and collecting the data from the electrochemical detector.

The isoflavone content of the supplements was analyzed on the basis of the method described by Penalvo et al. (15) with a platinum C18 column (Waters). Sample preparation for the analysis of isoflavones in plasma was performed following the method proposed by Saracino and Raggi (16) and measured with a Symmetry Shield C18 column (150 mm × 4.6 mm, 5μm) from Waters. For all analyses, a Brownlee Newguard precolumn (7μm; Shelton) was used. The isoflavones (daidzein, equol, genistein, and glycitein) were measured on 4 channels; 300, 500, 550, and 600 mV. Daidzein, equol, and genistein were quantified at 500 mV, whereas glycitein was quantified at 300 mV. The limit of detection for all isoflavones, except for glycitein, was 0.01 μg/mL, whereas the limit of quantification was 0.02μg/mL. For glycitein, these values were 0.03 and 0.06 μg/mL, respectively.

Serum hormone concentrations. To monitor the postmenopausal state of the participants, estradiol and FSH were measured in serum samples with an electrochemiluminescence immunoassay.

Data analysis. Two arrays for 2 different participants did not meet the quality criteria; therefore, arrays for 28 participants were analyzed by using MADMAX (Management and Analysis Database for Multiplatform Microarray Experiments) (17). Expression values were calculated with the Robust Multichip Average method and normalized by using quantile normalization (18,19). Genes with normalized expression values of >20 on >5 different arrays were defined as expressed and selected for further analysis. Filtered data were further analyzed with gene set enrichment analysis (GSEA) (20–22). Significantly regulated gene sets were defined with a false discovery rate of <0.25. The gene sets were visualized and clustered by using Cytoscape (23), which enabled the identification of clusters of gene sets.

A list of estrogen-responsive genes was derived from the Dragon Estrogen-Responsive Gene Database comprising 1069 genes from *Homo sapiens* [data downloaded December 2011 (24)]. This list was compared with the significantly changed genes in our study and visualized using a Venn diagram (25).

The statistical package SAS (version 9.2; SAS Institute) was used for statistical analysis of the data. Changes in plasma isoflavone concentrations after isoflavone supplementation and placebo were analyzed by paired *t* tests. Changes in macronutrient intake, as measured by FFQs, after both intervention periods were expressed as percentages of total energy intake and compared by paired *t* tests. All data were presented as means ± SDs unless indicated otherwise.

Results

In total, 30 postmenopausal, equol-producing women participated in the study, and all participants finished the study. To recruit this number of equol producers, initially 150 postmenopausal women were screened between September 2010 and March 2011. Forty-two women (27%) were equol producers according to their urinary $\log_{10}$-transformed equol:daidzein ratio of > −1.75 after a 3-d isoflavone challenge. After application of the exclusion criteria, 30 participants entered the study between October 2010 and October 2011. The participants were 61.1 ± 5.8 y old and had a BMI of 25.1 ± 3.7 kg/m^2 at the start of the study. FSH concentrations, measured after 8 wk of isoflavone and placebo supplementation, were above the cutoff for postmenopause of 40 IU/L for all participants; therefore, we regarded all women as postmenopausal (26). Estradiol concentrations at baseline were below the postmenopausal cutoff of 110 pmol/L (27) for all except for 1 participant. After the 8-wk isoflavone supplementation, estradiol concentrations in all participants were below the cutoff; after the placebo period, 1 participant had a concentration >110 pmol/L. Dietary habits were measured by FFQ, and a paired *t* test revealed that intakes of energy, fat, protein, and carbohydrate did not differ significantly between the isoflavone and placebo periods. During the isoflavone intervention, the participants consumed 36 ± 8% of their energy as fat, 15 ± 3% as protein, and 45 ± 8% as carbohydrates. During the placebo period, the corresponding figures were 36 ± 6% as fat, 15 ± 3% as protein, and 45 ± 7 % as carbohydrates.

Plasma concentrations of all 4 isoflavones were significantly higher after the 8-wk intervention compared with placebo ($P < 0.05$; **Fig. 1**). At the end of the placebo period, the concentrations of all isoflavones, except for daidzein (0.01 μg/mL), were below the detection limit of the method. As expected, daidzein and equol concentrations contributed most to the total plasma isoflavone concentrations after the isoflavone intervention. All participants remained equol producers during the study. The compliance based on returned pill counts after isoflavone supplementation was 97%, whereas in the placebo period the compliance was 95%.

Microarray analysis of the PBMC samples of 28 participants revealed that expression of a total of 357 genes was significantly changed after 8 wk of isoflavone treatment compared with placebo (**Supplemental Fig. 1**). Of those genes, the expression of 170 genes was upregulated and the expression of 187 genes was downregulated after isoflavone supplementation (**Supplemental Tables 1 and 2**). To elucidate which gene sets were regulated, GSEA was performed and revealed 1 gene set to be significantly upregulated and 91 gene sets to be significantly downregulated upon 8-wk isoflavone supplementation compared with placebo. The only upregulated gene set was the generic transcription pathway, which is based on positive enrichment of zinc finger (*ZNF*) genes. The 91 downregulated gene sets were grouped together with Cytoscape by means of overlapping genes. The clusters formed were related to inflammation (including complement and coagulation), oxidative phosphorylation (OXPHOS), and cell cycle (**Fig. 2**). Furthermore, some other significantly downregulated gene sets were of interest because of their high

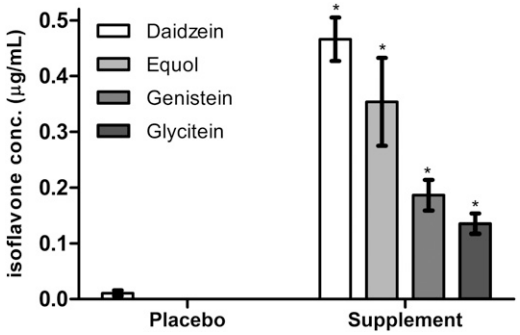

FIGURE 1 Plasma isoflavone concentrations in postmenopausal, equol-producing women after 8-wk exposure to placebo and isoflavone supplementation. Values are means ± SEMs, $n = 30$; *Different from placebo, $P < 0.05$. conc., concentration.

ranking in the GSEA, such as *PPARα* (peroxisome proliferator receptor *α*; false discovery rate *q* value = 0.029), oxidative stress (*q* = 0.034), *FOXM1* (forkhead box protein M1; *q* = 0.034), and nuclear receptor gene sets (*q* = 0.046), although expression of only a few genes within these gene sets was significantly changed. Individual changes in gene expression of the genes that significantly changed upon isoflavone supplementation are visualized per cluster in **Figure 3** (**Supplemental Fig. 2**). In summary, the inflammation cluster of gene sets mainly consisted of a reduced expression of Toll-like receptors (*TLR*s) and genes expressed in the complement and coagulation cascades such as *PLAUR* (plasminogen activator, urokinase receptor). Downregulation of several NADH dehydrogenase ubiquinone flavoprotein (*NDUF*) genes directed the downregulation of the OXPHOS cluster. The cell cycle cluster was directed by downregulation of the gene expression of histones in several gene sets.

Gene sets related to ER*α* and ER*β* activation were not changed after isoflavone supplementation compared with placebo. Subsequent checking of the expression level of *ERα* and *ERβ* revealed that expression levels of *ERα* were just sufficient to pass the filtering criteria, whereas the levels of *ERβ* were not. The low expression was confirmed by qPCR analysis; after normalization to human ribosomal protein LP0 expression, the 2-log ratio of *ERα* was −0.05 after isoflavone treatment and −0.14 after placebo treatment. For *ERβ*, this was −0.41 and −0.20, respectively, meaning that both *ERα* and *ERβ* expression was low in PBMCs of postmenopausal women.

To test the hypothesis of lack of isoflavone-induced ER signaling in PBMCs, we compared significantly changed genes in this study to estrogen-responsive genes from the Estrogen-Responsive Gene Database data set (24). Of the 1069 estrogen-responsive genes present in this database, we found an expression of only 17 estrogen-responsive genes to be significantly changed in our study (**Table 1**).

Discussion

With this 8-wk double-blind, randomized, placebo-controlled, crossover trial we showed that a daidzein-rich isoflavone supplement compared with placebo downregulated expression of genes involved in inflammation, OXPHOS, and cell cycle processes in PBMCs. The observed effects were not mediated by *ERα* and *ERβ*. The observed downregulation of gene sets related to inflammation are driven by diminished gene expression of

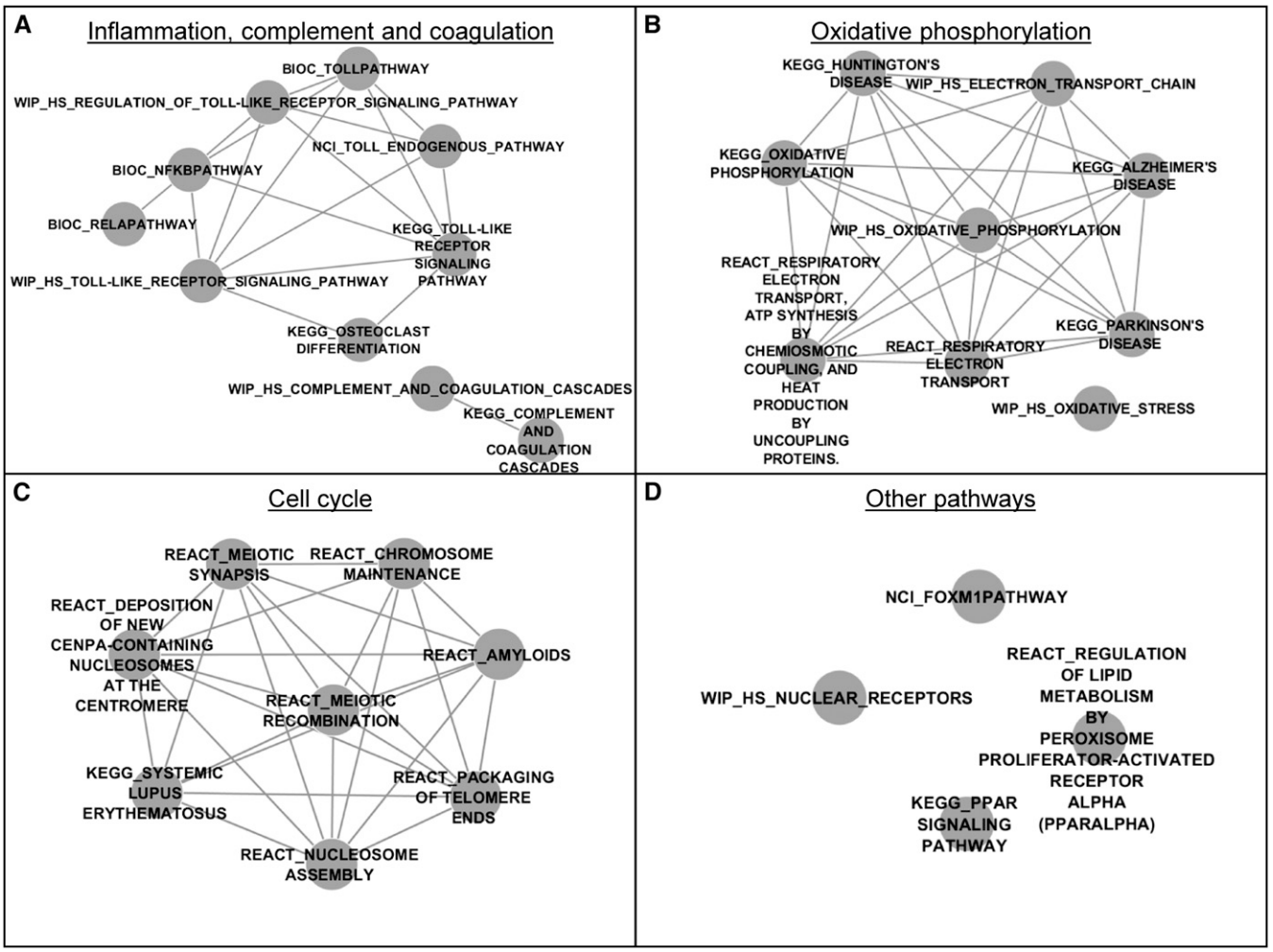

FIGURE 2 Enrichment maps of downregulated gene sets after 8-wk isoflavone supplementation compared with placebo in postmenopausal, equol-producing women identified with gene set enrichment analysis. Three main clustered gene sets were identified: (*A*) inflammation, complement, and coagulation; (*B*) oxidative phosphorylation; and (*C*) cell cycle. *Panel D* represents other gene sets. *Nodes* represent gene sets, whereas *edges* represent overlapping genes. Clusters of related gene sets were assigned a label on the basis of gene sets present in the cluster. BIOC, BioCarta Pathway Diagrams; CENPA, centromere protein A; FOXM1, forkhead box protein M1; KEGG, Kyoto Encyclopedia of Genes and Genomes database; NCI, Nature Pathway Interaction database; REACT, Reactome knowledgebase; WIP_HS, WikiPathways Homo Sapiens.

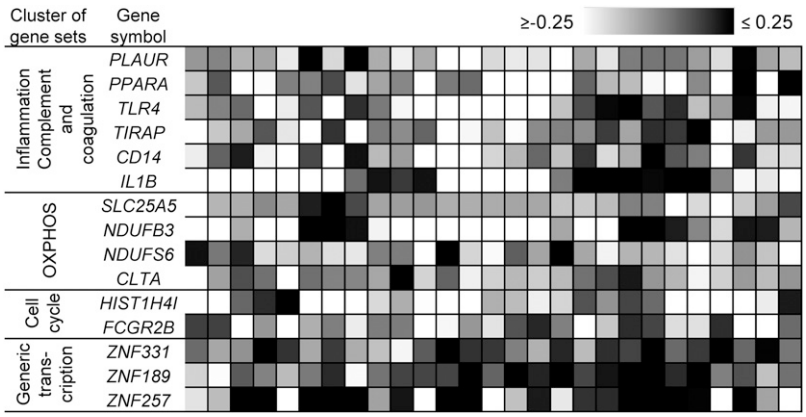

FIGURE 3 Heat map of significantly changed genes in peripheral blood mononuclear cells of postmenopausal, equol-producing women after 8 wk of isoflavone supplementation compared with placebo. Each *column* represents a participant; each *row* represents a single gene within the clusters of gene sets indicated in the blocks. Mean changes in expression (signal log ratios) are shown, ranging from ≥−0.25 (*white squares*) to ≤0.25 (*black squares*). Supplemental Figure 2 is a color version of this figure. *CD14*, monocyte differentiation antigen CD14; *CLTA*, clathrin light chain A; *FCGR2B*, low affinity IG γFc region receptor II-b; *HIST1H4I*, histone cluster 1, H4i; *IL1B*, interleukin 1β; *NDUF* (*B3* and *S6*), NADH dehydrogenase ubiquinone flavoprotein (B3 and S6); OXPHOS, oxidative phosphorylation; *PLAUR*, plasminogen activator, urokinase receptor; *PPARA*, peroxisome proliferator receptor α; *SLC25A5*, solute carrier family 25 member 5; *TIRAP*, Toll–interleukin 1 receptor domain containing adaptor protein; *TLR4*, Toll-like receptor 4; *ZNF* (*331*, *189*, and *257*), zinc finger (331, 189, and 257).

TLR4 (Toll-like receptor 4), *TIRAP* (Toll–interleukin 1 receptor domain containing adaptor protein), and *IL1β* (interleukin 1β) and downregulation of complement and coagulation gene sets. TLR and TIRAP proteins play a role in inflammation by enabling production of cytokines such as *IL1β* (28). Downregulation of inflammation by isoflavones in general has been repeatedly shown in animal and cell studies in a carcinogenic context (29).

In human studies, the results on inflammatory cytokines are inconsistent (30), but isoflavones have been shown to decrease concentrations of TNF-α (tumor necrosis factor α) and to a lesser extent IL-6 (interleukin 6) (31,32). The observed reduction in inflammation in the present study can be considered beneficial because it is known that chronic inflammation can potentially induce as well as promote cancer (33) and initiate diseases such as cardiovascular disease (34) and osteoporosis (35).

The regulation of OXPHOS was mainly driven by gene expression changes of several *NDUF* genes. The proteins encoded by *NDUF*s belong to the complex I molecules in mitochondria, which regulate mitochondrial function and can produce reactive oxygen species (ROS) (36). Downregulation of *NDUF* gene expression might result in reduced ROS production, and hence reduced oxidative stress. Interestingly, expression of superoxide dismutase 1 (*SOD1*), needed to discharge ROS, was also (borderline significantly) reduced after 8 wk of isoflavone supplementation. Both ROS and oxidative stress have the potential to induce DNA damage as a precursor for cancer (37).

In this study, the expression of histones and histone-like genes was downregulated within cell cycle–related gene sets. Histones are typically upregulated during the S phase of the cell cycle starting from the G1-S checkpoint (38). Because downregulation of histone transcription was observed, this could mean that

TABLE 1 List of 7 significantly upregulated and 10 downregulated estrogen-responsive genes in PBMCs of postmenopausal, equol-producing women after 8 wk of isoflavone supplementation compared with placebo[1]

Gene	Description	Regulation in current study[2]
BCL2L1	BCL2-like 1	↑
CACYBP	Calcyclin binding protein	↓
EDEM1	Endoplasmatic reticulum degradation enhancer, mannosidase alpha-like 1	↑
ERBB2	v-erb-b2 erythroblastic leukemia viral oncogene homolog 2, neuro/glioblastoma derived oncogene homolog (avian)	↑
FKBP5	FK506 binding protein 5	↑
FOXP1	Forkhead box P1	↑
HSPA1A	Heat shock 70 kDa protein 1A	↓
MYB	v-myb myeloblastosis viral oncogene homolog (avian)	↑
NME2	NME/NM23 nucleoside diphosphate kinase 2	↓
NRP1	Neuropilin 1	↓
PSMD8	Proteasome (prosome, macropain) 26S subunit, non-ATPase, 8	↓
PTPRO	Protein tyrosine phosphatase, receptor type, O	↓
SLC25A5	Solute carrier family 25 (mitochondrial carrier; adenine nucleotide translocator), member 5	↓
SPRED1	Sprouty-related, EVH1 domain containing 1	↓
STAB1	Stabilin 1	↓
STXBP1	Syntaxin binding protein 1	↑
TIMELESS	Timeless homolog (*Drosophila*)	↓

[1] As compared to the Estrogen-Responsive Gene Database (24). BCL2, B-cell CLL/lymphoma; PBMC, peripheral blood mononuclear cell; ↓, downregulated; ↑, upregulated.
[2] $P < 0.05$.

many cells remain in arrest at some point in the cycle, for instance at G0/G1 (39), after exposure to isoflavone supplementation. This was supported by the significantly downregulated enrichment of gene sets associated with mitotic G1-S phase and G1-S transition in this study. The results of the present study are in line with the review by Medjakovic et al. (40), who discussed effects of isoflavones on cell cycle arrest in several cell lines and concluded that daidzein seems to cause an arrest in the G0/G1 phase, whereas genistein induces G2/M arrest. In addition, the observation that the *FOXM1* pathway, which plays an important role in regulating histones and cell cycle (41), was downregulated in our study, strengthens the theory of cell cycle arrest after exposure to isoflavones (42). The observed cell cycle arrest provides time for the cell to repair any damaged DNA or to go into apoptosis and consequently prevents replication of cells with DNA damage and formation into tumors (43).

There are several strengths regarding the study design and results. First, a sample size of 28 participants is appropriate and larger than previous nutritional intervention studies that used whole-genome transcriptomics (9,12). Second, compliance to the study was good because the plasma concentrations of isoflavones showed a significant difference between the intervention and the placebo period, as supported by the capsule counts. An additional strength is that we used a commercially available supplement rich in daidzein for this study, which is representative in composition of the majority of isoflavone supplements on the market. Many supplements are made from soy germ, which contains relatively more daidzein than genistein and glycitein (daidzein:genistein:glycitein ratio of 4:1:3) (44). Furthermore, the used dosage was at the high end of the manufacturers' recommendations on isoflavone supplements. Finally, for this study a homogenous group of postmenopausal, equol-producing women was preselected by criteria such as absence of menstrual cycle and FSH concentrations for postmenopausal status and urinary equol concentrations after an isoflavone challenge for equol-producing capacity. These characteristics were stable in the participants during the trial, as monitored by FSH and equol concentrations in the blood. The stable features of the participants make the results of this study applicable to a very relevant potential risk group, but limit the extrapolation to other groups of people. The exposure time of 8 wk was chosen because we were interested in moderate to long-term effects.

In the current study, the expression of 357 genes (1.8%) was significantly changed after isoflavone supplementation versus placebo on a gene chip encoding 19,738 gene identifiers. This indicates that small effects were observed, compared with results from nutritional intervention studies with other compounds performed in PBMCs at the same laboratory with similar analysis techniques (9,12). Also, in a previous study with an isoflavone supplement (900 mg/d, high genistein) with a parallel design, only a limited number of genes [319 genes (2.0%)] were significantly changed on a 16K oligo array in PBMCs of postmenopausal equol producers compared with all participants (n = 10, of whom 2 were equol producers) who received placebo treatment (10). However, in that study, in total only 7 equol producers were included and therefore low numbers of significantly expressed genes could be expected, whereas our study included 28 participants and used a crossover design. In the previous study, 11 estrogen-responsive genes were found in equol-producing women that did not overlap with the 17 significantly expressed estrogen-responsive genes in the current study. Because of the use of PBMCs in the current study, the observed effects cannot easily be extrapolated to, for example, healthy breast or uterus tissue, mainly because of the difference in ERα and ERβ occurrence (45). Gene expression measured by microarrays and confirmed by qPCR revealed that, in our study, the expression of both *ERα* and *ERβ* in PBMCs was very low. Also, well-known estrogen-responsive genes, such as the progesterone receptor (*PR*), presenelin-2 (*pS2*), B-cell CLL/lymphoma (*BCL2*), and cyclin D1 (*CCND1*) genes (46), were not significantly expressed, which supports the lack of isoflavone-induced ER signaling. The lack of expression of *ERα* and *ERβ* in PBMCs contradicts recent literature in which these genes were found to be expressed in these cells (47–49). This might be explained by the postmenopausal status of the participants in this study. Postmenopausal women do not produce endogenous estradiol; therefore, expression of ERs might be less necessary and might even be silenced by mechanisms such as DNA methylation. However, expression levels of *ERα* and *ERβ* were not significant different between premenopausal women and postmenopausal women (unpublished results).

It is possible that other nuclear receptors known to be affected by isoflavones are involved in the regulation of the observed effects. Likely candidates from literature are PPARα, aryl hydrocarbon receptor (AhR), and G protein–coupled ER (GPER) (40,50). In the current study, the expression of *AhR* and *GPER* was observed in PBMCs, but was not significantly changed after exposure. On the other hand, expression of *PPARα* was significantly decreased after the 8-wk exposure to isoflavones compared with placebo. Another nuclear receptor, *LXR* (liver X receptor), was significantly changed in this study and therefore also a candidate for the regulation of the observed effects. Because the expression of target genes of these receptors was not significantly changed, it is not likely that effects were mediated by these nuclear receptors. Interpretation of the effects on gene expression in this study might have been facilitated by measuring circulating biomarkers for the observed effects. However, the gene expression results did not point to a specific marker that would have differentiated between either beneficial or harmful effects of isoflavones. Although PBMCs might not be the preferred model to study isoflavone-induced estrogenic effects, these cells circulate through the whole body and pervade tissues; therefore, they may reflect systemic changes (51). The observed effects on inflammation, OXPHOS, and cell cycle may be caused by direct effects on gene expression by the isoflavones itself but might also be systemic as result of effects in other tissues.

Together, the observed downregulation of inflammation, OXPHOS, and cell cycle gene sets point toward beneficial effects of isoflavone supplementation with respect to prevention of cancer and cardiovascular disease. However, whether ER-related effects of isoflavones can be expected to be beneficial or harmful should be studied in tissues that are more likely to express ERs, such as breast, uterus, or adipose tissue.

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