Molecular effects of isoflavone supplementation

Human intervention studies and quantitative models for risk assessment

Vera van der Velpen

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This research was conducted under the auspices of the Graduate School VLAG (Advanced studies in Food Technology, Agrobiotechnology, Nutrition and Health Sciences).

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Thesis

submitted in fulfilment of the requirements for the degree of doctor
at Wageningen University
by the authority of the Rector Magnificus
Prof. Dr M.J. Kropff,
in the presence of the
Thesis Committee appointed by the Academic Board
to be defended in public
on Friday 20 June 2014
at 13.30 p.m. in the Aula.

Vera van der Velpen

Molecular effects of isoflavone supplementation: Human intervention studies and quantitative models for risk assessment
154 pages

PhD thesis, Wageningen University, Wageningen, NL (2014)
With references, with summaries in Dutch and English
ISBN: 978-94-6173-952-0

ABSTRACT

Background: Risk assessment can potentially be improved by closely linked experiments in the disciplines of epidemiology and toxicology. This was explored for isoflavones in a case study. Isoflavones are suggested to have potential beneficial effects, but discussions on their safety are ongoing as well.

Aims and methods: Effects of isoflavone supplements on gene expression were studied in white blood cells (PBMCs) and adipose tissue, among postmenopausal women in two human intervention studies. To advance risk assessment, the dose-response relation between isoflavone intake and plasma concentrations was studied with a log-linear regression model. Furthermore, human gene expression profiles after isoflavone supplementation were quantified and compared to results from a rat experiment using multivariate analysis.

Results: In both PBMCs and adipose tissue, changes in gene expression profiles pointed at effects of isoflavones on energy metabolism, inflammation and cell cycle; these effects were modified by supplement composition and equol-producing phenotype. Hypothesized estrogen-responsive effects were not observed. For the intake range of 0-100 mg/day, the plasma concentrations of daidzein, equol, genistein and total isoflavones were quantified, but a large degree of inter-individual variation was observed. Effect sizes of estrogen-responsive gene profiles and other biological pathways could be quantitatively compared between PBMCs and adipose tissue, as well as between humans and rats.

Conclusion: Effects of isoflavone supplementation on gene expression in PBMCs and adipose tissue of postmenopausal women suggested mainly beneficial effects of a dose of ~100 mg/day. The absence of a distinct estrogen-like response suggested a limited role of the estrogen receptor in isoflavone induced gene expression in postmenopausal women. Modelling exposure marker and gene expression data from human and animal studies provided important tools for further exploration of intertissue and interspecies similarities and for the use of transcriptomics in improving risk assessment.

Contents

Chapter 1

Introduction

IMPROVEMENT OF RISK ASSESSMENT WITH ISOFLAVONES AS A CASE STUDY

The idea of this project originated from the need to improve risk assessment in the domain of food, nutrition and health. To this end, the Netherlands Food Safety Authority funded a study in 2009 on the improvement of risk assessment. This resulted in a Dutch report, representing an inventory of views of experts in toxicology and epidemiology on the current risk assessment process and on how toxicology and epidemiology can work together to improve risk assessment (1). It was concluded that both disciplines could complement each other in the risk assessment process with regard to research on dietary intake, exposure markers, early effect markers and disease endpoints (Figure 1.1). In this approach, measurement of exposure markers and early effect markers would need to be further standardized and aligned in a collaboration between the two disciplines, for instance with human intervention studies and animal experiments. Furthermore, epidemiology would provide insight in dietary intake across the population. While in toxicology, data on health endpoints could be obtained in animal experiments (Figure 1.1).

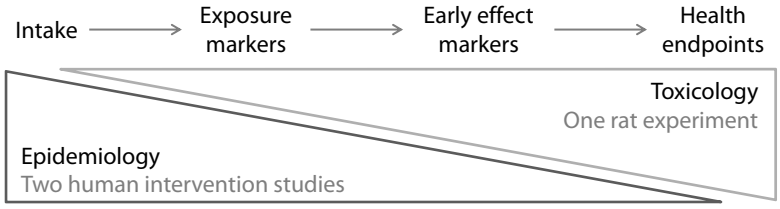

Figure 1.1 The concept of integrating toxicological and epidemiological approaches to improve risk assessment.

In this thesis, the practical application of this concept was further developed using isoflavones as a case study. This topic was chosen, because safety of isoflavone consumption at higher doses was under debate and concerns existed on effects of isoflavones on tumour promotion and thyroid function (2-4).

RISK ASSESSMENT

Assessment of risks and benefits in nutritional toxicology

Risk assessment has been defined by the EU as: 'a scientifically based process consisting of four steps; hazard identification, hazard characterisation, exposure assessment and

risk characterisation' (EC No 178/2002 (5)). These four steps are a framework in which scientific data on health risks are evaluated and translated for risk management (6). Ideally, information on associations with human health endpoints, like cancer risk, are derived from observational studies, as done for chromium by Goldbohm *et al*. (7, 8). In practice, human data on hard endpoints is often lacking or incomplete and instead data from animal experiments is extrapolated to the human situation with the use of uncertainty factors, typically one for intraspecies variability and one for interspecies variability (9). The traditional uncertainty factors – 10 for intraspecies and 10 for interspecies – were applied to a reference dose at which adverse health effects are considered absent in animal experiments. These uncertainty factors have been updated over the years starting with separate uncertainty factors for pharmacokinetics and pharmacodynamics (9). More recently, the concept of allometric scaling of metabolic rate between species was further developed, in which an interspecies factor of 4 for rat to human and 7 for mouse to human was derived (10). Other initiatives derived similar interspecies factors from No Observed Effect Level (NOEL) or Benchmark Dose (BMD) comparisons together with their factor distribution from different databases, like RepDose and technical reports from the National Toxicology Program (11, 12). The uncertainty caused by extrapolation is only one of several sources of uncertainty and variability in all steps of the risk assessment process (13) and underlines the need for improvement of this process.

Parallelogram approach for improvement of risk assessment

The Dutch report concluded that risk assessment could be improved by concentrating research on intake and health endpoints, along with further standardization of exposure markers and early effect markers measured in both human intervention studies and animal experiments (Figure 1.1 (1)). In a practical setting, this would imply the design of an animal experiment completely aligned to a human intervention study with regard to dose, duration and target group. This way, observed effects can be evaluated between species using a parallelogram approach with intake, exposure markers, early effect markers and hard endpoints as 'linking pins' enabling a comparison between species (Figure 1.2). With regard to early effects markers, toxicological research is currently exploiting high throughput techniques and computational models to detect early perturbations in biological systems to reduce animal experiments on disease endpoints (14). Detection of early perturbations of health is also key in human intervention studies (15), because toxicological studies on disease endpoints are not ethical in humans. Therefore, this detection of early perturbations, for instance by transcriptomics, represents a potentially

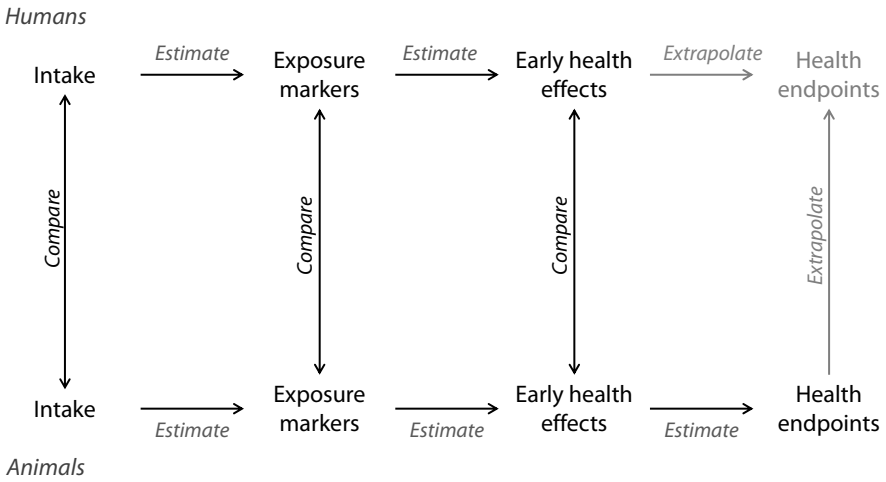

Figure 1.2 The practical parallelogram approach for improvement of risk assessment. Uncertainty in extrapolating human health endpoint from animal studies depends on the comparability of pathways from intake to health endpoints in both species. In the current thesis, this was estimated using similar markers and comparable methodologies.

new opportunity to fill a knowledge gap in risk assessment by comparing gene expression effects between animals and humans.

This practical setting for improvement of risk assessment was applied in a case study with isoflavones, in which two human intervention studies were conducted at the department of Human Nutrition (this thesis) as well as a rat experiment by the sub-department of Toxicology of Wageningen University.

ISOFLAVONES

From soy plants to the systemic circulation

The three major isoflavones, daidzein, genistein and glycitein, are phytoestrogens that are naturally present in soy and soy products. The soy plant (Glycine max) consists of different parts in which the isoflavone content and ratio between the individual isoflavones vary (16). The composition is further influenced by plant genotype and environmental factors during growth (17). Isoflavone molecules in soy plants are often bound to glucose and therefore denoted as daidzin, genistin and glycitin. These glucosides can be also be present

in esterified form, with either malonyl or acetyl groups (18). Methylated forms of daidzein and genistein, like formononetin and biochanin A, occur mainly in red clover and alfalfa sprouts (19).

Mean intake of isoflavones in European countries is estimated to be 0.5-0.8 mg/day for the general population, while vegetarians and vegans in the UK are reported to consume on average 22.4 mg isoflavones/day (20). In Japan, mean intakes of daidzein and genistein of respectively 18.3 and 31.4 mg/day have been observed (21). In Western countries, supplement users are reported to consume 50 mg isoflavones/day on average, but soy-based dietary supplements can contain up to 107 mg aglycone equivalents of isoflavones (22-24). Although exact market figures are lacking, a recent review of surveys indicated that about 50% of the women are seeking alternative therapies for their menopausal symptoms and that phytoestrogen supplements are the second most popular alternative after herbal medicine (25).

After consumption of soy isoflavones, the isoflavone glucosides are deglycosylated during their journey through the gastro-intestinal tract by lactase phlorizin hydrolase (LPH) in the enterocyte brush border (26). Isoflavone glucosides are more bioavailable than aglycones due to their water solubility and stability, but aglycones can be more rapidly absorbed because they do not need to undergo deglycosylation (27). Uptake of the aglycone isoflavones occurs in the small intestine and the colon via passive diffusion (28), after which the isoflavones are conjugated by intestinal P450 enzymes with glucuronides or sulfonate esters and enter the bloodstream (27).

One of the isoflavones, daidzein, can be converted into equol by intestinal bacteria present in 25-30% of the Western population and 50-60% of the Asian population (29). These people are called equol producers and this is known as a relatively stable phenotype (30). In intervention and cross-sectional studies, equol production was shown to be associated with intake of several nutrients (31-33). Furthermore, the equol-producing phenotype was suggested to be affected by antibiotic treatment in an observational study (33), but this effect was not found in an intervention study (34). Next to equol, O-desmethylangolensin (O-DMA) is also a gut metabolite of daidzein, which is found to be produced in 92% of the Caucasian and 84% of the Korean American population of Seattle (35).

From the systemic circulation to molecular effects of isoflavones

Isoflavones resemble the molecular structure of 17β estradiol (E2) and are known to bind to the estrogen receptors (ER) α and β, hence the name phytoestrogens (36). When ligands

bind to the ERs, homo- or heterodimers are formed with other ligand-bound ERs and this complex binds to the estrogen-responsive element (ERE) on the DNA and induces mRNA transcription. Moreover, the isoflavone-bound ERs can heterodimerize with specificity protein 1 (SP1) and activator protein-1 (AP-1) and bind to other promotor sequences to induce gene expression. Lastly, isoflavones can potentially induce non-genomic, rapid effects of membrane-bound ERs. These effects include changes in calcium and potassium signalling, increase in cAMP and phosphorylation of the cAMP response element binding protein (CREB) as well as activation of MAPK signalling (37).

In addition to the ERα and ERβ, the G-protein coupled receptor 30 (GPR30) was recently established to be an ER and is now denoted as G-protein coupled ER (GPER, (38)). Although the structure of the isoflavones allows binding to all three ERs, they preferentially bind to the ERβ. Genistein has the highest binding affinity for ERβ and equol is suggested to have a similar or slightly lower binding affinity to this receptor. Daidzein has a lower binding affinity than genistein and equol, but has a higher binding affinity than glycitein and O-DMA (39-41).

Isoflavones can also induce effects on gene expression via other nuclear receptors and pathways. For instance, they are known to activate the progesterone receptor (PR), androgen receptor (AR), peroxisome proliferator-activated receptors (PPARs), aryl-hydrocarbon receptor (AhR), but also the Vitamin D receptor (VDR) and Retinoic acid receptor (RAR) (42, 43).

Because of their estrogen-like structure, effects of isoflavones are expected to be predominantly mediated via the ERs, and tissues expressing these receptors are potential target tissues of isoflavone action. Thus, the main target tissues of interest are in the reproductive system, like breast, uterus and prostate tissue. However, other tissues like bone, liver and adipose tissue, and tissues in the cardiovascular and central nervous system are also known to express ERs (44, 45).

Beneficial effects of isoflavones

Adlercreutz *et al*. (46) suggested in 1992 that higher isoflavone concentrations in urine of Japanese compared to American and Finnish women could explain the lower frequency of hot flushes observed in Japan by Lock *et al*. (47). Other beneficial effects of isoflavone consumption suggested from epidemiological research were reduced incidence of osteoporosis, cardiovascular disease, and certain cancers (48). Because of these suggested beneficial effects, soy products are more often consumed in Western countries in recent

years and a market for isoflavone supplements has emerged, especially for women experiencing menopausal complaints (25). A recent meta-analysis on the efficacy of isoflavone supplements on hot flushes indicated that these supplements potentially reduce the severity and the frequency of hot flushes (49).

Another possible consequence of menopause is osteoporosis, which is enhanced by the decline in the production of endogenous estrogen (52). In epidemiological studies in Asian populations, isoflavone intake was suggested to decrease hip fracture risk and increase bone mineral density (53, 54). However, the effects of isoflavone supplementation in bone mineral density measured in a 3-year randomized controlled trial (RCT) suggested a modest protective effect only for neck bone mineral density (55). On the other hand, a meta-analysis showed protective effects only on lumbar spine bone mineral density after isoflavone supplementation (56). Finally, a recent systematic review showed a statistically significant increase in bone mineral density and the urinary bone resorption marker deoxypyridinoline (DPD), but these effects were dependent on menopausal status, supplement type, dose and duration of intervention (57).

Effects of isoflavones on cardiovascular disease (CVD) in RCTs point also towards beneficial effects, like a modest effect of isoflavones on flow mediated dilation (FMD) detected in a meta-analysis of RCTs (58). Another meta-analysis confirmed these effects, but only in women with low baseline FMD (59). Next to markers for endothelial function, effects on lipid profile and inflammation markers are often used as early cardiovascular health endpoints. A systematic review found lowering of triglycerides after isoflavone consumption, but no effects on other markers in the lipid profile (60). Furthermore, reported effects on inflammation markers related to CVD were not consistent, for instance lowering of C-reactive protein (CRP) was found in one study (61), while other studies reported positive effects on serum adiponectin concentrations (62), or found no effect on any of the markers measured (63).

Epidemiological studies in Asia showed that cancer incidence is lower in countries with higher isoflavone intake (64). Indeed, also a recent meta-analysis showed that soy food intake as consumed by the Asian population might be protective for breast cancer (65). It has been suggested that this protective effect mainly occurs due to soy consumption during childhood (3, 66). Although most of the research focusses on breast cancer, one observational study suggested that isoflavone intake also has beneficial effects on endometrial cancer occurrence (67). Suggestions of beneficial effects on other cancers, like in other tissues of the reproductive system, are limited (4).

Potential harmful effects of isoflavones

Epidemiological studies suggest that breast cancer incidence is lower in Asian countries due to isoflavone intake. On the other hand, isoflavones are hypothesized to activate ERs in the same way as estrogens and these are known to increase breast cancer risk (2). This contradiction raises concern about potential adverse effects of isoflavones on breast cancer risk at higher doses (4). In this respect, a recent meta-analysis of eight RCTs found no effect of isoflavone intake on breast density in postmenopausal women, as a surrogate marker for breast cancer risk, although an increase was suggested in premenopausal women (68). Similarly, concerns exist on the effect of isoflavones on endometrial cancer risk (3), supported by results from animal experiments and from one human study (69). However, in two recent 2- and 3-year human intervention studies, no effects of isoflavone intake on endometrial thickness, as an intermediate marker for endometrial cancer risk, were found (55, 70). The last issue of concern regarding isoflavones is their suggested effect on thyroid function; isoflavone intake might interfere with thyroid metabolism and with synthetic thyroid hormone in patients suffering from hypothyroidism. This makes isoflavone intake of concern for vulnerable groups, like infants with hypothyroidism consuming soy milk (71).

Rationale for human intervention studies

Altogether, beneficial and adverse effects of isoflavone intake are not yet established. Safety issues are of importance for subpopulations consuming high doses, like peri- and postmenopausal women consuming isoflavone supplements. Especially postmenopausal women, who do not produce endogenous estradiol to compete with isoflavones for the ERs, and those who produce equol, which is more active than daidzein, might be groups at risk of adverse effects of isoflavones.

Potential beneficial effects are also relevant in this age group. During menopause, the decline in endogenous estrogen production induces adipose tissue distribution changes, which may eventually lead to the development of the metabolic syndrome (45). The metabolic syndrome co-exists with a pro-inflammatory state of the adipose tissue (72) and is associated with a two-fold increased risk of cardiovascular disease (73). It is hypothesized that isoflavones may have positive effects on these unfavorable body fat distribution changes (74, 75), but also on glucose metabolism, lipid metabolism and inflammation markers.

THESIS OUTLINE

To study the potential adverse and beneficial effects of isoflavones, two intervention studies were conducted, in which effects were assessed using whole-genome gene expression. The first intervention study focussed on the most vulnerable population of equol-producing postmenopausal women; in this study gene expression effects of isoflavones were measured in peripheral blood mononuclear cells (PBMCs, **chapter 2**). The second intervention study included the general postmenopausal population, both equol producers and non-producers; in this study the effects of two different isoflavone supplements were studied in an estrogen-responsive tissue, i.e. adipose tissue (**chapter 3**).

The basic concept underlying this thesis was that alignment of human intervention studies and animal experiments would enable assessment of similar exposure markers and early effect markers (Figure 1.2), which would enable an integrated approach between epidemiology and toxicology for improvement of risk assessment (Figure 1.1). Therefore, in **chapter 4**, the relation between isoflavone intake and exposure markers was modelled using data from the two human intervention studies together with a third intervention study with a lower dose of isoflavones from soy foods. Finally, in **chapter 5**, data from the two human intervention studies was connected to a rat experiment conducted by the sub-department of Toxicology. In this last chapter, the similarity of isoflavone effects on early effect markers in PBMCs and adipose tissue of humans and rats was studied for improvement of risk assessment.

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Chapter 2

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

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Published in: Journal of Nutrition, June 2013

ABSTRACT

Background: 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.

Objective: 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.

Design: Thirty participants received an isoflavone supplement or a placebo for 8 wk each in a double-blind, randomized crossover design. The isoflavone supplement was rich in daidzein (60%) and provided 94 mg isoflavones (aglycone equivalents) daily.

Results: 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.

Conclusion: 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.

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, 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 women produce little endogenous estradiol to compete with isoflavones for the estrogen receptor (ER) (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 non-producers, 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 log10-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 anti-inflammatory 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, crossover, 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 *g* at a temperature of 4°C. Serum was collected in 8-mL BD SST tubes, centrifuged for 10 min at 1580 *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 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 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 x 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 log10-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 en% as fat, 15 ± 3 en% protein, and 45 ± 7 en% as carbohydrates.

Plasma concentrations of all 4 isoflavones were significantly higher after the 8-wk intervention compared with placebo ($P < 0.05$; Figure 2.1). 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 (Figure 2.2). Of those genes, the expression of 170 genes was upregulated and the expression of 187 genes was downregulated after isoflavone supplementation (Supplemental Tables 2.1 and 2.2). 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

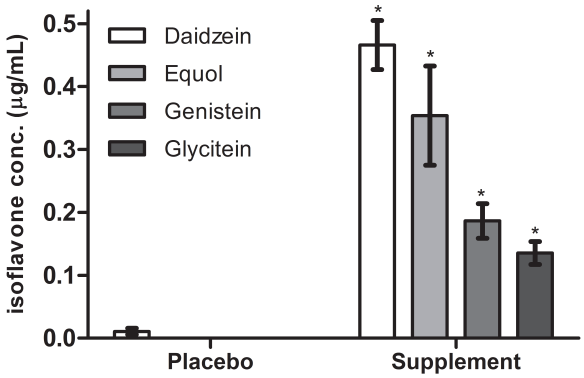

Figure 2.1 Plasma isoflavone concentrations in postmenopausal, equol-producing women after 8-wk exposure to placebo and isoflavone supplementation. Values are means ± SEMs, n = 30; * Different from placebo, P < 0.05. conc., concentration.

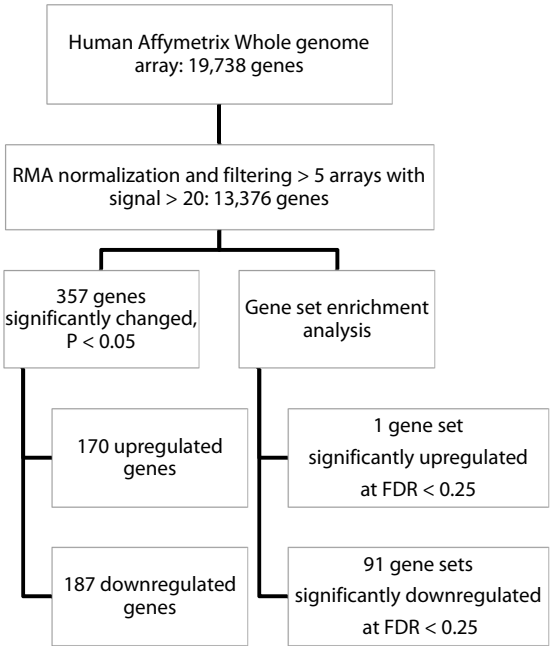

Figure 2.2 Flow chart of microarray analysis indicating significantly changed genes and results of gene set enrichment analysis (RMA, robust multichip average; expression level in arbitrary units; FDR, false discovery rate).

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 (Figure 2.3). Furthermore, some other significantly downregulated gene sets were of interest because of their high 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

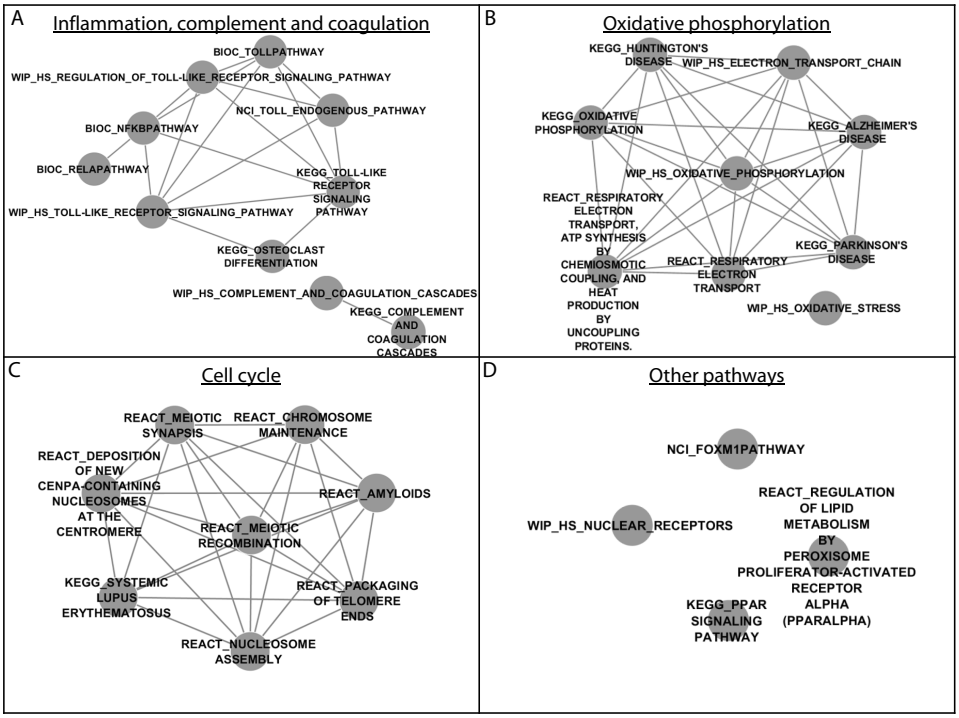

Figure 2.3 Enrichment maps of downregulated gene sets after 8-wk isoflavone supplementation compared with placebo in postmenopausal, equol-producing women identified with gene set enrichment analysis. Three main clustered gene sets were identified: (A) inflammation, complement, and coagulation; (B) oxidative 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.

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 2.4. In summary, the inflammation cluster of gene sets mainly consisted of a reduced expression of Toll-like receptors (TLRs) 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 ERα and ERβ 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,

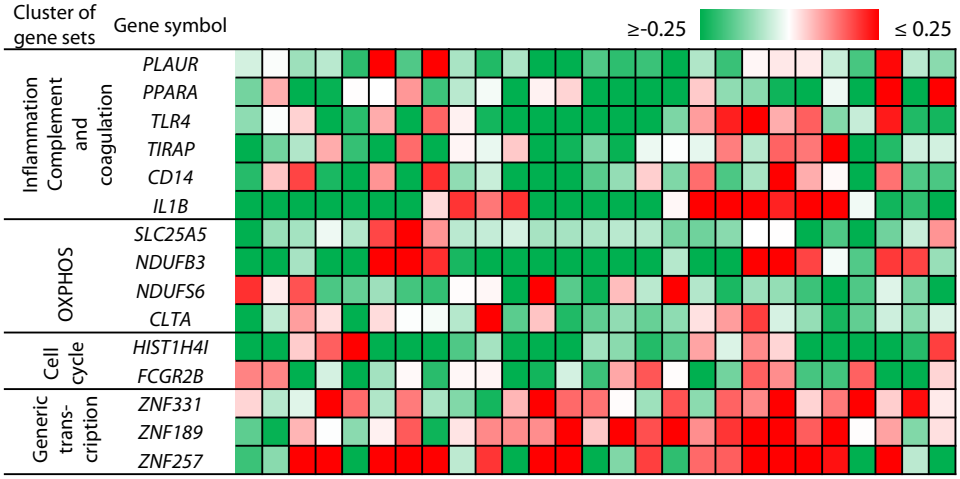

Figure 2.4 Heat map of significantly changed genes in peripheral blood mononuclear cells of postmenopausal, equol-producing women after 8 wk of isoflavone supplementation compared with placebo. Each column represents a participant; each row represents a single gene within the clusters of gene sets indicated in the blocks. Mean changes in expression (signal log ratios) are shown, ranging from ≤ -0.25 (green squares) to ≥ 0.25 (red squares). *CD14*, monocyte differentiation antigen CD14; *CLTA*, clathrin light chain A; *FCGR2B*, low affinity IG gFc region receptor II-b; *HIST1H4I*, histone cluster 1, H4i; *IL1β*, 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).

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 20.05 after isoflavone treatment and 20.14 after placebo treatment. For ERβ, this was 20.41 and 20.20, 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 2.1).

Table 2.1 List of 7 significantly upregulated and 10 downregulated estrogen-responsive genes in PMBCs 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	ER 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 70kDa 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; ↓, downregulated; ↑, upregulated.
[2] $P < 0.05$.

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 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 NDUFs 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 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 significantly 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 hydro-carbon 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.

ACKNOWLEDGEMENTS

The authors thank Dini Venema, Jenny Jansen, and Mechteld Grootte Bromhaar for excellent technical assistance.

V.v.d.V., A.G., P.C.H., E.G.S., L.A.A., and P.v.t.V. designed the research; and V.v.d.V. conducted the research, analyzed the data, and wrote the manuscript. All authors read and approved the final manuscript.

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Supplemental Table 2.1 Significantly downregulated genes after isoflavone supplementation compared to placebo

Gene	Description
AAMP	angio-associated, migratory cell protein
ABCB8	ATP-binding cassette, sub-family B (MDR/TAP), member 8
AFMID	arylformamidase
AKR1C1	aldo-keto reductase family 1, member C1 (dihydrodiol dehydrogenase 1 /// 20-alpha (3-alpha)-hydroxysteroid dehydrogenase)
AMPD2	adenosine monophosphate deaminase 2
ANKRD57	ankyrin repeat domain 57
ARPC3	actin related protein 2/3 complex, subunit 3, 21kDa
B4GALT2	UDP-Gal:betaGlcNAc beta 1,4- galactosyltransferase, polypeptide 2
C12orf45	chromosome 12 open reading frame 45
C14orf109	chromosome 14 open reading frame 109
C16orf87	chromosome 16 open reading frame 87
C19orf59	chromosome 19 open reading frame 59
C1orf53	chromosome 1 open reading frame 53
C21orf70	chromosome 21 open reading frame 70
C2orf76	chromosome 2 open reading frame 76
C6orf115	chromosome 6 open reading frame 115
C8orf76	chromosome 8 open reading frame 76
C9orf139	chromosome 9 open reading frame 139
C9orf24	chromosome 9 open reading frame 24
CACYBP	calcyclin binding protein
CALCRL	calcitonin receptor-like
CD101	CD101 molecule
CD14	CD14 molecule
CDC42EP1	CDC42 effector protein (Rho GTPase binding) 1
CHAF1A	chromatin assembly factor 1, subunit A (p150)
CIB2	calcium and integrin binding family member 2
CKB	creatine kinase, brain
CLEC4A	C-type lectin domain family 4, member A
CLTA	clathrin, light chain A
CRK	v-crk sarcoma virus CT10 oncogene homolog (avian)
CSF1R	colony stimulating factor 1 receptor
CSTA	cystatin A (stefin A)
CTF1	cardiotrophin 1
CTIF	CBP80/20-dependent translation initiation factor
CYP2S1	cytochrome P450, family 2, subfamily S, polypeptide 1
DAPK3	death-associated protein kinase 3
DCP2	DCP2 decapping enzyme homolog (S. cerevisiae)
DDX21	DEAD (Asp-Glu-Ala-Asp) box polypeptide 21
DEPDC1B	DEP domain containing 1B
DKFZP434F142	hypothetical DKFZp434F142
DNTTIP1	deoxynucleotidyltransferase, terminal, interacting protein 1

Gene	Description
DOC2B	double C2-like domains, beta
E2F6	E2F transcription factor 6
EIF2C1	eukaryotic translation initiation factor 2C, 1
EIF4A1	eukaryotic translation initiation factor 4A1
ELOVL1	elongation of very long chain fatty acids (FEN1/Elo2, SUR4/Elo3, yeast)-like 1
EMR3	egf-like module containing, mucin-like, hormone receptor-like 3
FAM108A1	family with sequence similarity 108, member A1
FAM70B	family with sequence similarity 70, member B
FBXO42	F-box protein 42
FCGR2B	Fc fragment of IgG, low affinity IIb, receptor (CD32)
FDXR	ferredoxin reductase
FGD6	FYVE, RhoGEF and PH domain containing 6
FSCN2	fascin homolog 2, actin-bundling protein, retinal (Strongylocentrotus purpuratus)
FTL	ferritin, light polypeptide
FTSJ2	FtsJ homolog 2 (E. coli)
FZD1	frizzled homolog 1 (Drosophila)
FZD5	frizzled homolog 5 (Drosophila)
GAS2L3	growth arrest-specific 2 like 3
GINS1	GINS complex subunit 1 (Psf1 homolog)
GLTP	glycolipid transfer protein
GNG5	guanine nucleotide binding protein (G protein), gamma 5
HAGH	hydroxyacylglutathione hydrolase
HIST1H4I	histone cluster 1, H4i
HK3	hexokinase 3 (white cell)
HLA-DQB2	major histocompatibility complex, class II, DQ beta 2
HSPA1A	heat shock 70kDa protein 1A
HTRA1	HtrA serine peptidase 1
IL1B	interleukin 1, beta
ISG20L2	interferon stimulated exonuclease gene 20kDa-like 2
ITPRIP	inositol 1,4,5-triphosphate receptor interacting protein
JPH4	junctophilin 4
KCNH3	potassium voltage-gated channel, subfamily H (eag-related), member 3
KIAA1143	KIAA1143
KIF1C	kinesin family member 1C
KLF4	Kruppel-like factor 4 (gut)
KLK5	kallikrein-related peptidase 5
KRTAP12-3	keratin associated protein 12-3
KRTAP5-10	keratin associated protein 5-10
LAMB2	laminin, beta 2 (laminin S)
LDLRAD3	low density lipoprotein receptor class A domain containing 3
LGALS2	lectin, galactoside-binding, soluble, 2
LMNB2	lamin B2
LOC100505818	afadin-like

Supplemental Table 2.1 continues on next page

Supplemental Table 2.1 *Continued*

Gene	Description
LOC283392	hypothetical LOC283392
LOC401127	WD repeat domain 5 pseudogene
LRG1	leucine-rich alpha-2-glycoprotein 1
LRRC56	leucine rich repeat containing 56
LRRC69	leucine rich repeat containing 69
MAFB	v-maf musculoaponeurotic fibrosarcoma oncogene homolog B (avian)
MCART1	mitochondrial carrier triple repeat 1
MIA	melanoma inhibitory activity
MICAL2	microtubule associated monoxygenase, calponin and LIM domain containing 2
MIR330	microRNA 330
MMP25	matrix metallopeptidase 25
MRGPRE	MAS-related GPR, member E
MRPL54	mitochondrial ribosomal protein L54
MTMR11	myotubularin related protein 11
NDUFAF2	NADH dehydrogenase (ubiquinone) 1 alpha subcomplex, assembly factor 2
NDUFB3	NADH dehydrogenase (ubiquinone) 1 beta subcomplex, 3, 12kDa
NDUFS6	NADH dehydrogenase (ubiquinone) Fe-S protein 6, 13kDa (NADH-coenzyme Q reductase)
NEURL	neuralized homolog (Drosophila)
NME2	non-metastatic cells 2, protein (NM23B) expressed in
NR1H3	nuclear receptor subfamily 1, group H, member 3
NRP1	neuropilin 1
NTAN1	N-terminal asparagine amidase
OAZ2	ornithine decarboxylase antizyme 2
PAQR4	progestin and adipoQ receptor family member IV
PDCD6	programmed cell death 6
PDE8B	phosphodiesterase 8B
PGM2	phosphoglucomutase 2
PID1	phosphotyrosine interaction domain containing 1
PIK3R6	phosphoinositide-3-kinase, regulatory subunit 6
PLAUR	plasminogen activator, urokinase receptor
PLB1	phospholipase B1
PLEKHJ1	pleckstrin homology domain containing, family J member 1
PMM1	phosphomannomutase 1
PNO1	partner of NOB1 homolog (S. cerevisiae)
PNPLA6	patatin-like phospholipase domain containing 6
PODXL	podocalyxin-like
POM121L9P	POM121 membrane glycoprotein-like 9, pseudogene
POU2F2	POU class 2 homeobox 2
PPARA	peroxisome proliferator-activated receptor alpha
PRDX1	peroxiredoxin 1
PRR11	proline rich 11
PSMD8	proteasome (prosome, macropain) 26S subunit, non-ATPase, 8
PSMG2	proteasome (prosome, macropain) assembly chaperone 2

Gene	Description
PSMG3	proteasome (prosome, macropain) assembly chaperone 3
PTPLB	protein tyrosine phosphatase-like (proline instead of catalytic arginine), member b
PTPRE	protein tyrosine phosphatase, receptor type, E
PTPRO	protein tyrosine phosphatase, receptor type, O
PTX3	pentraxin 3, long
QPRT	quinolinate phosphoribosyltransferase
RASSF4	Ras association (RalGDS/AF-6) domain family member 4
RELT	RELT tumor necrosis factor receptor
RLN3	relaxin 3
RNF139	ring finger protein 139
RNF215	ring finger protein 215
RPL13AP5	ribosomal protein L13a pseudogene 5
RPRD1B	regulation of nuclear pre-mRNA domain containing 1B
RPS26	ribosomal protein S26
RRP12	ribosomal RNA processing 12 homolog (S. cerevisiae)
S100A12	S100 calcium binding protein A12
SCAMP5	secretory carrier membrane protein 5
SCARF2	scavenger receptor class F, member 2
SDF2	stromal cell-derived factor 2
SDF4	stromal cell derived factor 4
SERPINB10	serpin peptidase inhibitor, clade B (ovalbumin), member 10
SERPINB2	serpin peptidase inhibitor, clade B (ovalbumin), member 2
SERPINB8	serpin peptidase inhibitor, clade B (ovalbumin), member 8
SGK1	serum/glucocorticoid regulated kinase 1
SH3D21	SH3 domain containing 21
SHISA9	shisa homolog 9 (Xenopus laevis)
SIGLEC10	sialic acid binding Ig-like lectin 10
SIRPA	signal-regulatory protein alpha
SLC25A5	solute carrier family 25 (mitochondrial carrier /// adenine nucleotide translocator), member 5
SLC46A1	solute carrier family 46 (folate transporter), member 1
SLC46A2	solute carrier family 46, member 2
SNORA5B	small nucleolar RNA, H/ACA box 5B
SNORA62	small nucleolar RNA, H/ACA box 62
SNX30	sorting nexin family member 30
SPATA6	spermatogenesis associated 6
SPC24	SPC24, NDC80 kinetochore complex component, homolog (S. cerevisiae)
SPRED1	sprouty-related, EVH1 domain containing 1
SRGAP3	SLIT-ROBO Rho GTPase activating protein 3
STAB1	stabilin 1
STRAP	serine/threonine kinase receptor associated protein
STYXL1	serine/threonine/tyrosine interacting-like 1
SUCLG1	succinate-CoA ligase, alpha subunit

Supplemental Table 2.1 continues on next page

Supplemental Table 2.1 *Continued*

Gene	Description
TCF24	transcription factor 24
TIGD5	tigger transposable element derived 5
TIMELESS	timeless homolog (Drosophila)
TIRAP	toll-interleukin 1 receptor (TIR) domain containing adaptor protein
TLR4	toll-like receptor 4
TNFRSF10B	tumor necrosis factor receptor superfamily, member 10b
TNIP3	TNFAIP3 interacting protein 3
TP53I11	tumor protein p53 inducible protein 11
TP53I3	tumor protein p53 inducible protein 3
TRIAP1	TP53 regulated inhibitor of apoptosis 1
TRIB1	tribbles homolog 1 (Drosophila)
TUBA1A	tubulin, alpha 1a
UCK2	uridine-cytidine kinase 2
USP46	ubiquitin specific peptidase 46
WBSCR16	Williams-Beuren syndrome chromosome region 16
ZNF324B	zinc finger protein 324B
ZNF697	zinc finger protein 697
ZNF710	zinc finger protein 710

Supplemental Table 2.2 Significantly upregulated genes after isoflavone supplementation compared to placebo

Gene	Description
A2LD1	AIG2-like domain 1
ABCF3	ATP-binding cassette, sub-family F (GCN20), member 3
ADD1	adducin 1 (alpha)
ADD3	adducin 3 (gamma)
AGGF1	angiogenic factor with G patch and FHA domains 1
AK3	adenylate kinase 3
AK5	adenylate kinase 5
AKIRIN1	akirin 1
AMIGO2	adhesion molecule with Ig-like domain 2
ANKRD55	ankyrin repeat domain 55
APITD1	apoptosis-inducing, TAF9-like domain 1
APOBEC3F	apolipoprotein B mRNA editing enzyme, catalytic polypeptide-like 3F
ARHGEF9	Cdc42 guanine nucleotide exchange factor (GEF) 9
ASPSCR1	alveolar soft part sarcoma chromosome region, candidate 1
B3GNT1	UDP-GlcNAc:betaGal beta-1,3-N-acetylglucosaminyltransferase 1
BACH2	BTB and CNC homology 1, basic leucine zipper transcription factor 2
BAG5	BCL2-associated athanogene 5
BBS4	Bardet-Biedl syndrome 4
BCL2L1	BCL2-like 1
BPHL	biphenyl hydrolase-like (serine hydrolase)
C10orf111	chromosome 10 open reading frame 111
C12orf41	chromosome 12 open reading frame 41
C17orf28	chromosome 17 open reading frame 28
C21orf128	chromosome 21 open reading frame 128
C21orf49	chromosome 21 open reading frame 49
C21orf63	chromosome 21 open reading frame 63
C22orf36	chromosome 22 open reading frame 36
C3orf39	chromosome 3 open reading frame 39
C9orf78	chromosome 9 open reading frame 78
CA11	carbonic anhydrase XI
CABP5	calcium binding protein 5
CACNA1H	calcium channel, voltage-dependent, T type, alpha 1H subunit
CDC14A	CDC14 cell division cycle 14 homolog A (S. cerevisiae)
CDHR1	cadherin-related family member 1
CDNF	cerebral dopamine neurotrophic factor
CLEC17A	C-type lectin domain family 17, member A
CLK2P	CDC-like kinase 2, pseudogene
CORT	cortistatin
CSRP2BP	CSRP2 binding protein
CYP2D7P1	cytochrome P450, family 2, subfamily D, polypeptide 7 pseudogene 1
DDX51	DEAD (Asp-Glu-Ala-Asp) box polypeptide 51
DENND2C	DENN/MADD domain containing 2C

Supplemental Table 2.2 continues on next page

Supplemental Table 2.2 *Continued*

Gene	Description
DHX34	DEAH (Asp-Glu-Ala-His) box polypeptide 34
DHX38	DEAH (Asp-Glu-Ala-His) box polypeptide 38
DSEL	dermatan sulfate epimerase-like
DUS4L	dihydrouridine synthase 4-like (S. cerevisiae)
EDAR	ectodysplasin A receptor
EDEM1	ER degradation enhancer, mannosidase alpha-like 1
EPB41	erythrocyte membrane protein band 4.1 (elliptocytosis 1, RH-linked)
ERBB2	v-erb-b2 erythroblastic leukemia viral oncogene homolog 2, neuro/glioblastoma derived oncogene homolog (avian)
ERCC5	excision repair cross-complementing rodent repair deficiency, complementation group 5
EXT2	exostosin 2
FAM134C	family with sequence similarity 134, member C
FITM2	fat storage-inducing transmembrane protein 2
FKBP11	FK506 binding protein 11, 19 kDa
FKBP5	FK506 binding protein 5
FLJ36031	hypothetical protein FLJ36031
FLJ44606	glutaredoxin-like protein YDR286C homolog
FOXP1	forkhead box P1
FUBP1	far upstream element (FUSE) binding protein 1
GALNT12	UDP-N-acetyl-alpha-D-galactosamine:polypeptide N-acetylgalactosaminyltransferase 12 (GalNAc-T12)
GATAD2B	GATA zinc finger domain containing 2B
GOLGA8A	golgin A8 family, member A
GPA33	glycoprotein A33 (transmembrane)
GPATCH2	G patch domain containing 2
GUSBL1	glucuronidase, beta-like 1
HEMK1	HemK methyltransferase family member 1
HINFP	histone H4 transcription factor
HIST1H2BO	histone cluster 1, H2bo
IGLV3-10	immunoglobulin lambda variable 3-10
IGLV7-46	immunoglobulin lambda variable 7-46 (gene/pseudogene)
IKZF5	IKAROS family zinc finger 5 (Pegasus)
KCTD9	potassium channel tetramerisation domain containing 9
KIAA0319L	KIAA0319-like
KIAA0748	KIAA0748
KLC4	kinesin light chain 4
LOC100133207	hypothetical LOC100133207
LOC100286979	anaphase-promoting complex subunit 1-like
LOC100289388	hypothetical LOC100289388
LOC100499177	hypothetical LOC100499177
LOC100507418	hypothetical LOC100507418
LOC100507457	hypothetical LOC100507457
LOC145757	hypothetical LOC145757
LOC158572	hypothetical LOC158572

Gene	Description
LOC441208	zinc and ring finger 2 pseudogene
LOC646813	DEAH (Asp-Glu-Ala-His) box polypeptide 9 pseudogene
LOC93622	hypothetical LOC93622
LONRF3	LON peptidase N-terminal domain and ring finger 3
LUC7L2	LUC7-like 2 (S. cerevisiae)
MAGED1	melanoma antigen family D, 1
MAX	MYC associated factor X
MOB2	Mps one binder kinase activator-like 2
MPHOSPH10	M-phase phosphoprotein 10 (U3 small nucleolar ribonucleoprotein)
MYB	v-myb myeloblastosis viral oncogene homolog (avian)
NAPG	N-ethylmaleimide-sensitive factor attachment protein, gamma
NECAB3	N-terminal EF-hand calcium binding protein 3
NELL2	NEL-like 2 (chicken)
NEURL3	neuralized homolog 3 (Drosophila) pseudogene
NLRP6	NLR family, pyrin domain containing 6
NMB	neuromedin B
NUB1	negative regulator of ubiquitin-like proteins 1
NUDT6	nudix (nucleoside diphosphate linked moiety X)-type motif 6
OCRL	oculocerebrorenal syndrome of Lowe
ODF2L	outer dense fiber of sperm tails 2-like
PARK2	parkinson protein 2, E3 ubiquitin protein ligase (parkin)
PDE3B	phosphodiesterase 3B, cGMP-inhibited
PDE4D	phosphodiesterase 4D, cAMP-specific
PDE7A	phosphodiesterase 7A
PDIK1L	PDLIM1 interacting kinase 1 like
PIGC	phosphatidylinositol glycan anchor biosynthesis, class C
PKIG	protein kinase (cAMP-dependent, catalytic) inhibitor gamma
PM20D2	peptidase M20 domain containing 2
PPAT	phosphoribosyl pyrophosphate amidotransferase
PPCS	phosphopantothenoylcysteine synthetase
PPP2R5C	protein phosphatase 2, regulatory subunit B', gamma
PRH1	proline-rich protein HaeIII subfamily 1
RASGRF2	Ras protein-specific guanine nucleotide-releasing factor 2
RCC2	regulator of chromosome condensation 2
RFC1	replication factor C (activator 1) 1, 145kDa
RINL	Ras and Rab interactor-like
RNF144A	ring finger protein 144A
RNF157	ring finger protein 157
RNF165	ring finger protein 165
RNY4P8	RNA, Ro-associated Y4 pseudogene 8
RPL10	ribosomal protein L10
RPL34	ribosomal protein L34
RPLP2	ribosomal protein, large, P2

Supplemental Table 2.2 continues on next page

Supplemental Table 2.2 *Continued*

Gene	Description
RUFY4	RUN and FYVE domain containing 4
SAMD10	sterile alpha motif domain containing 10
SBDS	Shwachman-Bodian-Diamond syndrome
SDC4	syndecan 4
SEL1L3	sel-1 suppressor of lin-12-like 3 (C. elegans)
sep06	septin 6
SERPINE2	serpin peptidase inhibitor, clade E (nexin, plasminogen activator inhibitor type 1), member 2
SLC10A7	solute carrier family 10 (sodium/bile acid cotransporter family), member 7
SLC35B4	solute carrier family 35, member B4
SPRR2G	small proline-rich protein 2G
STXBP1	syntaxin binding protein 1
SUMO1	SMT3 suppressor of mif two 3 homolog 1 (S. cerevisiae)
SVIP	small VCP/p97-interacting protein
SYTL3	synaptotagmin-like 3
TBC1D25	TBC1 domain family, member 25
TC2N	tandem C2 domains, nuclear
THEM4	thioesterase superfamily member 4
TLK1	tousled-like kinase 1
TMEM161A	transmembrane protein 161A
TRIM22	tripartite motif containing 22
TRIM33	tripartite motif containing 33
TRIM39	tripartite motif containing 39
TRIM4	tripartite motif containing 4
TRIM40	tripartite motif containing 40
TSEN2	tRNA splicing endonuclease 2 homolog (S. cerevisiae)
TTC16	tetratricopeptide repeat domain 16
TUBG2	tubulin, gamma 2
TXNDC16	thioredoxin domain containing 16
USP18	ubiquitin specific peptidase 18
ZCCHC17	zinc finger, CCHC domain containing 17
ZFP161	zinc finger protein 161 homolog (mouse)
ZMYM3	zinc finger, MYM-type 3
ZNF189	zinc finger protein 189
ZNF257	zinc finger protein 257
ZNF329	zinc finger protein 329
ZNF331	zinc finger protein 331
ZNF470	zinc finger protein 470
ZNF549	zinc finger protein 549
ZNF569	zinc finger protein 569
ZNF674	zinc finger protein 674
ZNF681	zinc finger protein 681
ZNF84	zinc finger protein 84
ZNF880	zinc finger protein 880

Chapter 3

Isoflavone supplement composition and equol producer status affect gene expression in adipose tissue: A randomized controlled trial in postmenopausal women

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ABSTRACT

Background: Isoflavone supplements, consumed by women experiencing menopausal complaints, are suggested to positively affect menopause-related adiposity and cardiovascular risk profile but discussions about their safety are still ongoing.

Objective: To study the effects of 8 wk consumption of two different isoflavone supplements compared to placebo on whole-genome gene expression in adipose tissue of postmenopausal women.

Design: This randomized double-blind placebo-controlled crossover intervention consisted of two sub studies; one with a low genistein supplement (LG, 56% daidzein, 16% genistein), the other with a high genistein supplement (HG, 49% daidzein, 41% genistein). Both supplements provided ~100 mg isoflavones/day (aglycone equivalents). After the 8 wk isoflavone and placebo period, whole-genome arrays were performed in subcutaneous adipose tissue of postmenopausal women (n = 24 after LG, n = 31 after HG). Data was analysed per sub study for equol producers and non-producers separately.

Results: GSEA showed downregulation of expression of energy metabolism-related genes after LG supplementation in both equol-producing phenotypes and oppositely regulated expression for equol producers (down) and non-producers (up) after HG supplementation. Expression of inflammation-related genes was upregulated in equol producers, while downregulated in non-producers, independent of supplement type. Body weight, adipocyte size and plasma lipid profile were not affected by isoflavone supplementation.

Conclusion: Effects of isoflavones on adipose tissue gene expression were influenced by supplement composition and equol-producing phenotype, while estrogen-responsive effects were limited. LG isoflavone supplementation resulted in a caloric restriction-like gene expression profile for both producer phenotypes and pointed towards a potential beneficial effect, while both supplements induced anti-inflammatory gene expression in equol producers.

INTRODUCTION

Isoflavones, naturally present in soy, are known for their alleged positive health effects in the Asian, predominantly soy-consuming, population (1). The potential positive effects on menopausal complaints makes isoflavones interesting for Western postmenopausal women and therefore for the supplement industry (2). Soy isoflavone supplements are on the market in different compositions (3, 4) in which the ratio between daidzein, genistein and glycitein is depending on the part of the soy plant used for isoflavone extraction (5). Next to the variation in composition, a large variation in doses exists of 5 to 107 mg/day (3, 4). This raises questions regarding the safety and functionality of the different isoflavones (6) at higher doses. Because isoflavones resemble the molecular structure of 17β estradiol, part of the health effects of isoflavones are hypothesized to be induced via α, β or G-coupled estrogen receptor (ER)-mediated gene expression (7, 8). Isoflavones may also affect processes independent of ERs, as we demonstrated in our previous study with peripheral blood mononuclear cells (PBMC), where we found no changes related to activation of the ER. We did observe a downregulation in expression of genes related to oxidative phosphorylation (OXPHOS), inflammation and cell cycle (9). To unravel the total and ER-mediated effects of isoflavone supplementation in postmenopausal women, studies are needed in estrogen-responsive tissues such as adipose tissue. Adipose tissue is of interest because its distribution changes during menopause due to the decline in endogenous estrogen production and it is hypothesized that isoflavones may have positive effects on these unfavourable body fat distribution changes (10, 11). The potential mechanisms have not been evaluated in humans, but results from animal studies suggest that isoflavones may affect glucose and lipid metabolism, for instance via increased energy expenditure as demonstrated in mice (12) and decreased release of adipokines as found in Huanjiang minipigs (13). It has also been suggested that in this respect activation of PPAR (14) and AMPK signaling (15) might play an important role.

It is not yet elucidated whether individual isoflavones have differential effects, or whether the total isoflavone content is responsible for the health effects. In addition, it has been suggested that equol, the more active metabolite of daidzein produced by gut bacteria of 20 to 30% of the general Western population (16), affects gene expression response (17).

In the current study, we aimed to identify the effects of two different types of isoflavone supplements on whole-genome gene expression in adipose tissue of postmenopausal women, taking into account the potential influence of the equol-producing phenotype.

PARTICIPANTS AND METHODS

Subjects

Fifty-eight postmenopausal women were recruited for the intervention study at Wageningen University from November 2011 to January 2012. Equol producer phenotype was determined before the start of the study according to the protocol of Setchell *et al*. (18) and performed as previously described (9). The participants were defined as postmenopausal when they had their last menses more than one year before the start of the study. Exclusion criteria were regular intake of soy products or isoflavone supplements (> 1x/wk), current use of hormone-containing contraceptives, hormone replacement therapy, sex hormones-containing or sex hormone-triggering medication, anti-inflammatory medicines, and use of antibiotics in the past 3 months. Furthermore, women with severe heart disease, thyroid disorders, a removed thyroid gland, removed ovaries or prior diagnosis of cancer were excluded. Lifestyle-related exclusion criteria included alcohol and drug abuse, smoking, a BMI above 35 kg/m2 and self-reported allergy to soy. All subjects consented to participate in the study after oral and written information was provided. The study was approved by the Medical Ethical Committee of Wageningen University and registered at clinicaltrials.gov under NCT01556737.

Study design

This randomized double-blind crossover placebo-controlled study included two 8 wk intervention periods with one 8 wk washout period in between and was conducted in the period from February to October 2012. The study consisted of two parallel sub studies, one for a low genistein isoflavone supplement (LG) vs. placebo and the other for a high genistein isoflavone supplement (HG) vs. placebo. The participants were randomly allocated to one of the sub studies and to either receive the supplement or placebo first. Stratified randomization was performed by an independent research assistant using a computer-generated table of random numbers. The strata used were based on equol producer phenotype and participation to a previous study at the same university (ISO study, (9)). Researchers as well as participants were blinded to randomization until after data analysis.

On the basis of previous nutritional intervention studies using whole-genome transcriptomics a sample size of 20 participants in each sub study was assumed to be sufficient to detect significant effects on whole-genome gene expression in adipose tissue (19).

Isoflavone supplement and placebo

Both supplements consisted of a mix of daidzein, genistein and glycitein, but contained comparable amounts of daidzein. Therefore, the supplement with the lowest genistein content was defined as a 'low genistein supplement' or LG supplement and the other supplement, which contained more genistein as a 'high genistein supplement' or HG supplement. The LG supplement was commercially available as Phytosoya forte 35mg and purchased from Arkopharma (Carros Cedex, France). For the placebo supplement identical empty capsules were provided by Arkopharma, which consisted of hydroxypropylmethyl cellulose (HPMC) dyed with iron oxide and titanium oxide. These were filled by Fagron (Nieuwerkerk a/d IJssel, the Netherlands) with microcrystalline cellulose. The participants in this sub study consumed 100 mg isoflavone/day (in aglycone equivalents; 56 mg daidzein, 16 mg genistein and 28 mg glycitein) for which the participants were asked to consume four capsules a day: two in the morning and two in the evening. The HG supplement was provided by Springfield Nutraceuticals (Oud-Beijerland, the Netherlands) based on the commercially available supplement Mega Soja. The identical-looking placebo capsules were also provided by this supplier; these consisted of HPMC, were not coloured and were filled with microcrystalline cellulose. These participants consumed 104 mg isoflavones daily (in aglycone equivalents; 51 mg daidzein, 43 mg genistein and 10 mg glycitein) for which the participants consumed two capsules a day: one in the morning and one in the evening. The supplement composition was measured at our lab and is reported in Table 3.1. All participants were asked not to eat soy foods for 4 wk before the trial as well as during the trial. To this aid, 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 a 125-item semi-quantitative food frequency questionnaire (FFQ) to monitor their eating habits during these periods (20).

Table 3.1 Isoflavone supplement composition in aglycone equivalents (mg/day)

	Low genistein supplement (mg/day)	High genistein supplement (mg/day)
Daidzin	52.0	50.8
Daidzein	3.7	0.5
Genistin	16.5	42.0
Genistein	0	0.7
Glycitin	26.3	9.8
Glycitein	1.5	0.4
Total isoflavones	100.1	104.2

Blood sampling and adipose tissue biopsies

The participants came to the research venue at Wageningen University four times after an overnight fast; at the start and end of each intervention period. At each visit, venous plasma and serum samples were obtained as described before (9). The subcutaneous adipose tissue samples were obtained by needle biopsy from the periumbilical area under local anaesthesia. The samples were rinsed to eliminate blood and were immediately frozen in liquid nitrogen. All samples were stored in aliquots at -80°C.

Plasma samples were analysed for isoflavone concentrations using HPLC with electrochemical detection as previously described (9, 21). For this method the limit of detection and limit of quantification were 0.11 and 0.22 µmol/L for glycitein and 0.04 and 0.08 µmol/L for the other isoflavones.

Serum samples were analysed for lipid profile (total cholesterol, HDL cholesterol and triglycerides) at the Centre for Medical Diagnostics (SHO, Velp, The Netherlands) and RBP4 concentration at Wageningen University with ELISA and the Quantikine RBP4 immunoassay kit from R&D systems (R&D systems Inc., Minneapolis, USA).

RNA extraction and microarray processing

RNA was isolated from all adipose samples using Qiagen miRNeasy Mini kit (Qiagen, Venlo, The Netherlands) after homogenization with Trizol (Invitrogen, Breda, The Netherlands) and extraction with chloroform and ethanol. RNA yield was quantified and integrity was measured as previously described (9). Of 57 subjects, adipose tissue samples yielded enough RNA of sufficient quality to perform microarray analysis, resulting in a total of 114 microarrays. RNA labeling and hybridization to GeneChip® Human Gene 1.1 ST Array was performed according to the manufacturers' instructions as previously described (9).

Adipocyte size

Because of the observed effects of the LG supplement on energy metabolism-related gene expression in our study, adipocyte size was studied for the LG supplement sub study (n = 19). Frozen (-80°C) adipose tissue biopsy samples were transferred to 4% formaldehyde in phosphate buffered saline (PBS) for at least 24h and embedded in paraffin. Slices of 8 µm were cut using a microtome (model RM2355, Leica Microsystems B.V., Rijswijk, Netherlands), a hematoxylin and eosin staining was performed and cell size was determined using a Olympus BX41 microscope with CellSens Imaging software (Olympus Nederland B.V., Zoetermeer, Netherlands).

Data analysis

Pairs of arrays performed for 55 participants yielded good quality microarray results; 24 participants from the LG supplement sub study and 31 participants from the HG supplement sub study. Expression values of the microarray data were calculated using the Robust Multichip Average (RMA) method and normalized using quantile normalization (22, 23) in MADMAX (management and analysis database for multi-platform microarray experiments, (24)). Genes with normalized signals of > 20 on > 15 arrays were defined as expressed and selected for analysis. Genes were regarded as significantly changed at $P < 0.05$. Further data analysis was performed using Gene Set Enrichment Analysis (GSEA, (25, 26)) with a false discovery rate (FDR) of < 0.25 and results were visualized and clustered using Cytoscape (27). Upstream regulator analysis was performed using Ingenuity Pathway Analysis (Ingenuity Systems, Redwood City, CA, (28)). A list of estrogen-responsive genes derived from the Dragon Estrogen-Responsive Gene Database (ERGDB) comprising 1069 genes from Homo Sapiens (data downloaded September 2013, (29)) was compared to the significantly changed genes in both sub studies.

For the 55 participants with good quality microarrays, plasma isoflavone concentrations and changes in eating habits (FFQ data, one missing for LG supplement) were reported. To further examine the effect of the isoflavone supplement on energy metabolism, data on serum lipid profile, serum RBP4 concentrations and body weight data were reported. Samples of 19 participants in the LG supplement sub study were analysed for adipocyte size, as this sub study showed the most pronounced effects on energy metabolism-related gene expression. Mean perimeters (in µm) from 20 different cells were averaged and used for statistical analysis. Statistical analysis for these variables was performed using paired t-tests ($P < 0.05$) for differences between supplement and placebo for the two supplements and also for the two supplements and the two producer phenotypes separately (SAS version 9.3, SAS Institute, Inc., Cary, NC).

RESULTS

In this study, 7 equol producers were identified in the LG supplement sub study (n = 24) and 8 in the HG supplement sub study (n = 31). Baseline age, body weight and BMI of participants were not significantly different between the two sub studies (Table 3.2). Mean total isoflavone plasma concentrations after intake of the supplements ranged from 2.18 to 2.57 µmol/L in the two sub studies per producer phenotype (Table 3.3). Plasma daidzein concentrations did not significantly differ between the supplements, while

genistein concentrations were significantly higher after intake of the HG supplement. Glycitein concentrations were significantly higher for non-producers after intake of the LG supplement, and equol concentrations were only detected in equol-producers.

Table 3.2 Baseline characteristics of participants with good quality arrays per sub study

	Low genistein supplement	High genistein supplement
N	24	31
N equol producers (%)*	7 (29)	8 (26)
Age (years)	63.2 ± 5.5	63.0 ± 5.5
BMI (kg/m^2)	25.3 ± 3.0	25.0 ± 3.7
Body weight (kg)	71.8 ± 10.1	69.7 ± 11.2

Values are means ± SDs for age, BMI and bodyweight.
There were no significant differences in subject characteristics for the two sub studies.
* Number of equol producers as determined at the end of the intervention period with the isoflavone supplement.

Table 3.3 Isoflavone plasma concentrations in the two sub studies after the isoflavones intervention separate for equol producers and non-producers with good quality arrays

	Low genistein supplement		High genistein supplement	
	EP (n = 7)	NP (n = 17)	EP (n = 8)	NP (n = 23)
Daidzein	1.06 (± 0.84)	1.54 (± 0.74)	0.97 (± 0.50)	1.21 (± 0.65)
Equol	0.73 (± 0.61)	NA	0.64 (± 0.28)	NA
Genistein	0.31 (± 0.17)	0.50 (± 0.37)	0.93 (± 0.56)*	1.29 (± 0.94)*
Glycitein	0.18 (± 0.18)	0.12 (± 0.12)	0.03 (± 0.05)	0.05 (± 0.07)*
Total**	2.27 (± 0.99)	2.18 (± 0.96)	2.57 (± 1.16)	2.55 (± 1.48)

Values are means ± SDs in µmol/L, EP equol producer, NP non-producer, NA not applicable.
** Total isoflavones as the sum of daidzein, equol, genistein and glycitein.
* $P < 0.05$ for comparison between supplements separately for producer status.

Low genistein vs. High genistein supplement

Microarray analysis of adipose tissue after intake of the LG supplement showed a significant change in expression of 1245 genes in adipose tissue of 24 postmenopausal women as compared to placebo (Figure 3.1). In adipose tissue of the 31 postmenopausal women taking the HG supplement, expression of 528 genes was significantly changed. The overlap of significantly changed genes between the two supplements was 53 genes. GSEA of this data showed that after exposure to the LG supplement compared to placebo, gene sets

related to energy metabolism, amino acid metabolism and cell cycle were downregulated, whereas after the HG supplement these gene sets were upregulated. In addition, after intake of the latter supplement an upregulation of gene sets involved in inflammation and estrogen signaling was observed (Figure 3.2)

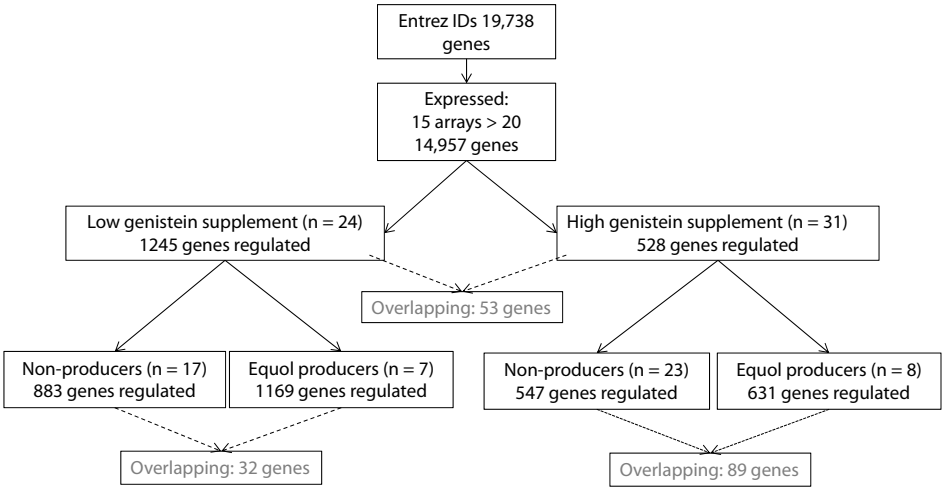

Figure 3.1 Flow chart of genes from Affymetrix gene chip to genes with significantly changed expression after exposure to each supplement compared to placebo and further separated for producer status.

Gene sets in GSEA	Low genistein			High genistein		
	All	NP	EP	All	NP	EP
Glucose metabolism	↓	↓	↓	↑	↑	↓
OXPHOS	↓	↓	↓	↑	↑	↓
TCA cycle	↓	↓	↓	↑	↑	
Fatty acid metabolism	↓	↓	↓	↑	↑	
Inflammation		↓	↑	↑	↓	↑
Cell cycle	↓	↑	↓	↑	↑	
Amino acid metabolism	↓		↓	↑		
Protein processing				↑	↑	↓
Complement & coagulation					↓	
Estrogen signaling				↑	↑	

Figure 3.2 Results of GSEA of both supplements for all participants and separately for equol producers (EP) and non-producers (NP).

Effect of the equol-producing phenotype

To identify the effect of the equol-producing phenotype on gene expression changes in adipose tissue, data analysis were performed separately for equol producers and non-producers for each supplement (Figure 3.1) and showed different gene expression changes in both sub studies. GSEA, performed separately for each producer phenotype, showed similar effects of the two phenotypes on energy-related gene expression after intake of the LG supplement and opposite effects on inflammation and cell cycle (Figure 3.2). Effects on amino acid metabolism were only observed for equol producers. For the HG supplement, opposite effects for the two phenotypes were observed for energy metabolism, inflammation and protein processing. Effects on cell cycle, complement and coagulation and estrogen signaling were only observed for non-producers after intake of the HG supplement.

Gene sets related to energy metabolism and inflammation were selected for further analysis as these had lowest FDR values (FDR < 0.25) for both producer phenotypes in the two sub studies. Individual changes in expression of energy metabolism-related genes are visualized in heatmaps for both supplements (Supplemental Figures 3.1a and 3.1b). The number of genes with significantly changed expression involved in energy metabolism was 121 for the LG supplement and 19 for the HG supplement, indicating a more pronounced effect after intake of the LG supplement. To illustrate the effect of LG supplement on energy metabolism, a schematic representation of an adipocyte with the effects of this supplement on genes in pathways of glucose metabolism, TCA cycle, OXPHOS, fatty acid synthesis, triglyceride synthesis and cholesterol biosynthesis is shown in Figure 3.3. Individual changes in expression of genes in the inflammation-related gene sets are visualized in heatmaps in Supplemental Figures 3.2a and 3.2b.

With Ingenuity software, potential upstream regulators of the genes with significantly changed expression were identified. Several upstream regulators were identified for equol producers after intake of the LG supplement while only a few or none were identified for the other groups (Table 3.4). Most upstream regulators were involved in activation or inhibition of energy metabolism and inflammation-related genes.

Estrogen-related effects

The estrogen-responsive effects on gene expression after isoflavone supplementation were further explored by comparison of the genes with significantly changed expression to the ERGDB database. As a result, between 4.4 and 7.0% (30 to 82) of genes with significantly

changed expression after intake of both supplements for both producer phenotypes were identified as estrogen-responsive (Supplemental Figure 3.3).

Table 3.4 Upstream regulators derived from Ingenuity analysis (-1.5 > z > 1.5, P < 0.05) with effects on their downstream pathways

		Upstream regulator	z-value	Overlap P-value	Effects on downstream pathways	
					Energy metabolism	Inflammation
Low genistein supplement	EP	BRD4	-2.63	0.04	↓	
		IL13	-2.27	0.00	↓	↑
		SYVN1	-2.14	0.03	↓	↓
		PPARGCA1	-1.95	0.01	↓	
		KRAS	-1.94	0.00		↑
		MAPK1	-1.53	0.01		↑/↓
		NEDD9	1.63	0.01	↓	
		GATA1	1.98	0.00		↑
		EPO	1.98	0.00		
		CD40LG	2.19	0.00		↑
		FOXO3	2.59	0.00		↑
	NP	KDM5D	-1.67	0.04		↓
		CD24	2.45	0.04		↑
High genistein	EP	IFNB1	2.00	0.00		↑
	NP	none				

EP, equol producer; NP, non-producer.

Effects on physiological markers

Because the isoflavone-induced gene expression pointed towards a downregulation of glucose metabolism, TCA cycle, OXPHOS, fatty acid synthesis, triglyceride synthesis and cholesterol biosynthesis in adipose tissue, effects of isoflavone supplement intake on body weight, adipocyte size and plasma lipid profiles were also evaluated. No significant differences on body weight and adipocyte size were observed for supplement vs. placebo in producers and non-producers (Table 3.5). Furthermore, no significant differences after intake of the two supplements vs. placebo were observed for total, HDL and LDL cholesterol and triglycerides in serum of equol producers and non-producers. Additionally, expression of RPB4, a gene coding for an adipokine associated with energy metabolism, was significantly downregulated in adipose tissue of equol producers after intake of the LG supplement.

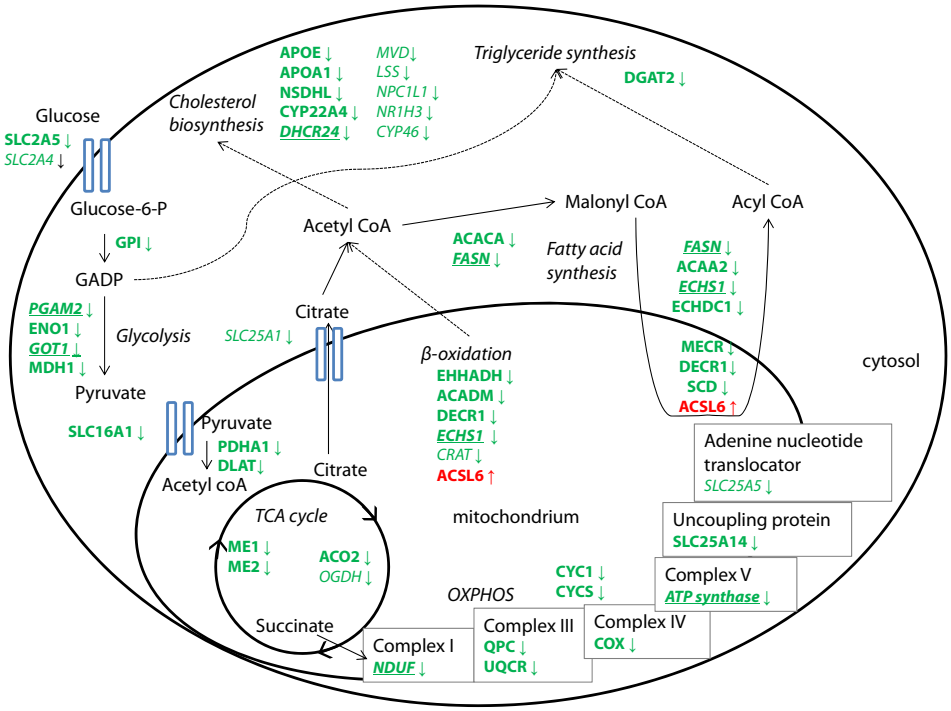

Figure 3.3 Significantly changed genes related to energy metabolism, i.e. the pathways of glucose metabolism, TCA cycle, oxidative phosphorylation, fatty acid synthesis, triglyceride synthesis and cholesterol biosynthesis, after exposure to the low genistein supplement depicted in a schematic adipocyte. Bold genes are significantly changed in adipose tissue of equol producers, italic genes in adipose tissue of non-producers and underlined genes in both phenotypes, $P < 0.05$.

ACAA2, acetyl-CoA acyltransferase 2; ACACA, acetyl-CoA carboxylase alpha; ACADM, acyl-CoA dehydrogenase; ACO2, aconitase 2, mitochondrial; ACSL6, acyl-CoA synthetase long-chain family member 6; APOA1, apolipoprotein A-I; APOE, apolipoprotein E; COX , cytochrome c oxidase; CRAT, carnitine O-acetyltransferase; CYC1, cytochrome c-1; CYCS, cytochrome c, somatic; CYP22A4 and CYP46, cytochrome P450 22A4 and 46; DECR1, 2,4-dienoyl CoA reductase 1, mitochondrial; DGAT2, diacylglycerol O-acyltransferase 2; DHCR24, 24-dehydrocholesterol reductase; DLAT, dihydrolipoamide S-acetyltransferase; ECHDC1, enoyl CoA hydratase domain containing 1; ECHS1, enoyl CoA hydratase, short chain, 1, mitochondrial; EHHADH, enoyl-CoA, hydratase/3-hydroxyacyl CoA dehydrogenase; ENO1, enolase 1, (alpha); FASN, fatty acid synthase; GOT1, glutamic-oxaloacetic transaminase 1, soluble (aspartate aminotransferase 1); GPI, glucose-6-phosphate isomerase; LSS, lanosterol synthase (2,3-oxidosqualene-lanosterol cyclase); MDH1, malate dehydrogenase 1, NAD (soluble); ME1, malic enzyme 1, NADP(+)-dependent, cytosolic; ME2, malic enzyme 2, NAD(+)-dependent, mitochondrial; MECR, mitochondrial trans-2-enoyl-CoA reductase; MVD, mevalonate (diphospho) decarboxylase; NDUF, NADH dehydrogenase; NPC1L1, Niemann-Pick disease, type C1-like 1; NR1H3, nuclear receptor subfamily 1, group H, member 3; NSDHL, NAD(P) dependent steroid dehydrogenase-like; OGDH, oxoglutarate (alpha-ketoglutarate) dehydrogenase (lipoamide); PDHA1, pyruvate dehydrogenase (lipoamide) alpha 1; PGAM2, phosphoglycerate mutase 2 (muscle); QPC, glutaminyl-peptide cyclotransferase; SCD, stearoyl-CoA desaturase (delta-9-desaturase); SLC16A1, SLC25A1, SLC25A14, SLC25A5 , SLC2A4, SLC2A5, solute carrier family; UQCR, ubiquinol-cytochrome c reductase.

Therefore, serum RPB4 was measured in the total study population. No statistical significant effects of the isoflavone supplements vs. placebo were observed on this marker for each producer phenotype (Table 3.5).

Table 3.5 Plasma markers for lipid profile and RBP4

		Low genistein supplement (EP n = 7, NP n = 17)		High genistein supplement (EP n = 8, NP n = 23)	
		Supplement	Placebo	Supplement	Placebo
Cholesterol (mmol/L)	EP	6.52 ± 0.58	6.81 ± 1.49	6.04 ± 0.80	5.98 ± 0.57
	NP	5.98 ± 0.91	6.09 ± 0.90	5.84 ± 0.92	5.81 ± 0.95
HDL (mmol/L)	EP	1.99 ± 0.49	2.05 ± 0.60	1.74 ± 0.35	1.79 ± 0.57
	NP	1.75 ± 0.59	1.80 ± 0.59	1.84 ± 0.43	1.88 ± 0.50
LDL (mmol/L)	EP	3.98 ± 0.63	4.12 ± 1.35	3.67 ± 0.78	3.64 ± 0.62
	NP	3.67 ± 0.85	3.72 ± 0.85	3.51 ± 0.97	3.42 ± 0.93
Triglycerides (mmol/L)	EP	1.23 ± 0.28	1.43 ± 0.92	1.40 ± 0.51	1.23 ± 0.44
	NP	1.24 ± 0.53	1.27 ± 0.51	1.09 ± 0.47	1.12 ± 0.45
RBP4 (µmol/L)	EP	1.16 ± 0.44	1.50 ± 0.42	1.10 ± 0.38	1.10 ± 0.38
	NP	1.19 ± 0.40	1.13 ± 0.38	1.07 ± 0.44	1.02 ± 0.34
Body weight (kg)	EP	65.9 ± 10.0	65.5 ± 10.3	70.7 ± 14.7	70.8 ± 14.1
	NP	73.7 ± 9.7	73.5 ± 10.1	68.2 ± 10.5	68.3 ± 10.3
Adipocyte size* (µm)	EP	357.8 ± 33.9	378.0 ± 25.3	-	-
	NP	331.4 ± 42.0	330.0 ± 44.1	-	-

Values are means ± SDs for all variables.
No significant differences were observed after supplement compared to placebo for both supplements and producer phenotypes (paired t-test, $P < 0.05$).
*Adipocyte size was only measured in the sub study with the low genistein supplement for n = 4 equol producers and n=15 non-producers.
RPB4, Retinol Binding Protein 4; EP, equol producer; NP, non-producer.

FFQ results

There were no significant differences in energy-adjusted carbohydrate and fat intake by the participants during the supplement and placebo intervention periods for both supplements (results not shown). Mean energy intake by the participants was significantly higher (+ 579kJ) during the supplement than the placebo intervention period for the HG supplement sub study. Energy-adjusted protein intake was significantly higher (+ 1 energy%) during the supplement than the placebo intervention period for the LG supplement sub study.

DISCUSSION

Isoflavone supplement intake for 8 weeks affected gene expression in adipose tissue of postmenopausal women. The effects were dependent on the isoflavone supplement-type consumed and the equol-producing phenotype of the participants. Expression of energy metabolism-related genes was downregulated after intake of the LG supplement for both phenotypes and oppositely regulated for equol producers (down) and non-producers (up) after intake of the HG supplement. Expression of inflammation-related genes was upregulated in equol producers, while downregulated in non-producers, independent of supplement type (Figure 3.2).

After consumption of the LG supplement, the downregulated expression of several genes within connecting metabolic pathways of glucose metabolism, TCA cycle, OXPHOS, fatty acid synthesis, triglyceride and cholesterol metabolism pointed towards a reduction in energy metabolism. One of the identified upstream regulators was PGC-1α, which was significantly downregulated in equol producers and is known to be involved in the regulation of OXPHOS (30). A previous study examining adipose tissue of female OVX Sprague-Dawley rats after intake of an isoflavone supplement, similar to the LG supplement, in combination with a high fat diet as compared to effects after a high fat diet only, also resulted in a downregulation of gene expression related to energy metabolism (31). The observed downregulation in gene expression of PGC-1α- and energy-related pathways in adipose tissue is also very well known as a consequence of caloric restriction (32, 33). In caloric restriction, these expression changes are paralleled by a reduction in adipocyte size and subsequent weight loss. In our study we could not identify a significant decrease in adipocyte size or body weight. This could be due to the relatively short 8 wk exposure period, because the mild effects of isoflavones might require longer exposure to isoflavones to induce changes in adipocyte size and body weight. In addition, adipocyte size was assessed in subcutaneous adipose tissue, while effects might have shown in visceral adipose tissue. Furthermore, changes in body fat distribution were not assessed, while this might have been affected without changing body weight. Also, isoflavone-induced effects on gene expression of RBP4 and of genes related to cholesterol metabolism were not reflected by changes in serum RBP4 and cholesterol (total, HDL and triglycerides) concentrations.

Besides a role as energy-storing organ, adipose tissue is an active tissue in the regulation of immunity and inflammation (34-36). Intake of the two supplements also resulted in the significant upregulation of genesets involved in inflammation for the equol producers. However, further analysis identified an inhibition of upstream regulators known to play

a role in activating inflammation, such as MAPK1 and KRAS. In addition, expression of the receptor for the anti-inflammatory interleukin 10 (IL10RA) and NFKBIA were upregulated, while pro-inflammatory IRAK2 was downregulated. Although first analyses suggested an upregulation of inflammation, more in depth analysis pointed towards a predominance of the anti-inflammatory response after intake of the LG supplement in equol producers. Intake of the HG supplement by equol producers resulted in activation of the upstream regulator interferon β (IFNB1). Activation of this cytokine is suggested to lead to inhibition of the NLRP1b and NLRP3 inflammasomes via upregulation of STAT1 (37). Although expression of specific inflammasome-related genes was not changed, STAT1 was significantly upregulated which may point towards an anti-inflammatory response after intake of the HG supplement by equol producers. In contrast to this more anti-inflammatory response in adipose tissue of equol producers after intake of both supplements, specific pro- or anti-inflammatory responses could not be elucidated in adipose tissue of non-producers.

Isoflavones are known to induce estrogen-related effects because of their structural similarity with 17β-estradiol. In this study, the effects of isoflavone supplementation were studied in adipose tissue, an endocrine organ (38), therefore estrogen receptor-mediated effects were expected to be prominent. We observed that only about 4.4 to 7% of the genes with significantly changed expression in our study were known to be estrogen-responsive. This indicates that, next to estrogen-responsive effects, the majority of isoflavone-induced gene expression effects (93%) are most likely regulated via other ways than activation of the ERs in these postmenopausal women. The limited effects of isoflavones via the ERs might be due to lower expression of the estrogen receptor in postmenopausal women compared to premenopausal women, although we previously did not find differences in the gene expression levels of pre- and postmenopausal women (9). However, their protein levels might still be reduced. Furthermore, isoflavones might induce more estrogen-responsive effects in other estrogen-responsive tissues such as breast and uterus tissue.

The gene expression effects in this study were separately analysed for both producer phenotypes, which explained some of the variation in response, but large inter-individual differences in response were still observed. This might be explained by other effect modifiers for isoflavones that were not taken into account, such as production of other gut-metabolites of daidzein; for instance production of O-desmethylangolensin (O-DMA) (39). But other factors might also be responsible for the individual variation in response, like ER polymorphisms (40, 41) and DNA methylation of certain genes (42).

To our knowledge, effects of isoflavone supplementation on whole-genome gene expression in human adipose tissue have not been published so far. In this study, the two supplements were well-characterized with regard to isoflavone content and the equol-producing phenotypes were identified prior to the study to allow stratification. Another strength of our study was the sample size (n = 24 and 31), as crossover studies involving whole-genome gene expression are often performed with lesser participants (43, 44). No differences in baseline characteristics between the two sub studies and the producer phenotypes were observed. However, the estimated energy and protein intake based upon FFQ during the intervention periods differed between supplement and placebo for the HG and LG supplement, respectively. The daily dose of both commercially-available supplements (100 mg/day) was relevant with regard to effects of over-the-counter isoflavone supplements. Compliance to the study was confirmed by returned pill count (> 94.5% compliant, results not shown) and by the reflection of the isoflavone content of the supplement in plasma concentrations. The two commercial supplements were soy extracts, differing not only in genistein content but also in glycitein content. Considering that the estrogen-responsive effects were limited in our study, the higher glycitein concentration might also be responsible for the more prominent effects of the LG supplement. Furthermore, potential competition or interaction of the four isoflavones for other receptors or transcriptional regulators might also play a role in the observed effects. Because the responsible isoflavone, isoflavone ratio or molecular pathway cannot be elucidated from this study, further investigation on these is required before any conclusion can be drawn regarding health effects of isoflavone supplementation.

Altogether, the current study showed that isoflavone supplementation induced molecular effects on energy metabolism and inflammation which were only partly induced via the classical ER-related pathways, but were highly influenced by supplement composition and equol-producing phenotype. Consumption of the LG isoflavone supplement by postmenopausal women resulted in a caloric restriction-like gene expression profile in adipose tissue and pointed towards a potential beneficial effect of this supplement, while intake of both supplements induced anti-inflammatory gene expression in equol producers.

ACKNOWLEDGEMENTS

We thank Dini Venema and Shohreh Keshtar for their excellent technical assistance, Mark Boekschoten for statistical assistance for the micro array analysis, Rinke Stienstra and Polyxeni Papanastasiou for their work on adipocyte size. Furthermore, we thank all participants, dieticians and students for their contribution to the study.

The authors' responsibilities were as follows-VvdV, AG, EGS, PvtV, LAA: designed the trial; VvdV: conducted the trial; VvdV: analyzed the data; VvdV: wrote the manuscript; and AG, EGS, PCH, PvtV and LAA: critically reviewed the manuscript. None of the authors declared a conflict of interest.

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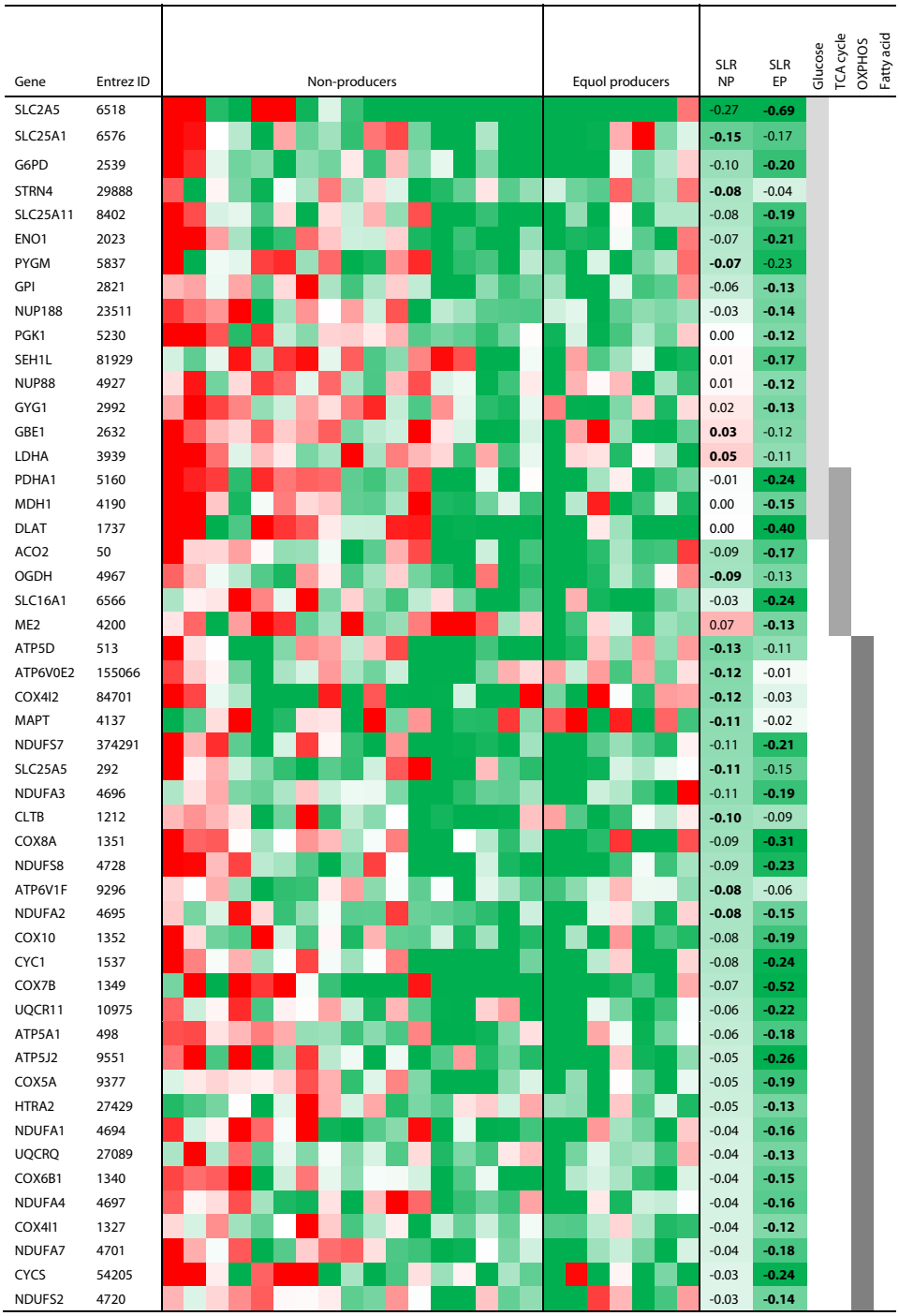
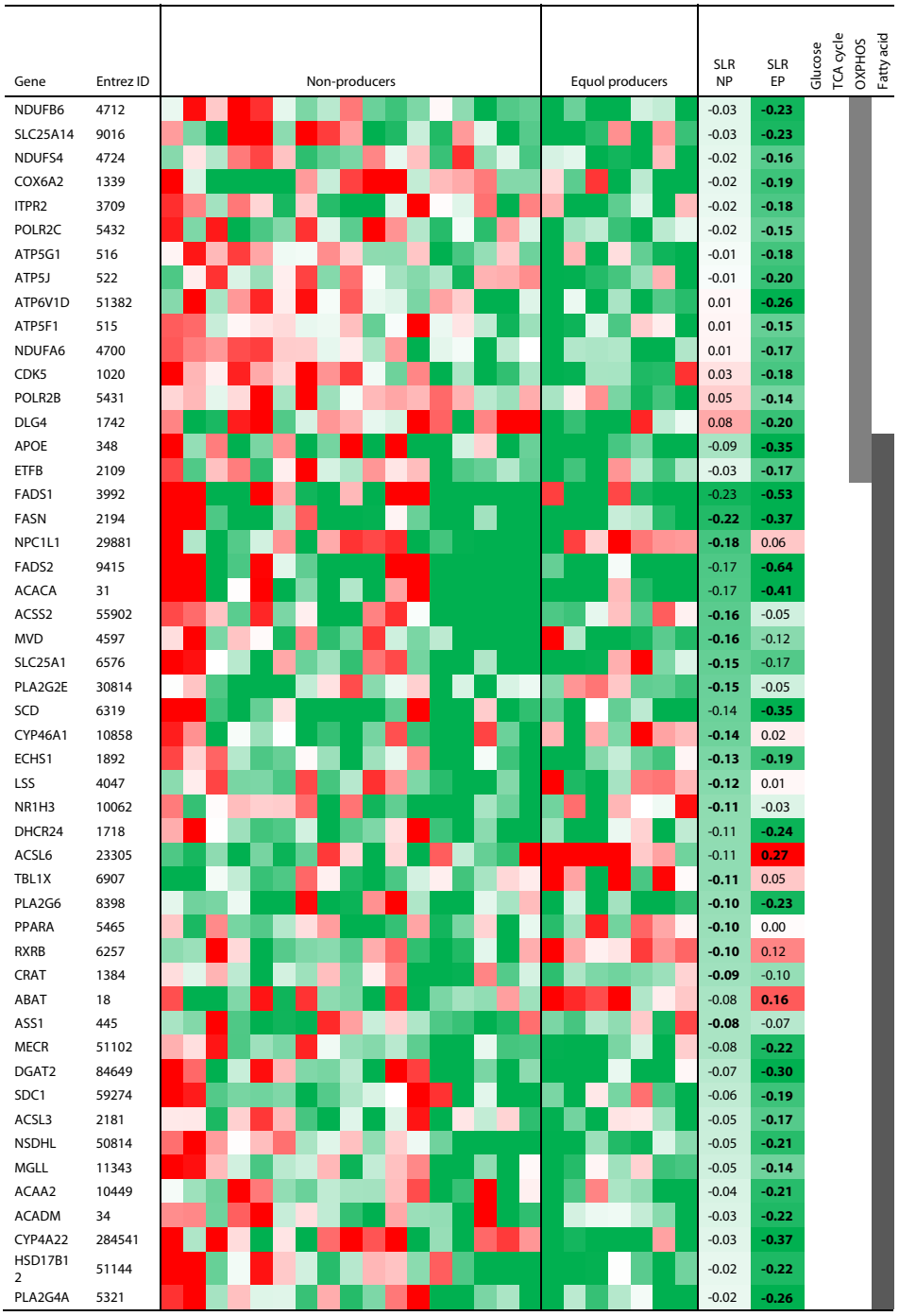
Supplemental Figure 3.1a Energy metabolism Low genistein supplement



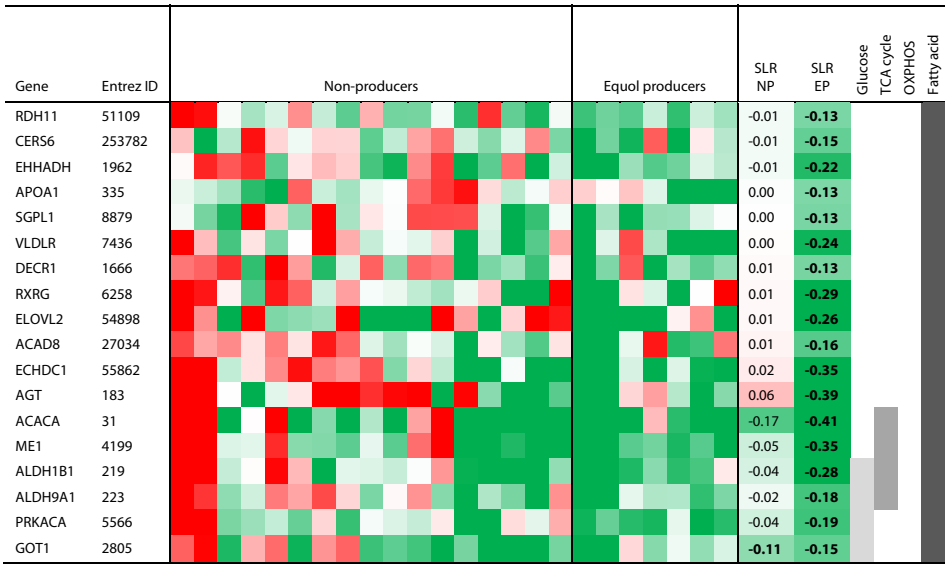
Gene	Entrez ID	SLR NP	SLR EP
SLC2A5	6518	-0.27	**-0.69**
SLC25A1	6576	**-0.15**	-0.17
G6PD	2539	-0.10	**-0.20**
STRN4	29888	**-0.08**	-0.04
SLC25A11	8402	-0.08	**-0.19**
ENO1	2023	-0.07	**-0.21**
PYGM	5837	**-0.07**	-0.23
GPI	2821	-0.06	**-0.13**
NUP188	23511	-0.03	**-0.14**
PGK1	5230	0.00	**-0.12**
SEH1L	81929	0.01	**-0.17**
NUP88	4927	0.01	**-0.12**
GYG1	2992	0.02	**-0.13**
GBE1	2632	**0.03**	-0.12
LDHA	3939	**0.05**	-0.11
PDHA1	5160	-0.01	**-0.24**
MDH1	4190	0.00	**-0.15**
DLAT	1737	0.00	**-0.40**
ACO2	50	-0.09	**-0.17**
OGDH	4967	**-0.09**	-0.13
SLC16A1	6566	-0.03	**-0.24**
ME2	4200	0.07	**-0.13**
ATP5D	513	**-0.13**	-0.11
ATP6V0E2	155066	**-0.12**	-0.01
COX4I2	84701	**-0.12**	-0.03
MAPT	4137	**-0.11**	-0.02
NDUFS7	374291	-0.11	**-0.21**
SLC25A5	292	**-0.11**	-0.15
NDUFA3	4696	-0.11	**-0.19**
CLTB	1212	**-0.10**	-0.09
COX8A	1351	-0.09	**-0.31**
NDUFS8	4728	-0.09	**-0.23**
ATP6V1F	9296	**-0.08**	-0.06
NDUFA2	4695	**-0.08**	**-0.15**
COX10	1352	-0.08	**-0.19**
CYC1	1537	-0.08	**-0.24**
COX7B	1349	-0.07	**-0.52**
UQCR11	10975	-0.06	**-0.22**
ATP5A1	498	-0.06	**-0.18**
ATP5J2	9551	-0.05	**-0.26**
COX5A	9377	-0.05	**-0.19**
HTRA2	27429	-0.05	**-0.13**
NDUFA1	4694	-0.04	**-0.16**
UQCRQ	27089	-0.04	**-0.13**
COX6B1	1340	-0.04	**-0.15**
NDUFA4	4697	-0.04	**-0.16**
COX4I1	1327	-0.04	**-0.12**
NDUFA7	4701	-0.04	**-0.18**
CYCS	54205	-0.03	**-0.24**
NDUFS2	4720	-0.03	**-0.14**

Supplemental Figure 3.1a Energy metabolism Low genistein supplement



Gene	Entrez ID	SLR NP	SLR EP
NDUFB6	4712	-0.03	**-0.23**
SLC25A14	9016	-0.03	**-0.23**
NDUFS4	4724	-0.02	**-0.16**
COX6A2	1339	-0.02	**-0.19**
ITPR2	3709	-0.02	**-0.18**
POLR2C	5432	-0.02	**-0.15**
ATP5G1	516	-0.01	**-0.18**
ATP5J	522	-0.01	**-0.20**
ATP6V1D	51382	0.01	**-0.26**
ATP5F1	515	0.01	**-0.15**
NDUFA6	4700	0.01	**-0.17**
CDK5	1020	0.03	**-0.18**
POLR2B	5431	0.05	**-0.14**
DLG4	1742	0.08	**-0.20**
APOE	348	-0.09	**-0.35**
ETFB	2109	-0.03	**-0.17**
FADS1	3992	-0.23	**-0.53**
FASN	2194	**-0.22**	**-0.37**
NPC1L1	29881	**-0.18**	0.06
FADS2	9415	-0.17	**-0.64**
ACACA	31	-0.17	**-0.41**
ACSS2	55902	**-0.16**	-0.05
MVD	4597	**-0.16**	-0.12
SLC25A1	6576	**-0.15**	-0.17
PLA2G2E	30814	**-0.15**	-0.05
SCD	6319	-0.14	**-0.35**
CYP46A1	10858	**-0.14**	0.02
ECHS1	1892	**-0.13**	**-0.19**
LSS	4047	**-0.12**	0.01
NR1H3	10062	**-0.11**	-0.03
DHCR24	1718	-0.11	**-0.24**
ACSL6	23305	-0.11	**0.27**
TBL1X	6907	**-0.11**	0.05
PLA2G6	8398	**-0.10**	**-0.23**
PPARA	5465	**-0.10**	0.00
RXRB	6257	**-0.10**	0.12
CRAT	1384	**-0.09**	-0.10
ABAT	18	-0.08	**0.16**
ASS1	445	**-0.08**	-0.07
MECR	51102	-0.08	**-0.22**
DGAT2	84649	-0.07	**-0.30**
SDC1	59274	-0.06	**-0.19**
ACSL3	2181	-0.05	**-0.17**
NSDHL	50814	-0.05	**-0.21**
MGLL	11343	-0.05	**-0.14**
ACAA2	10449	-0.04	**-0.21**
ACADM	34	-0.03	**-0.22**
CYP4A22	284541	-0.03	**-0.37**
HSD17B12	51144	-0.02	**-0.22**
PLA2G4A	5321	-0.02	**-0.26**

Supplemental Figure 3.1a Energy metabolism Low genistein supplement

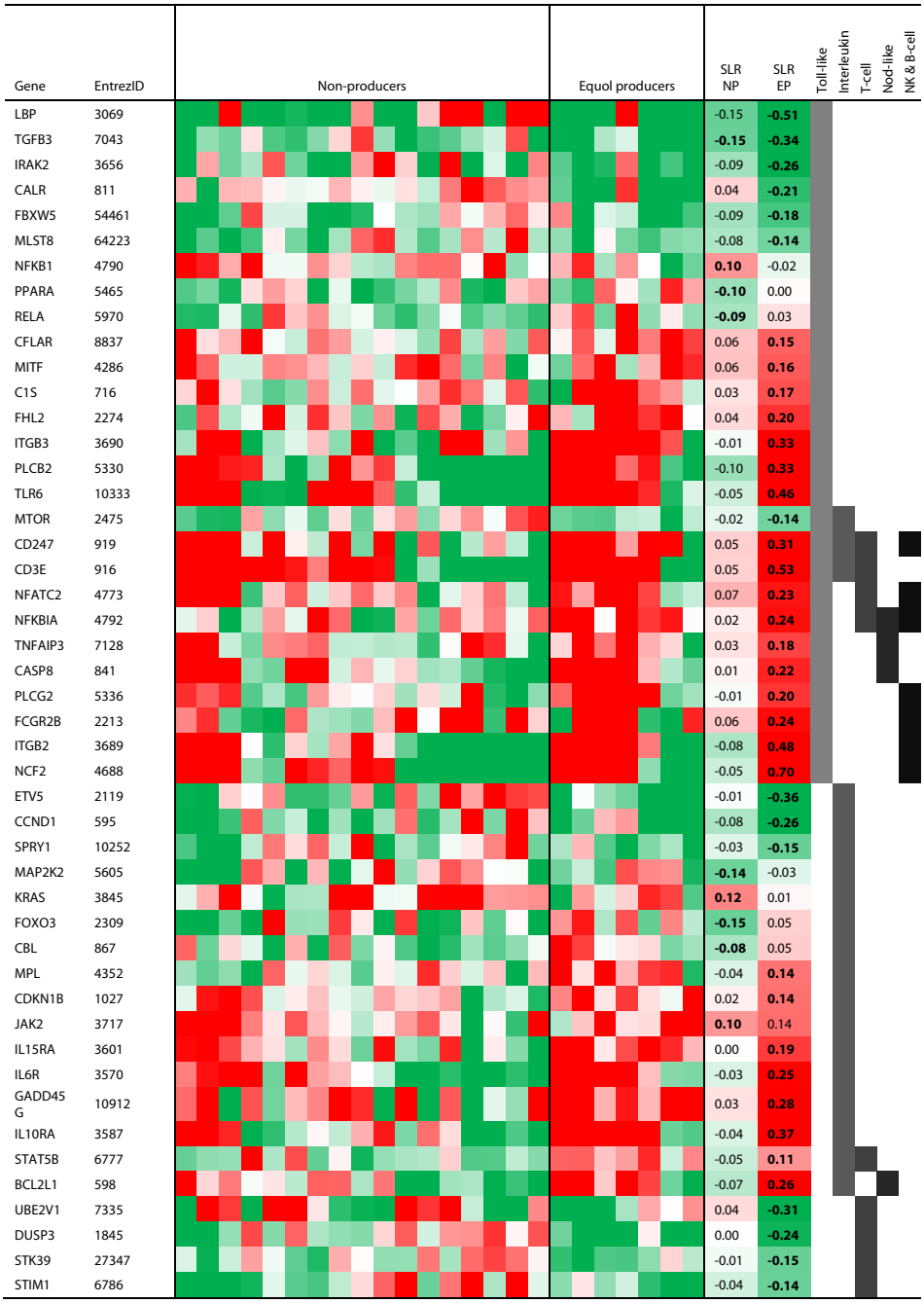


Supplemental Figure 3.1a Heatmap of significantly expressed genes in postmenopausal women involved in energy metabolism after eight weeks exposure to the low genistein supplement compared to placebo. Bold SLRs are significantly expressed, SLR signal log ratio, EP equol producer, NP non producer.

Supplemental Figure 3.1b Energy metabolism High genistein supplement

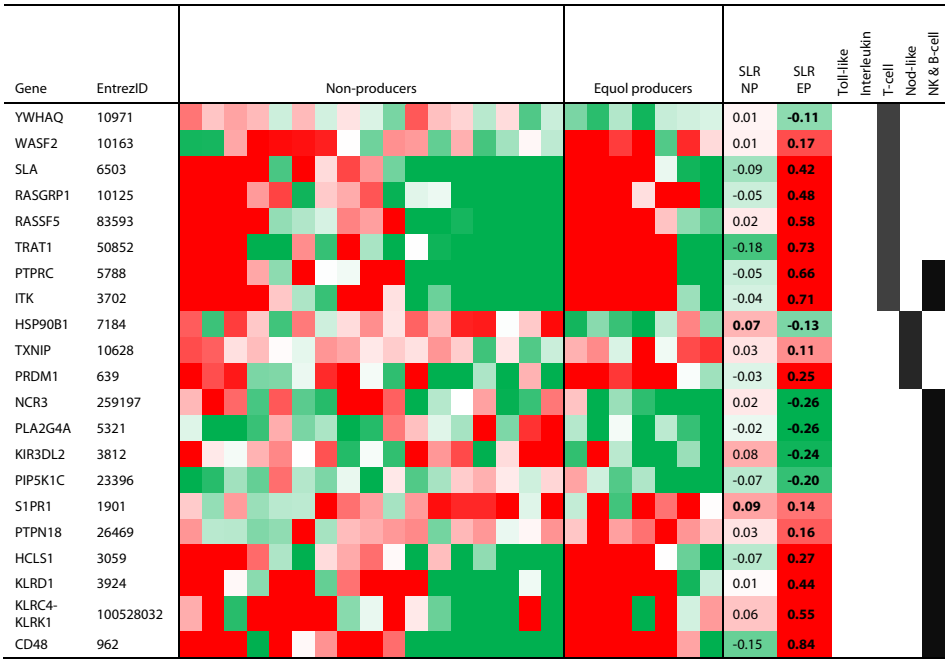
Supplemental Figure 3.1b Heatmap of significantly expressed genes in postemenopausal women involved in energy metabolism after eight weeks exposure to the high gensitein supplement compared to placebo. Bold SLRs are signficantly expressed, SLR signal log ratio, EP equol producer, NP non producer.

Supplemental Figure 3.2a Inflammation Low genistein supplement



Gene	EntrezID	SLR NP	SLR EP
LBP	3069	-0.15	**-0.51**
TGFB3	7043	**-0.15**	**-0.34**
IRAK2	3656	-0.09	**-0.26**
CALR	811	0.04	**-0.21**
FBXW5	54461	-0.09	**-0.18**
MLST8	64223	-0.08	**-0.14**
NFKB1	4790	**0.10**	-0.02
PPARA	5465	**-0.10**	0.00
RELA	5970	**-0.09**	0.03
CFLAR	8837	0.06	**0.15**
MITF	4286	0.06	**0.16**
C1S	716	0.03	**0.17**
FHL2	2274	0.04	**0.20**
ITGB3	3690	-0.01	**0.33**
PLCB2	5330	-0.10	**0.33**
TLR6	10333	-0.05	**0.46**
MTOR	2475	-0.02	**-0.14**
CD247	919	0.05	**0.31**
CD3E	916	0.05	**0.53**
NFATC2	4773	0.07	**0.23**
NFKBIA	4792	0.02	**0.24**
TNFAIP3	7128	0.03	**0.18**
CASP8	841	0.01	**0.22**
PLCG2	5336	-0.01	**0.20**
FCGR2B	2213	0.06	**0.24**
ITGB2	3689	-0.08	**0.48**
NCF2	4688	-0.05	**0.70**
ETV5	2119	-0.01	**-0.36**
CCND1	595	-0.08	**-0.26**
SPRY1	10252	-0.03	**-0.15**
MAP2K2	5605	**-0.14**	-0.03
KRAS	3845	**0.12**	0.01
FOXO3	2309	**-0.15**	0.05
CBL	867	**-0.08**	0.05
MPL	4352	-0.04	**0.14**
CDKN1B	1027	0.02	**0.14**
JAK2	3717	**0.10**	0.14
IL15RA	3601	0.00	**0.19**
IL6R	3570	-0.03	**0.25**
GADD45G	10912	0.03	**0.28**
IL10RA	3587	-0.04	**0.37**
STAT5B	6777	-0.05	**0.11**
BCL2L1	598	-0.07	**0.26**
UBE2V1	7335	0.04	**-0.31**
DUSP3	1845	0.00	**-0.24**
STK39	27347	-0.01	**-0.15**
STIM1	6786	-0.04	**-0.14**

Supplemental Figure 3.2a Inflammation Low genistein supplement



Supplemental Figure 3.2a Heatmap of significantly expressed genes involved in inflammation in adipose tissue of postmenopausal women after eight weeks exposure to the low genistein supplement compared to placebo. Bold SLRs are significantly expressed, SLR signal log ratio, EP equol producer, NP non producer. Genes are identified in genesets related to Toll-like receptor signaling, T-cell receptor signaling, Nod-like receptor signaling, Natural killer cell and B-cell signaling.

Supplemental Figure 3.2b Inflammation High genistein supplement

Supplemental Figure 3.2b Inflammation High genistein supplement

Supplemental Figure 3.2b Heatmap of significantly expressed genes in postmenopausal women involved in inflammation after eight weeks exposure to the high genistein supplement compared to placebo. Bold SLRs are signficantly expressed, SLR signal log ratio, EP equol producer, NP non producer. Genes are identified in gene sets related to interleukin, Toll like receptor, G-protein coupled receptor signalling and immune response.

Supplemental Figure 3.3 Estrogen-responsive genes

		Low genistein supplement		High genistein supplement	
Gene	Description	SLR NP	SLR EP	SLR NP	SLR EP
KRT6B	keratin 6B	-0.25			
TPX2	TPX2, microtubule-associated, homolog (Xenopus laevis)	-0.22			
NR4A1	nuclear receptor subfamily 4, group A, member 1	-0.21	-0.35		
ADRB1	adrenoceptor beta 1	-0.21			
ZWINT	ZW10 interactor	-0.19			
NME2	NME/NM23 nucleoside diphosphate kinase 2	-0.18			
RET	ret proto-oncogene	-0.18	-0.21		
MUC1	mucin 1, cell surface associated	-0.16			
EGR3	early growth response 3	-0.16			
TK1	thymidine kinase 1, soluble	-0.15	-0.21		
TGFB3	transforming growth factor, beta 3	-0.15	-0.34		-0.18
CORO2B	coronin, actin binding protein, 2B	-0.15			
SSNA1	Sjogren syndrome nuclear autoantigen 1	-0.13			
NOS3	nitric oxide synthase 3 (endothelial cell)	-0.13	-0.25		
BIK	BCL2-interacting killer (apoptosis-inducing)	-0.12			
GOT1	glutamic-oxaloacetic transaminase 1, soluble (aspartate aminotransferase 1)	-0.11	-0.15		
FGF18	fibroblast growth factor 18	-0.11		0.12	
SLC25A5	solute carrier family 25 (mitochondrial carrier /// adenine nucleotide translocator),	-0.11			
CTBP1	C-terminal binding protein 1	-0.10			
CELSR2	cadherin, EGF LAG seven-pass G-type receptor 2 (flamingo homolog, Drosophila)	-0.08			
TMED2	transmembrane emp24 domain trafficking protein 2	0.06			
sep-02	septin 2	0.07			
MAN1A1	mannosidase, alpha, class 1A, member 1	0.07			
RIF1	RAP1 interacting factor homolog (yeast)	0.08			
WDR43	WD repeat domain 43	0.09			
NR2C1	nuclear receptor subfamily 2, group C, member 1	0.09			
LRRFIP2	leucine rich repeat (in FLII) interacting protein 2	0.09			
KIFAP3	kinesin-associated protein 3	0.09			
OLFM1	olfactomedin 1	0.10			
APOA2	apolipoprotein A-II	0.10			
RPL15	ribosomal protein L15	0.11			
EIF5	eukaryotic translation initiation factor 5	0.12			
LRRC49	leucine rich repeat containing 49	0.12			
TMED7	transmembrane emp24 protein transport domain containing 7	0.12			
ABCE1	ATP-binding cassette, sub-family E (OABP), member 1	0.12			
RBBP8	retinoblastoma binding protein 8	0.13			
ZNF230	zinc finger protein 230	0.16			
FXYD2	FXYD domain containing ion transport regulator 2	0.20			
GPRC5A	G protein-coupled receptor, family C, group 5, member A	0.21			
TNC	tenascin C		-0.44		
IGF2	insulin-like growth factor 2 (somatomedin A)		-0.44		
PPIF	peptidylprolyl isomerase F		-0.36		
ME1	malic enzyme 1, NADP(+)-dependent, cytosolic		-0.35		
NME1	NME/NM23 nucleoside diphosphate kinase 1		-0.34		
TUBG1	tubulin, gamma 1		-0.31		
THBD	thrombomodulin		-0.31		
NCAM2	neural cell adhesion molecule 2		-0.30		
KITLG	KIT ligand		-0.27		

Supplemental Figure 3.3 Estrogen-responsive genes

		Low genistein supplement		High genistein supplement	
Gene	Description	SLR NP	SLR EP	SLR NP	SLR EP
ELOVL2	ELOVL fatty acid elongase 2		-0.26		
CCND1	cyclin D1		-0.26		
CNKSR3	CNKSR family member 3		-0.25		
CYCS	cytochrome c, somatic		-0.24		
DHCR24	24-dehydrocholesterol reductase		-0.24		
KIR3DL2	killer cell immunoglobulin-like receptor, three domains, long cytoplasmic tail, 2		-0.24		
PAICS	phosphoribosylaminoimidazole carboxylase, phosphoribosylaminoimidazole succinocarboxami		-0.23		
TFF1	trefoil factor 1		-0.23		
HSPD1	heat shock 60kDa protein 1 (chaperonin)		-0.23		
KPNA2	karyopherin alpha 2 (RAG cohort 1, importin alpha 1)		-0.22		
ARMCX3	armadillo repeat containing, X-linked 3		-0.22		
ENO1	enolase 1, (alpha)		-0.21		
SGCD	sarcoglycan, delta (35kDa dystrophin-associated glycoprotein)		-0.20		
SEMA5B	sema domain, seven thrombospondin repeats (type 1 and type 1-like), transmembrane domai		-0.20		
G6PD	glucose-6-phosphate dehydrogenase		-0.20		
RAMP3	receptor (G protein-coupled) activity modifying protein 3		-0.19		
AURKB	aurora kinase B		-0.19		
ESR2	estrogen receptor 2 (ER beta)		-0.18		
C1QBP	complement component 1, q subcomponent binding protein		-0.18		
CENPA	centromere protein A		-0.18		
ACO2	aconitase 2, mitochondrial		-0.17		
RUNX1	runt-related transcription factor 1		-0.17		
MCM4	minichromosome maintenance complex component 4		-0.17		
NR4A3	nuclear receptor subfamily 4, group A, member 3		-0.16		
FOXF1	forkhead box F1		-0.16		
ORMDL2	ORM1-like 2 (S. cerevisiae)		-0.15		
MARCKS	myristoylated alanine-rich protein kinase C substrate		-0.15		
SLC12A2	solute carrier family 12 (sodium/potassium/chloride transporters), member 2		-0.15		
SPRY1	sprouty homolog 1, antagonist of FGF signaling (Drosophila)		-0.15		
GARS	glycyl-tRNA synthetase		-0.15		
RPA3	replication protein A3, 14kDa		-0.14		
STMN1	stathmin 1		-0.14		
IARS	isoleucyl-tRNA synthetase		-0.14		
ITGAV	integrin, alpha V		-0.12		
NUP88	nucleoporin 88kDa		-0.12		
TXNIP	thioredoxin interacting protein		0.11		
NRF1	nuclear respiratory factor 1		0.13		
ECE1	endothelin converting enzyme 1		0.13		
EFEMP1	EGF containing fibulin-like extracellular matrix protein 1		0.13		
MPL	myeloproliferative leukemia virus oncogene		0.14		
GSTO1	glutathione S-transferase omega 1		0.15		
HIP1R	huntingtin interacting protein 1 related		0.16		-0.19
PAX8	paired box 8		0.16		
PTPN18	protein tyrosine phosphatase, non-receptor type 18 (brain-derived)		0.16		
WSB1	WD repeat and SOCS box containing 1		0.17	-0.11	
IGF1R	insulin-like growth factor 1 receptor		0.17		-0.15
GNG7	guanine nucleotide binding protein (G protein), gamma 7		0.17		

Supplemental Figure 3.3 Estrogen-responsive genes

		Low genistein supplement		High genistein supplement	
Gene	Description	SLR NP	SLR EP	SLR NP	SLR EP
SFRP1	secreted frizzled-related protein 1		0.18		
MCM7	minichromosome maintenance complex component 7		0.18		
SATB1	SATB homeobox 1		0.18		
INPP4B	inositol polyphosphate-4-phosphatase, type II, 105kDa		0.20		
THBS2	thrombospondin 2		0.20		
PTGER2	prostaglandin E receptor 2 (subtype EP2), 53kDa		0.21		
WISP2	WNT1 inducible signaling pathway protein 2		0.22		-0.21
BCL2L11	BCL2-like 11 (apoptosis facilitator)		0.22		
ABCB1	ATP-binding cassette, sub-family B (MDR/TAP), member 1		0.23		
TSC22D3	TSC22 domain family, member 3		0.24		
NFKBIA	nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, alpha		0.24		
ANKRD44	ankyrin repeat domain 44		0.25		
CTNND1	catenin (cadherin-associated protein), delta 1		0.25		
BCL2L1	BCL2-like 1		0.26		
TNFSF8	tumor necrosis factor (ligand) superfamily, member 8		0.26		
CCNG2	cyclin G2		0.26		
FKBP8	FK506 binding protein 8, 38kDa		0.26		
ZFP36L2	zinc finger protein 36, C3H type-like 2		0.27		
SLA	Src-like-adaptor		0.42		
S100P	S100 calcium binding protein P		0.51		
CD86	CD86 molecule			-0.15	
GNRH1	gonadotropin-releasing hormone 1 (luteinizing-releasing hormone)			-0.14	
PLAT	plasminogen activator, tissue			-0.12	
HMGB2	high mobility group box 2			-0.12	
TFPI	tissue factor pathway inhibitor (lipoprotein-associated coagulation inhibitor)			-0.11	
ICAM1	intercellular adhesion molecule 1			-0.10	
PDLIM5	PDZ and LIM domain 5			-0.09	
LRCH3	leucine-rich repeats and calponin homology (CH) domain containing 3			-0.09	
PTPRO	protein tyrosine phosphatase, receptor type, O			-0.09	
NRAS	neuroblastoma RAS viral (v-ras) oncogene homolog			-0.09	
PPP2CA	protein phosphatase 2, catalytic subunit, alpha isozyme			-0.08	
PSMD11	proteasome (prosome, macropain) 26S subunit, non-ATPase, 11			0.05	
EIF2S1	eukaryotic translation initiation factor 2, subunit 1 alpha, 35kDa			0.06	0.10
TSN	translin			0.08	
CSTF2	cleavage stimulation factor, 3' pre-RNA, subunit 2, 64kDa			0.08	
PVR	poliovirus receptor			0.08	
SND1	staphylococcal nuclease and tudor domain containing 1			0.09	
ACTN1	actinin, alpha 1			0.09	
MRPL3	mitochondrial ribosomal protein L3			0.09	
VPS37C	vacuolar protein sorting 37 homolog C (S. cerevisiae)			0.09	
HOXD4	homeobox D4			0.10	
CACYBP	calcyclin binding protein			0.10	
SLC22A1	solute carrier family 22 (organic cation transporter), member 1			0.10	
BLVRB	biliverdin reductase B (flavin reductase (NADPH))			0.11	
PRKCD	protein kinase C, delta			0.12	0.23
HSPB8	heat shock 22kDa protein 8			0.13	
PGR	progesterone receptor			0.14	
FKBP4	FK506 binding protein 4, 59kDa			0.15	

Supplemental Figure 3.3 Estrogen-responsive genes

		Low genistein supplement		High genistein supplement	
Gene	Description	SLR NP	SLR EP	SLR NP	SLR EP
APOB	apolipoprotein B (including Ag(x) antigen)			0.19	
EDN1	endothelin 1				-0.57
NTRK3	neurotrophic tyrosine kinase, receptor, type 3				-0.40
MAB21L1	mab-21-like 1 (C. elegans)				-0.26
FST	follistatin				-0.24
PGM5	phosphoglucomutase 5				-0.23
ENO3	enolase 3 (beta, muscle)				-0.22
LAMA3	laminin, alpha 3				-0.21
CXCL12	chemokine (C-X-C motif) ligand 12				-0.20
SYTL2	synaptotagmin-like 2				-0.18
PLXNA3	plexin A3				-0.18
PRKCE	protein kinase C, epsilon				-0.18
CACNA1C	calcium channel, voltage-dependent, L type, alpha 1C subunit				-0.18
CITED2	Cbp/p300-interacting transactivator, with Glu/Asp-rich carboxy-terminal domain, 2				-0.18
SVIL	supervillin				-0.17
TOP3B	topoisomerase (DNA) III beta				-0.16
KLF6	Kruppel-like factor 6				-0.15
PDIA4	protein disulfide isomerase family A, member 4				-0.14
EXOSC10	exosome component 10				-0.14
PDE3A	phosphodiesterase 3A, cGMP-inhibited				-0.13
ZFYVE26	zinc finger, FYVE domain containing 26				-0.13
HDAC6	histone deacetylase 6				-0.12
DLC1	deleted in liver cancer 1				-0.11
HUWE1	HECT, UBA and WWE domain containing 1, E3 ubiquitin protein ligase				-0.10
GNB1	guanine nucleotide binding protein (G protein), beta polypeptide 1				-0.10
DNAJA2	DnaJ (Hsp40) homolog, subfamily A, member 2				0.13
PHF7	PHD finger protein 7				0.14
CDKN1A	cyclin-dependent kinase inhibitor 1A (p21, Cip1)				0.17
RHOU	ras homolog family member U				0.20
PLOD2	procollagen-lysine, 2-oxoglutarate 5-dioxygenase 2				0.24
ZNF267	zinc finger protein 267				0.26
ISG20	interferon stimulated exonuclease gene 20kDa				0.31
BAK1	BCL2-antagonist/killer 1				0.33
JUNB	jun B proto-oncogene				0.36
CCR2	chemokine (C-C motif) receptor 2				0.38
CCR1	chemokine (C-C motif) receptor 1				0.48
Number of significantly regulated estrogen responsive genes		39	82	30	41
% of estrogen responsive genes relative to all regulated genes		4.4	7.0	5.5	6.5

Supplemental Figure 3.3 Significantly changed estrogen responsive genes. Expression of estrogen-responsive genes according to the ERGDB signficantly changed after eight weeks exposure to each supplement compared to placebo.

Chapter 4

Large inter-individual variation in isoflavone plasma concentration limits use of isoflavone intake data for risk assessment

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Accepted for publication in revised form:
European Journal of Clinical Nutrition

ABSTRACT

Background: Isoflavones are present in soy foods and soy-based supplements. Despite low plasma isoflavone concentrations in the general Western population, concentrations in supplement users exceed those suggested to be beneficial for health in Asian populations, raising concerns for adverse effects. To aid risk assessment, quantification of the relation between isoflavone intake and plasma concentrations is essential.

Methods: Plasma samples were collected from postmenopausal women in three placebo-controlled crossover studies with eight-week periods for supplements (2 studies, ~100 mg isoflavones/day, n = 88) or four-week periods for soy foods (1 study, ~48 mg isoflavones/day, n = 15). Plasma isoflavone concentrations (daidzein, equol, genistein and glycitein) were quantified with HPLC and electrochemical detection. The association between plasma concentrations and isoflavone intake, equol producer status, intake-producer interaction and background dietary intake was assessed based on the assumption of a log-linear relation.

Results: Median plasma total isoflavone concentrations after the soy food and supplement interventions were respectively 2.16 and 3.47 µmol/L for equol producers and 1.30 and 2.39 µmol/L for non-producers. Regression analysis showed that doubling isoflavone intake increased plasma concentrations by 55-62% (± SE 1-2%, $R^2 > 0.87$) for daidzein, genistein, equol (only for producers) and total isoflavones; for glycitein the association was weaker (15 ± 1%, $R^2 = 0.48$). Adjustments for energy, carbohydrate and fat intake did not affect these estimates. Inter-individual variation, estimated based on repeated measures in one of the studies, was 30-96%.

Conclusions: Although the relation between isoflavone intake and plasma concentrations was adequately quantified, the use of isoflavone intake data for risk assessment needs caution due to large inter-individual variation in plasma concentrations.

INTRODUCTION

Isoflavones, present in soy products, are suggested to relieve menopausal complaints (1, 2) and to have a number of other beneficial health effects, such as prevention of osteoporosis and cardiovascular disease (3-5). At higher doses, uncertainty exists regarding potentially detrimental effects on thyroid function and risk of breast and endometrium cancer, because long-term human trials are currently lacking (6-8).

The glucosides daidzin, genistin and glycitin are the main isoflavones in soy, whereas their malonyl and acetyl equivalents as well as the aglycones daidzein, genistein and glycitein occur in much lower quantities (9). The various isoflavones have specific pharmacokinetic characteristics: e.g. isoflavone glucosides are more bioavailable than their aglycones (10); daidzein is rapidly excreted in urine, whereas genistein enters enterohepatic recycling (10, 11). Additionally, 20-30% of the Western population are so-called equol producers; they can convert daidzein into the more active metabolite equol by their gut bacteria (12). Furthermore, isoflavone plasma concentrations can be influenced by isoflavone source, food matrix, diet, frequency of ingestion, gender and age (10).

Mean isoflavone intake is low (0.5-0.8 mg/day) across the European population and remains well below the intake in Asian countries even for vegetarians and vegans (22.4 mg/day) (13). In Japan, mean intakes of daidzein and genistein are 18.3 and 31.4 mg/day resulting in plasma concentrations of 0.12 and 0.48 µmol/L, respectively (14). On average, supplement users in Western countries consume 50 mg of isoflavones/day, but soy-based supplements can contain up to 107 mg aglycone equivalents of isoflavones (15-17). Two intervention studies demonstrated that an intake of 100 mg isoflavones/day resulted in total circulating isoflavones of 1.12 and 4.50 µmol/L (18, 19). This illustrates that postmenopausal women taking isoflavone supplements e.g. to relieve their menopausal complaints can be exposed to higher isoflavone concentrations than Asians who regularly consume soy products.

For risk assessment, insight in isoflavone plasma concentrations resulting from these higher isoflavone intake ranges is important. Isoflavone pharmacokinetics as well as positive correlations and dose-response curves between intake and circulating isoflavones have previously been thoroughly characterized (20-25). However, because of the small sample sizes (n = 10-18) these data are not suitable for risk assessment and a quantitative description of the relation between isoflavone intake and plasma concentrations in a much larger group of postmenopausal women is needed. We aimed to quantify this relation based on intakes of two different isoflavone supplements and a soy food, taking into account relevant factors like equol-producing phenotype and background dietary intake.

SUBJECTS AND METHODS

Trials and subjects

For this study, data from three randomized crossover trials were used: the ISO study, the ISO II study and the SOY study. All studies were conducted at the department of Human Nutrition of Wageningen University and were performed according to the guidelines laid down in the Declaration of Helsinki. All procedures were approved by the medical ethical review board of this University and written informed consent was obtained from all subjects. Thirty postmenopausal women participated in the double-blind ISO study of which all participants were equol producers, characterized at screening. The study was registered at clinicaltrials.gov under NCT01232751. The ISO II study, hereafter named the GD (genistein/daidzein) study, was a double-blind trial with two arms in which 72 postmenopausal women participated, 36 in each arm. The two arms were either a low genistein, high daidzein supplement (LG) or a high genistein, high daidzein supplement (HG), both against placebo. Equol producers, characterized at screening, were randomized over the two arms. The study was registered at clinicaltrials.gov under NCT01556737. Both double-blind trials included two eight-week intervention periods with one eight-week washout period in between and had the same in- and exclusion criteria, previously reported in (26). In the ISO study, postmenopausal status was defined as 3 months absence of menses and if shorter than 1 year this was complemented with an FSH test. For the GD study 1 year absence of menses defined postmenopausal status (see Table 4.1 for details on subjects). The SOY study was a strictly-controlled dietary intervention with a soy protein diet. Sixteen postmenopausal women with abdominal obesity, as defined by a waist circumference ≥ 80 cm, were included. During each four week intervention period the participants consumed a soy protein-rich diet or a mixed protein diet (control diet), with a four week washout period in between. The study was registered at clinicaltrials.gov under NCT01694056.

Equol-producing capacity of the participants of the ISO and the GD study was verified before entering the study by a three-day challenge with the daidzein-rich supplement, after which equol was detected in a spot urine sample. For exact criteria used, see van der Velpen *et al*., 2013 (26). In the ISO study, 42 equol producers (27%) out of 150 postmenopausal women were characterized of which 30 producers entered the study. In the GD study, 73 postmenopausal women were screened and 17 (23%) were equol producers; 16 producers and 54 non-producers were included in the study. At the end of the isoflavone intervention of the GD study 2 non-producers switched producer status. In the SOY study, 4 of 15 women (27%) were characterized as equol producers after the soy diet intervention period.

Isoflavone intervention

The isoflavone supplements of the ISO study (October 2010) which were also used in the LG-arm of the GD study (November 2011) were previously described (26). The capsules from the two different batches contained 14 mg daidzein, 3-4 mg genistein and 6-7 mg glycitein (aglycone equivalents) per supplement, as quantified in our laboratory (Table 4.1). The participants were asked to consume four capsules/day, two in the morning and two in the evening.

Table 4.1 Subject characteristics, isoflavone content of supplements and soy foods and background dietary intake

	ISO (n = 21)		GD LG (n = 32)		GD HG (n = 35)		SOY (n = 15)	
	Mean	SD	Mean	SD	Mean	SD	Mean	SD
Age (years)[1]	60.3	6.2	62.2	5.3	62.7	5.4	61.2	5.4
BMI (kg/m^2)	25.7	4.0	25.2	3.1	25.0	3.6	25.4	4.1
Body weight (kg)	73.0	13.3	71.4	9.8	69.5	11.0	69.4	12.6
No of equol producers (%)[2]	21	100	8	25	9	26	4	27
Total isoflavone intake (mg/day)[3]	93.9		100.1		104.2		48.3	
Daidzin	53.2		52.0		50.8		15.9	
Daidzein	3.2		3.7		0.5		2.3	
Genistin	11.4		16.5		42.0		21.4	
Genistein	0.4		0		0.7		6.0	
Glycitin	23.8		26.3		9.8		2.3	
Glycitein	2.0		1.5		0.4		0.5	
Energy intake[4] (kJ/day)								
Intervention	8726	2304	7626	2244	7869	1946	9126	1801
Placebo	8377	2264	7819	2675	7359	1938	9021	1674
Carbohydrate intake (g/day)								
Intervention	237	62	197	67	217	65	263	57
Placebo	228	60	208	80	203	62	274	55
Fat intake (g/day)								
Intervention	87	32	73	29	73	27	67	13
Placebo	81	32	76	32	68	24	63	12

[1] Age, BMI and body weight are determined at the start of the studies.
[2] No of equol producers as determined at the end of the intervention.
[3] Glucosides daidzin, genistin and glycitin calculated as aglycone equivalents.
[4] Diet data for GD LG arm for n = 31.

The supplements in the HG-arm of the GD study were provided by Springfield Nutraceuticals (Oud-Beijerland, the Netherlands) based on the commercially available supplement Mega Soja with an isoflavone content of 25 mg daidzein, 21 mg genistein and 5 mg glycitein per

supplement (aglycone equivalents, Table 4.1). The identical-looking placebo capsules were provided by the same supplier; these consisted of hydroxypropylmethyl cellulose (HPMC), were not coloured and were filled with microcrystalline cellulose. The participants in this study consumed two capsules/day, one in the morning and one in the evening. A run-in period of 4 weeks proceeded both studies. The participants were asked not to eat soy foods during the run-in and the study period.

Before the strictly controlled dietary intervention (SOY study), participants were assigned to an energy group based on the results of a validated 177-item FFQ (27) and their height and weight. The soy and mixed protein diet were iso-energetic with the same macronutrient composition (21 energy% protein, 26 energy% fat and 51 energy% carbohydrates). The soy protein diet contained approximately 30 gram of soy protein/day, in the form of soy based meat analogues and soy nuts provided by Alpro (Gent, Belgium) containing ~48 mg isoflavones (18 mg daidzein, 26 mg genistein, 3 mg glycitein, Table 4.1). During the one week run-in period and the 4 week washout period participants were not allowed to eat soy foods.

Background dietary intake

To monitor background dietary intake during the ISO and GD study, the participants were asked to fill out a semi-quantitative validated FFQ consisting of 125 items at the end of each eight week intervention period (27). Background dietary intake in the SOY study was derived from duplicate portions of both experimental diets in which energy, fat, protein, ash and dry matter content was analysed. Carbohydrate content was calculated by subtracting protein, fat, ash and moisture content from the total sample weight. Mean energy and nutrient intake per participant was calculated from food tables (28) and adjusted for the duplicate diet analysis.

Sample collection

In the ISO study, plasma samples were collected halfway and at the end of each 8 week intervention period. During the GD study and SOY study, plasma was collected after each intervention period. Fasted venous blood samples were collected into 6 ml EDTA vacutainers (Becton Dickinson (BD), Breda, the Netherlands) and centrifuged for ten minutes at 1190 *g* at a temperature of 4°C to obtain plasma. The samples were stored at -80°C in aliquots until analysis.

HPLC analysis of isoflavones

Isoflavones in plasma, supplements and soy foods were determined after enzymatic deconjugation using reversed phase HPLC with electrochemical detection as previously described for the ISO study (26). For all isoflavones, except glycitein, the limit of detection (LOD) and limit of quantification were 0.04 and 0.08 µmol/L, respectively. For glycitein these values were 0.11 and 0.22 µmol/L, respectively. The within-run and between-run variability coefficients of variation (CV in %) were 7.6 and 16.8% respectively for daidzein, 7.6 and 23.8% for equol, 14.7 and 30.6% for genistein and 24.2 and 28.0% for glycitein.

Data analysis

In the ISO study, one outlier was detected for all plasma concentrations (8 SD from mean) and excluded from the analysis. The GD study had three dropouts and the SOY study had one dropout. Ten subjects participated in more than one of the studies; these were excluded from the study with most equol producers, i.e. ISO study, resulting in 103 subjects. Each subject contributed two data points (206 observations), one after the placebo or mixed protein period (unexposed) and one after the intervention period of each trial (exposed). Although these data are paired, they were considered statistically independent because plasma concentrations for unexposed were close to zero and not correlated to the concentration for exposed and there was no overlap in study subjects.

Linear regression analysis was used to explain total and component-specific concentrations of isoflavones in plasma by intake (in µmol/kg bw). Both dose and plasma concentrations (µmol/L) were $\log_e$ transformed and all models were adjusted for study (ISO study, GD study, SOY study). For daidzein, equol and total isoflavones the model also accounted for equol producer status (prod; 1 for producer, 0 for non-producer) and its interaction with intake:

$$\log_e(\text{conc}) = \beta_0 + \beta_1 * \log_e(\text{dose}) + \beta_2 * \text{prod} + \beta_3 * \text{prod} * \log_e(\text{dose}) + \varepsilon$$

When original values for concentration were zero, 0.5 times the LOD was used to enable $\log_e$ transformation of the data, i.e. 0.02 for daidzein, equol, genistein and total isoflavones and 0.055 for glycitein. When intake was zero, a dose of 0.01 µmol/kg bw was used. To account for the role of the background diet, a second model was further adjusted for energy intake (continuous in kJ/day), carbohydrate intake (g/day) and fat intake (g/day). Dietary intake data from 1 participant in the GD LG-arm was missing.

To estimate inter- and intra-individual variation at a high supplement dose, $\log_e$ transformed plasma isoflavone concentrations after four and eight weeks of isoflavone supplementation

were used (ISO study, n = 29). Variance between subjects and total variance was obtained by the varcomp procedure and the mean square error of the regression model (MSE_{model}). Coefficients of variation were obtained as $CV_{between}$ (%) = sqrt(exp$^{(Variancebetween)}$ – 1)) * 100 and analogously for CV_{total} and MSE_{model} (29). Differences between plasma concentrations after four and eight weeks of exposure to 94 mg isoflavones/day in the ISO study were tested with a paired t-test (P-value < 0.05, SAS, v9.2, SAS Institute Inc., Cary, NC, USA).

RESULTS

In the studies with isoflavone supplements (~100 mg/day), the mean total isoflavone concentration after eight weeks exposure to isoflavones (daidzein, equol, genistein and glycitein) was 3.47 µmol/L for equol producers (n = 38) and 2.39 µmol/L for non-producers (n = 50). After the four week intervention with soy protein (~48 mg isoflavones/day) the mean total isoflavone concentration was 2.16 µmol/L for equol producers (n = 4) and 1.30 µmol/L for non-producers (n = 11, Table 4.2). For all studies at the end of the placebo or mixed protein period, 86% of the measured daidzein concentrations were below the quantification limit of the method, this was respectively 99%, 96%, and 100% for equol, genistein and glycitein.

Table 4.2 Mean (± SD) plasma isoflavone concentrations in equol producers and non-producers after the interventions

		ISO study[2] (EP n = 21)		GD study LG (EP n = 8, NP n = 24)		GD study HG (EP n = 9, NP n = 26)		SOY study (EP n = 4, NP n = 11)	
		Mean (µmol/L)	SD	Mean (µmol/L)	SD	Mean (µmol/L)	SD	Mean (µmol/L)	SD
Daidzein	EP	1.75	0.79	1.22	1.06	1.07	0.56	0.60	0.40
	NP	NA		1.53	0.64	1.27	0.69	0.43	0.19
Equol	EP	1.20	0.52	0.72	0.58	0.61	0.28	0.39	0.15
	NP	NA		NA		NA		NA	
Genistein	EP	0.69	0.52	0.40	0.30	1.19	0.95	1.16	1.08
	NP	NA		0.43	0.33	1.33	0.90	0.87	0.64
Glycitein	EP	0.44	0.40	0.19	0.17	0.02	0.05	0.00	0.00
	NP	NA		0.15	0.12	0.06	0.08	0.00	0.00
Total[1]	EP	4.08	1.55	2.53	1.25	2.90	1.48	2.16	1.48
	NP	NA		2.11	0.84	2.65	1.49	1.30	0.80

[1] Total isoflavones is the sum of daidzein, equol, genistein and glycitein.
[2] The ISO and GD study provided ~100 mg isoflavones/d (aglycone equivalents) as supplements for 8 wk, the SOY study provided ~48 mg/d as soy protein diet for 4 wk.
EP, equol producer; NP, non-producer.

Significant linear associations between natural logarithm (log_e) of plasma concentration versus log_e of intake per kg bw (dose) were observed in non-producers for daidzein (β_1 = 0.66), genistein (β_1 = 0.70), glycitein (β_1 = 0.20) and total isoflavones (β_1 = 0.67, Table 4.3). In these non-producers the regression coefficient of equol concentration after daidzein intake was β_1 = 0.0, while in equol producers this was $\beta_1 + \beta_3$ = 0.63. Furthermore for equol producers, the association between plasma daidzein and intake was $\beta_1 + \beta_3$ = 0.64 and for total isoflavones it was $\beta_1 + \beta_3$ = 0.74. This linear model on the log_e-log_e scale can be interpreted on a normal scale as concentration = $e^{\beta_0+\beta_2}$ * $dose^{\beta_1+\beta_3}$, with β_0 (intercept) and β_1 (dose, Figure 4.1). β_2 (producer status) and β_3 (intake-producer status interaction) were only relevant for equol producers for daidzein, equol and total isoflavone plasma concentrations. When isoflavone intake doubles from 1.5 to 3 µmol/kg bw, the equation is as follows $(1.5/3.0)^{\beta_1} = (2)^{0.67}$ = 1.59 fold or 59%. So, total isoflavone plasma concentrations increase with 59% when its intake doubles.

Except for glycitein (R^2 = 0.48), the explained variances of the models were higher than 0.87.

Inclusion of energy (kJ/day), carbohydrate and fat intake (g/day) in the model as background dietary intake did not change the associations (data not shown).

Figure 4.2 shows that the relation between intake and plasma concentration, calculated as the concentration divided by the dose, for soy foods is not different than for isoflavone supplements.

Table 4.3 Outcomes of the linear regression model on log_e transformed intake (µmol/kg bw) and log_e transformed plasma concentrations (µmol/L)[1]

	Non-producers[2]				Producers[3]				
	β_0	95% CI	β_1 * dose	95% CI	$\beta_0 + \beta_2$	95% CI	$(\beta_1 + \beta_3)$ * dose	95% CI	R^2
Daidzein	-0.54	-0.70, -0.38	0.66	0.61, 0.71	-0.49	-0.74, -0.24	0.64	0.59, 0.70	0.87
Equol	-3.92	-3.98, -3.85	0.00	-0.02, 0.02	-1.12	-1.23, -1.02	0.63	0.61, 0.66	0.96
Genistein	-0.66	-0.78, -0.54	0.70	0.67, 0.74					0.90
Glycitein	-2.14	-2.26, -2.01	0.20	0.17, 0.24					0.48
Total	-0.39	-0.55, -0.23	0.67	0.63, 0.72	-0.06	-0.31, 0.18	0.74	0.68, 0.79	0.90

[1] The log-linear regression model: $log_e(conc) = \beta_0 + \beta_1 * log_e(dose) + \beta_2 * prod + \beta_3 * prod * log_e(dose) + \varepsilon$.
[2] The model was adjusted for study (ISO, GD or SOY).
[3] The model was adjusted for study (ISO, GD or SOY), equol producer status (β_2) and log_e-transformed intake * producer status interaction (β_3).

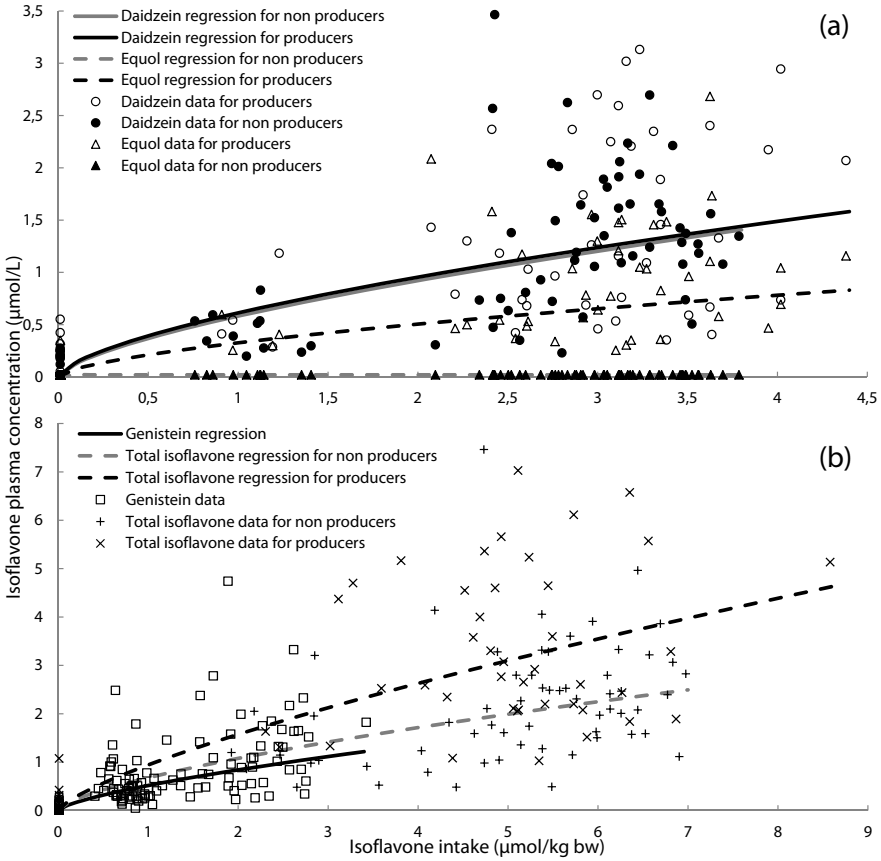

Figure 4.1 Exponential regression lines for the association between isoflavone intake (µmol/kg bw) and plasma concentration (µmol/L) combined with individual data. Isoflavone intake and plasma concentration on the x- and y-axis refer to specific isoflavones; panel A shows daidzein intake with daidzein plasma concentration and daidzein intake with plasma equol concentration; panel B shows genistein intake with genistein concentration and total isoflavone intake with total isoflavone concentration.

Among the equol-producing women in the ISO study, plasma concentrations of the individual isoflavones after four and eight weeks of exposure were similar (results not shown). In this study, within person CV is smaller than between person CV for all individual and total isoflavones, resulting in a large intraclass correlation coefficient (ICC, Table 4.4). The CVs between persons were 30-45% for all isoflavones except for genistein (96%). The CVs for MSEmodel, comprising the inter- and intra-individual variation, was 31% for equol and ranged from 59 to 87% for the other isoflavones.

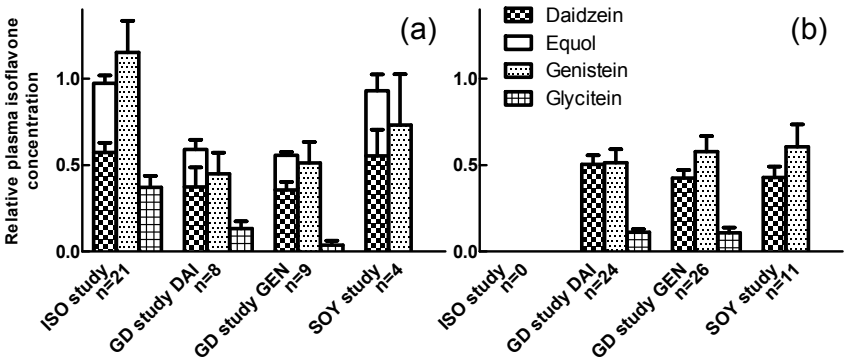

Figure 4.2 Relative plasma isoflavone concentrations of equol producers (panel A) and non-producers (panel B) in the three studies. Relative concentrations were calculated by dividing plasma concentrations in µmol/L by intake in µmol/kg bw. The bar for equol concentration is placed on top of the bar for daidzein concentration. DAI, low genistein (LG) arm; GEN, high genistein (HG) arm.

Table 4.4 Between person and total variance, coefficient of variation (%) and ICC of isoflavone concentrations 4 and 8 wk after supplementation with 94 mg isoflavones (aglycone equivalents) in the ISO study in 29 equol-producing postmenopausal women

	$Var_{between}$		Var_{total}		ICC	MSE model[2]	
	$CV_{between}$[1]	%	CV_{total}	%		CV	%
Daidzein	0.18	44	0.27	56	0.67	0.52	83
Equol	0.18	45	0.27	56	0.67	0.09	31
Genistein	0.65	96	0.81	112	0.80	0.32	61
Glycitein	0.19	46	0.56	87	0.34	0.30	59
Total	0.09	30	0.14	39	0.64	0.56	87

[1] Coefficient of variation between persons from log_e-transformed concentration data, calculated as the square root of the exponent of the variance minus 1.
[2] For comparison of the coefficients of the ISO study, the MSE of the linear regression model is shown.
ICC, intra class correlation; CV, coefficient of variation; MSE, mean square error.

DISCUSSION

In this study, plasma isoflavone concentrations from 103 postmenopausal women participating in three intervention studies were evaluated after exposure to either isoflavone supplements or soy foods. A log-linear regression model showed that over a range of total isoflavone intakes from 0 to 100 mg/day, doubling of the dose (per kg bw) increased plasma concentrations with 55-62% (± SE 1-2%) for daidzein, genistein, equol (only in producers)

and total isoflavones ($R^2 > 0.87$). For glycitein, with an intake range of 0 to 28 mg/day, the observed increase was much smaller (15% ± 1%, $R^2 = 0.47$). Including background dietary intake in the model did not affect the associations. Both visual inspection of the regression model and quantification with ISO study data, indicated large inter-individual variation of isoflavone plasma concentrations at this intake range.

We studied the isoflavone dose-concentration relation among 103 postmenopausal women, which is a relatively large sample size compared to other studies (n = 39-76) (18, 19). The percentage of equol producers in the studies (23-27%) was similar to literature data (12). Results from three intervention studies are presented, covering isoflavone intake from 0 to 100 mg/day. The three studies, having small differences in duration and dose, are comparable because they were conducted in postmenopausal women using similar determination of isoflavones with regard to lab, methods and reference materials. We demonstrated that data from four and eight weeks of exposure could be combined in the repeated measurements of the ISO study, also supported by calculations from literature (30), and that food matrix did not influence the comparability of the studies.

The outcomes of our model suggested that production of equol did not affect daidzein plasma concentrations as these concentrations increased equally for producers and non-producers (56 and 58%, respectively) at doubling daidzein intake (Table 4.3). Equol plasma concentrations, only relevant for producers, did depend on daidzein intake as doubling intake increased plasma equol concentration by 55%. Together this resulted in higher increases in total plasma isoflavone concentrations in equol producers (67%) than in non-producers (59%) at doubling daidzein intake. Previous literature is not consistent on whether equol is produced at the expense of daidzein plasma concentrations (31-33), which might partly be explained by differences in the pharmacokinetics of daidzein and equol (34).

In the model we assumed that the ability to produce equol would influence daidzein, equol and total isoflavone concentrations and that a linear relation between the natural logarithms of intake and plasma concentrations existed. By using $\log_e$ transformations of both intake and concentration, homogeneity of the variance of error terms was allowed for, while the back transformed curve flattened down at higher doses. Without these $\log_e$ transformations and inclusion of the unexposed data the model fit would be less (data not shown). The high explained variances of the models ($R^2 > 0.87$) indicated a good model fit except for glycitein (Table 4.3), although plasma isoflavone concentrations varied substantially between individuals (Figure 4.1). This variation proved to be large when quantified by CV and ICC with ISO study data and the MSE_{model} for the regression model

(Table 4.4). Differences between these measures of variance can be explained by the number of studies and therefore supplements included. In previous studies with isoflavone supplements, inter-individual variation in plasma isoflavone concentrations was quantified as 162 to 1596-fold for the individual isoflavones (18, 19). Inter-individual variation might be caused by differences in uptake efficiency and metabolism, which in turn might be influenced by microbiota (10) or genetic variation in transporter genes (35). Other studies hypothesized that fat, carbohydrate and fibre intake could explain inter-individual variation (36, 37). Therefore we complemented our model with energy, fat and carbohydrate intake data. Fibre intake data could not be extracted from the FFQ used. This adjustment did not change the observed associations and suggests that background dietary intake did not explain the observed inter-individual variation.

Postmenopausal women produce little endogenous estradiol and are therefore considered susceptible to the potentially beneficial or even adverse health effects of isoflavones when using these supplements. Our study confirmed that supplement intake by postmenopausal women led to high concentrations of circulating isoflavones compared with soy food intake in Japanese populations (14). For risk assessment purposes, plasma isoflavone concentrations from 103 postmenopausal women in this study could be explained by isoflavone intake and equol producer status at a relevant intake range of 0-100 mg isoflavones/day.

Despite the adequate explanation of plasma isoflavone concentrations over this intake range, the large inter-individual variation will restrict the use of this model for future risk assessment to the population level and cannot be applied to predict plasma concentrations in individuals.

ACKNOWLEDGEMENTS

The authors would like to thank Dini Venema for her excellent technical assistance with the HPLC analysis and Els Siebelink, Corine Perenboom, Saskia Meyboom and Karin Borgonjen for providing the FFQ data. Furthermore thanks to Springfield Nutraceuticals and Alpro for providing the supplements and soy foods.

The ISO and ISO II (GD) study were funded by the Dutch Food Safety Authority (nVWA), the SOY study was funded by the Alpro Foundation.

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Chapter 5

Quantitative comparison of gene expression profiles of humans and rats after isoflavone supplementation

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ABSTRACT

Background: In risk assessment, potential adverse health effects in humans are often extrapolated from animal experiments, this extrapolation represents an important source of uncertainty. Transcriptomics may be a powerful tool to evaluate effects in different tissues and species and might reduce this uncertainty in risk assessment.

Objective: We aimed to quantify gene expression effects of isoflavone supplementation and to compare these in peripheral blood mononuclear cells (PBMCs) and white adipose tissue (WAT) of postmenopausal women and ovariectomized F344 rats.

Methods: Gene expression effects were quantified after 8wk isoflavone supplementation compared to placebo. The women were exposed to a supplement containing ~100 mg isoflavones/day (~1.5 mg/kg body weight) and the rats to ~0.42 mg/day (~2.1 mg/kg body weight) with the following composition: ~58% daidzein, ~14% genistein and ~28% glycitein. Gene expression, measured using Affymetrix gene chips, was compared between tissues and species with gene set enrichment analysis (GSEA) and multivariate regression modeling.

Results: Multivariate analysis showed a change in gene expression after isoflavone supplementation of 8% in human PBMCs and 7% in human WAT and of 24% and 34% in these tissues in rats, respectively. For both tissues and species, GSEA revealed changes in similar gene sets, i.e. energy metabolism, inflammation and cell cycle. Interspecies correlations were only significant for estrogen-responsive genes in WAT ($r = 0.31$) and for OXPHOS genes in PBMCs ($r = 0.33$). Intertissue correlations were only significant for estrogen-responsive genes and cell cycle-related genes in both tissues.

Conclusion: Our model can be used to quantify gene expression effect size for selected gene sets and to quantitatively compare these between tissues and species. This model provides an important tool to contribute to advancing use of transcriptomics in the parallelogram approach for risk assessment.

INTRODUCTION

In current risk assessment practices, estimation of potential adverse health effects in humans is often based on results of animal experiments. Extrapolation of effects from animal to man is one of several sources of variability and uncertainty in risk assessment (1). Traditionally used uncertainty factors for extrapolation are 10 for intraspecies differences and 10 for interspecies differences, applied to a dose experimentally determined as safe in animals (2). Application of new techniques, like quantitative structure-activity relationship modelling, physiologically based biokinetic modelling and transcriptomics, might enable better quantification of interspecies differences (3). Especially the latter is promising, because transcriptomics can be used in animal experiments as well as in human intervention studies. Furthermore, transcriptomics can be considered as a powerful tool for detection of early effect markers, especially when changes in gene expression are considered within biological pathways (4) as done with computational methods, like gene set enrichment analysis (GSEA (5)). However, the interpretation is often restricted to qualitative evaluation of up- versus downregulation of gene expression, instead of considering effect size. In two recent studies, transcriptomics were qualitatively compared for risk assessment using the parallelogram approach (6, 7). The parallelogram approach enables comparison of effects in two species, for instance humans and animals, and a subsequent extrapolation to an intermediate or clinically relevant outcome in humans. For transcriptomics, this would imply comparison of gene expression data from similar tissues in animals and humans and animal target tissue to predict the effects in human target tissue. To reduce uncertainty in extrapolation from animal to human, it is important to quantitatively compare gene expression effects within and between species. Therefore, our study focusses on quantification of these effects after isoflavone supplementation in humans and animals for use in risk assessment.

Isoflavones are phytoestrogens present in soy (products) and the main isoflavones are daidzein, genistein and glycitein. Epidemiological studies in Asian countries suggest that isoflavones are beneficial for health, because in these studies high soy consumption was associated with lower incidence of several types of cancer, osteoporosis and cardiovascular disease (8). However, results from in vitro and animal studies still raise doubts about the safety of isoflavone intake (9-11), especially because isoflavones are hypothesized to activate estrogen receptors (ER) in the same way as estrogens and estrogens are known to increase breast cancer risk (9). Peri- and postmenopausal women are a potentially vulnerable group for these adverse effects because they have reduced competition by endogenous estrogens for the ERs and potentially frequently use isoflavone supplements with high doses to relieve their menopausal complaints. This vulnerability might be enhanced in a subgroup of so-

called equol-producing individuals. These individuals, comprising 20-30% of the Western population, harbour specific intestinal bacteria that can convert daidzein into the more active metabolite equol (12).

In this paper, isoflavones are studied to explore the intertissue and interspecies comparability of gene expression, in peripheral blood mononuclear cells (PBMCs) and white adipose tissue (WAT), by quantitative analysis of aligned experiments in rats and humans (Figure 5.1).

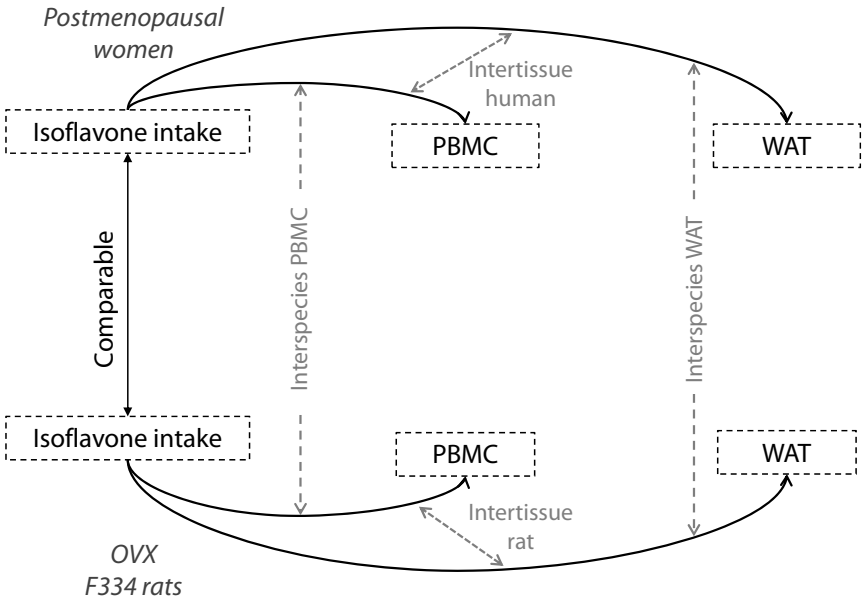

Figure 5.1 Quantification of gene expression effects after isoflavone supplementation and intertissue and interspecies comparisons in peripheral blood mononuclear cells (PBMCs) and white adipose tissue (WAT) in postmenopausal women and OVX F344 rats. Solid lines indicate effects of isoflavone intake on PBMCs and WAT, dotted lines indicate correlations between the estimates of these effects, i.e. intertissue (between tissues, within species) and interspecies (within tissues, between species).

METHODS

Quantification and comparison of gene expression effects after isoflavone intake was performed using data from two human intervention studies and one rat experiment. These studies were aligned for dose, duration and target group, and standardized laboratory methods were used.

Human intervention studies

The two human intervention studies were conducted at the Division of Human Nutrition of Wageningen University, approved by the Medical Ethical Committee of this university and have been described earlier by Van der Velpen *et al*. (13, 14). Both studies were double-blind placebo-controlled crossover studies with two eight week intervention periods and an eight week washout period in between. All participants received both supplement and placebo treatment.

In the ISO study, the effects of a low genistein, high daidzein (LG) on PBMC gene expression were studied in 27 equol-producing postmenopausal women. The ISO II study consisted of 2 sub studies that compared the same LG supplement vs. placebo in one sub study. This sub study was conducted in 36 postmenopausal women, both equol producers and non-producers. For our analysis, data from 24 postmenopausal women was used. The exact in- and exclusion criteria for both the ISO study and the ISO II study, as well as the screening procedure for equol producers have previously been described (13, 14). The studies were registered at clinicaltrials.gov under NCT01232751 (ISO study) and NCT01556737 (ISO II study).

Rat experiment

The rat experiment was performed at the Centre for Laboratory Animals (CKP, Wageningen) in compliance with the Dutch Act on animal experimentation (Stb, 1977, 67; Stb 1996, 565, revised February 5, 1997) and was approved by the ethical committee on animal experimentation of Wageningen University. All procedures were considered to avoid and minimize animal discomfort. For our analysis, data from 10 eight-week-old female inbred ovariectomized (OVX) F344 rats were used, all equol producers. Further detailed experimental conditions are described by Islam *et al*. (15).

Oral gavage stock of the LG supplement was daily and freshly prepared in 10 ml water containing 1% dimethyl sulfoxide. After eight weeks, the animals were anesthetized with a mixture of isofluorene and oxygen and blood was removed from the dorsal aorta. PBMCs were isolated from the blood and stored at -20°C and abdominal WAT was collected in liquid nitrogen during sacrifice and stored at -80°C until further analysis.

Supplements and doses

The studies were performed with the same LG supplement which contained 60% daidzein, 13% genistein and 27% glycitein for the supplements used in the ISO study, originating

from a batch bought in October 2010. The supplements from the ISO II study and the rat experiment originated from a batch bought in November 2011 and contained 56% daidzein, 16% genistein and 28% glycitein (absolute contents in Table 5.1).

The postmenopausal women in the two studies received ~100 mg isoflavones/day calculated in aglycone equivalents, for which two supplements were administered twice daily. For the animal study, the intake was scaled to body weight. For the women, the daily dose was 0.88-1.81 mg/kg bw/day as their body weights ranged from 55 to 107 kg. For the rats this was ~2.1 mg/kg bw/day, which is equivalent to ~0.42 mg/day for a 200 g rat.

Table 5.1 Daily isoflavone intake in the two human intervention studies (ISO study and ISO II study) and the rat experiment in mg isoflavone per day and mg/kg bw calculated as aglycone equivalents

	ISO study[1]		ISO II study[1]		Rat experiment[2]	
	mg/day	mg/ kg bw/day	mg/day	mg/ kg bw/day	mg/day	mg/ kg bw/day
Total isoflavone	93.9	0.88-1.70	100.1	0.94-1.81	0.42	2.10
Daidzin	53.2	0.50-0.96	52.0	0.49-0.94	0.22	1.10
Daidzein	3.2	0.03-0.06	3.7	0.03-0.07	0.02	0.10
Genistin	11.4	0.11-0.21	16.5	0.15-0.30	0.07	0.35
Genistein	0.4	0.00-0.01	0	0	0	0.00
Glycitin	23.8	0.22-0.43	26.3	0.25-0.48	0.11	0.55
Glycitein	2.0	0.02-0.04	1.5	0.01-0.03	0.01	0.05

[1] In the human intervention studies, the absolute amount per day was the same for every participant; the dose per kg bw is shown for the range between the lowest (i.e. 55.2 kg) and the highest bodyweight (i.e.107.0 kg).
[2] In the rat experiment, the dose per kg bw was similar for each rat; for calculation of the total dose a bodyweight of 200 g was assumed.

Transcriptomics

In the human studies, gene expression after both supplement and placebo treatment was measured in PBMCs (n = 27, all equol producers of the ISO study) and WAT (n = 24; 7 equol producers and 17 non-producers of the ISO II study) by Affymetrix human gene 1.1 ST arrays (13, 14). In the rat experiment, the gene expression in PBMCs and WAT (n = 10, all equol producers) was measured by Affymetrix rat gene 1.1 ST arrays. Five rats were exposed to the supplement, five other rats to the placebo and both PBMCs and WAT were collected from all rats.

Data analysis

Data from the Affymetrix chips were normalized using CDF17 with MADMAX software (16). Filtering of results was done according to number of arrays in the original study; for the ISO study with intensity 20 on > 5 arrays, for the ISO II study this was intensity 20 on > 15 arrays, for the rat data this was intensity 20 on > 4 arrays. To compare rat and human gene expression, the rat genes were recoded into human genes using the Homologene database (http://www.ncbi.nlm.nih.gov/homologene) and all duplicate genes were removed. After this, 80.7% of the rat genes in PBMCs and 81.5% genes in WAT that passed filtering remained as human homologs for analysis.

To study whether effects on gene expression in humans were similar to rats, genes with significantly changed expression in PBMCs (n = 27) and WAT (n = 7) from equol-producing postmenopausal women were compared to the differentially expressed human homologs in PBMCs and WAT from OVX F344 rats (n = 10). Subsequently, GSEA was performed on gene expression results for the same four groups of PBMCs and WAT in humans and rats. The significant gene sets (FDR < 0.25) were visualised using Cytoscape.

To study alleged estrogen-responsive effects of isoflavone supplementation, the human genes with significantly changed expression (separate for equol producers and non-producers in human WAT) and differentially expressed human homologs in rats were compared to the human estrogen-responsive genes registered in the estrogen-responsive gene database (ERGDB, data downloaded September 2013, (17)).

To quantify gene expression effects in PBMCs and WAT of postmenopausal women and OVX rats, multivariate regression models were run with sets of log-transformed gene expression intensities from human and rat PBMCs and WAT as dependent variables. The multivariate analysis was performed using redundancy analysis (RDA), also known as least-squares reduced-rank regression (18, 19). Like principal component analysis (PCA), RDA can handle any number of response variables (20), but has the advantage over PCA that it can focus on the effects of explanatory variables by constraining the components (axes) by the explanatory variables. In our case, the explanatory variables were the isoflavone supplement and the two tissues. All constrained components were used in reporting the results, and thus reduced-rank was not used because treatment effects were present on all axes, as judged by the relative magnitude of the eigenvalues of the constrained axes. The human model focused on the within-person tissue-dependent effects of isoflavone supplementation compared to placebo with the following model formula (21):

[Expression of group of genes] = (supplement + supplement.tissue) | person, tissue

where . denotes "interaction" and | means "conditional on".

Supplement and tissue have two levels each; LG and placebo for *supplement* and PBMC and WAT for *tissue*. *Person* is used as a covariate to correct for the paired measurements (supplement and placebo) within persons. *Tissue* is contained in the factor *person*, because each person provided only one of the two tissues, therefore *tissue* is superfluous as a covariate.

The rat model also focused on the tissue-dependent effects of the isoflavone supplement compared to placebo:

[Expression of group of genes] = (supplement + supplement.tissue) | tissue

This model has the same levels for *supplement* and *tissue* as the human model and *tissue* as a covariate to correct for the PBMCs and WAT collected from the same rats.

The models were run separately for humans and rats using Canoco 5.03 (20); both jointly and separately for PBMC and WAT samples. In the joint analysis, RDA estimated coefficients for each gene (dependent variable) and each explanatory variable for each (constrained) axis. This resulted in an explained variance (R^2 in %) of the model. The effect sizes of the intake on gene expression were summarized by the root mean square effect (RMSE) on the $\log_e$-scale and shown as the summary effect size (SES in % = 100 * (e^{RMSE}-1)). The separate analysis for each tissue was used to evaluate significance of the SES using permutation tests producing a pseudo-F test statistic ($P < 0.05$ and $P < 0.10$). In the permutation tests, the human samples were shuffled within person, while for the rat model unrestricted permutations were used. In addition, intertissue correlations were derived to compare gene expression effects between PBMC and WAT within each of the species. Similarly, interspecies correlations were determined to compare gene expression effects between rats and humans for each tissue separately. Correlation coefficients were calculated between the regression coefficients of the individual genes estimated by the separate models for PBMCs and WAT using SAS (SAS, version 9.3, SAS Institute, Inc., Cary, NC, USA).

Four different sets of genes were used as dependent variables in the multivariate analysis; 1) all genes with significantly changed expression in human PBMCs, 2) all genes with significantly changed expression in human WAT, 3) all differentially expressed human homologs in rat PBMCs, and 4) all differentially expressed human homologs in WAT of rats. Furthermore, genes from the ERGDB with significant gene expression in both species were used as dependent variable in the multivariate analysis. This was also done for three gene

sets identified with GSEA, i.e. energy metabolism, inflammation and cell cycle. Therefore, groups of genes with significantly changed expression in women or differentially expressed genes in rats related to oxidative phosphorylation (OXPHOS), interleukin signalling and mitotic cell cycle were selected as representative for these biological pathways.

RESULTS

The overlap between significant gene expression effects in PBMCs of equol-producing postmenopausal women (n = 339 genes) and PBMCs of rats (n = 540), was only 12 genes. Between significant gene expression effects in WAT of equol-producing postmenopausal women (n = 1169) and rats (n = 1237), the overlap was 86 genes (Figure 5.2 and Supplemental Table 5.1).

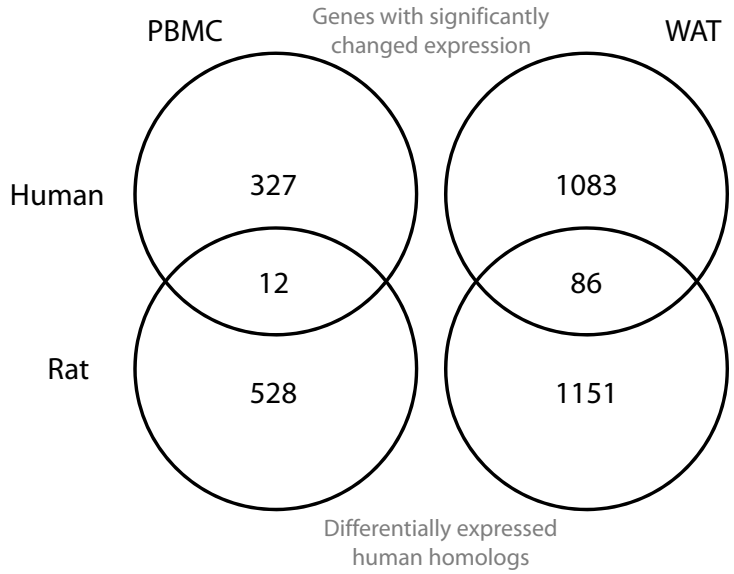

Figure 5.2 Overlap between genes with significantly changed expression in peripheral blood mononuclear cells (PBMCs, n = 27) of postmenopausal equol-producing women and differentially expressed human homologs in OVX F344 rat PBMCs (n = 10) and the overlap between these genes in white adipose tissue (WAT) of equol-producing women (n = 7) and rat (n = 10).

Next to evaluating effects of isoflavones on expression of single genes, the overall gene expression effect was quantified in a multivariate regression model. When genes with significantly changed expression in human PBMCs were used as dependent variables in

this model, the SES indicated 8.1% change of gene expression ($P < 0.05$) in these PBMCs. Similarly, a significant SES was obtained for human WAT (SES = 7.0%) with significant gene expression WAT as dependent variable, which was also observed for differentially expressed human homologs in the rat model for both PBMCs (SES = 23.9%) and WAT (SES = 34.0%). For the four analyses with significant gene expression as dependent variable, the SES was smaller and did not reach statistical significance in the other tissue in the same model, i.e. the tissue on which the selection of genes was not based (Table 5.2).

Table 5.2 Summary effect sizes (SES) of isoflavone supplementation for significantly changed gene expression in PBMCs and WAT, separate for the human intervention trials and the rat experiment

Significant gene expression in[1]	Within-subject changes in gene expression in human intervention studies[2]				Between-group differences in gene expression in rat experiment[3]			
	N_{genes}	PBMC[4] (%)	WAT (%)	R^2_{model} (%)	N_{genes}	PBMC (%)	WAT (%)	R^2_{model} (%)
PBMC	328	8.14‡	4.18	11.0	536	23.9‡	19.2	30.3
WAT	1158	3.16	7.01‡	6.9	1231	10.9	34.0‡	35.1

[1] Significant gene expression in PBMCs and WAT in humans and rats as mentioned in Figure 5.2.
[2] Based on two human crossover intervention trials, with 27 equol producing women for PBMCs, and 24 women (7 equol producers) for WAT.
[3] Two groups (placebo and supplement) with 5 rats each, PBMCs and WAT collected from all rats.
[4] Summary effect size (SES) in % calculated as $\exp((\tau * \sqrt{(\beta_{1st\ axis}^2 + \beta_{2nd\ axis}^2)}/SD)-1)*100)$ and interpreted as follows: if the exposure changes from placebo to supplement, gene expression for this selected group of genes changes with SES %.
‡ Significance determined by the separate models for each tissue ($P < 0.05$).
PBMC, peripheral blood mononuclear cell; WAT, white adipose tissue.

GSEA on the gene expression in the two tissues of equol producers in both species resulted in enriched gene sets related to energy metabolism, inflammation, cell cycle and nuclear receptor signalling, but not in gene sets related to estrogen signalling (Table 5.3). Gene sets which were significantly enriched, but could not be categorized in one of the previous pathways, were specified as 'other'. Gene sets involving estrogen signalling were not identified with GSEA, but all genes with significantly changed expression (in humans) and differentially expressed human homologs (in rats) were compared to the 1069 estrogen-responsive genes from the ERGDB (Supplemental Table 5.2). This analysis showed that 19 of the 339 genes (5.6%) with significantly changed expression in human PBMCs were estrogen-responsive. In WAT of equol producers, 82 out of 1169 genes (7.0%) with significantly changed expression were estrogen-responsive, whereas 39 out of 883 genes (4.4%) in WAT of non-producers were estrogen-responsive. In rats, 35 out of the 540 differentially expressed human homologs

Table 5.3 Results of gene set enrichment analysis on gene expression after isoflavone supplement intake performed for both PBMCs and WAT in postmenopausal women and OVX F344 rats

		Human[1]		Rat[2]	
Groups	Groups of gene sets	PBMC	WAT	PBMC	WAT
Energy metabolism	Glucose metabolism	*↓	↓		↓
	OXPHOS	↓	↓	↑	↓
	TCA cycle	*↓	↓	↑	
	Lipid metabolism		↓		
	Cholesterol biosynthesis				↓
	Amino acid metabolism		↓		
Inflammation	T-cell signalling		↑		↓
	B-cell signalling		↑	↓	
	Toll-like receptor signalling	↓	↑		↓
	Interleukin signalling		↑	↓	↓
	Nod-like signalling	↓	↑		
	Cytokine signalling		↑	↓	*↓
	MAPK signalling				↓
	GPCR signalling	↓			*↓
	NFκB signalling	↓			
	Interferon signalling			↓	↓
	NGF signalling				*↓
	Auto immune response	↓		↓	↓
	Adaptive immunity		↑		↓
Cell cycle	Mitotic cell cycle	↓	↓	↑	
	Proteasome	*↓	↓	↑	↓
	Cyclin signalling		↓	↑	↓
	mRNA metabolism			↑	
	mRNA processing			↑	
	Nucleosome assembly			↑	
	tRNA amino acylation		↓		
	Nucleotide excision repair		*↓	↑	
	Metabolism of proteins			↑	
	Apoptosis		↓		↓
	Protein folding		↓	↑	
	WNT signalling			↑	
Nuclear receptors	Nuclear receptor signalling	↓		↓	↓
	PPAR signalling	↓	↓		
Other	Synaptic transmission				↓
	Complement and coagulation	↓			
	VEGF / Integrin pathway	↓	↑		↓
	Hemostasis and platelet function			*↑	

[1] GSEA on human data is performed for PBMCs with n = 27 and for WAT with n = 7, all equol producers.
[2] GSEA on rat data is performed for n = 10 (n = 5 supplement, n = 5 placebo) for both PBMCs and WAT.
↓ downregulation of effects, ↑ upregulation of effects as shown by GSEA, * effects upregulated or downregulated in only one or two within the group of gene sets.
PBMC, peripheral blood mononuclear cell; WAT, white adipose tissue; ER, estrogen responsive; OXPHOS, oxidative phosphorylation; TCA cycle, tricarboxylic acid cycle; MAPK, mitogen-activated protein kinase; GPCR, G protein-coupled receptor; NFκB, nuclear factor kappa B; NGF, nerve growth factor; PPAR, peroxisome proliferator-activated receptors; VEGF, vascular endothelial growth factor.

(6.5%) in PBMCs and 84 out of 1237 genes (6.8%) in WAT were estrogen-responsive. Between the tissues and the species, only few overlapping genes were observed.

In the multivariate analysis, genes in the biological pathways from Table 5.3 were selected to obtain a pathway-specific effect size of gene expression in PBMCs and WAT of humans and rats (Table 5.4). As observed for the gene sets with significantly changed gene expression (Table 5.2), the SES in the biological pathways in rats was larger than the SES in humans. For the estrogen-responsive effects, the SES was 5.5% ($P < 0.05$) in WAT of postmenopausal women and in OVX rats the SES in this tissue was 26.7%. For genes related to OXPHOS, the SES were not significant. Effects on interleukin genes were only significant for rats with an effect size of 20.2% for PBMCs and 18.2% for WAT. Effects on mitotic cell cycle were only marginally significant ($P < 0.1$) for human WAT (4.0%) and rat PBMCs (11.5%) and WAT (10.6%).

Table 5.4 Summary effect sizes (SES) of gene expression in PBMCs and WAT after isoflavone supplementation for estrogen-responsive genes and genes related to energy metabolism, inflammation and cell cycle

Genes in biological pathway[1]	N_{genes}	Within-subject changes in gene expression in human intervention studies[2]			Between-group differences in gene expression in rat experiment[3]		
		PBMC[4] (%)	WAT (%)	R^2_{model} (%)	PBMC (%)	WAT (%)	R^2_{model} (%)
ER	170	3.80*	5.52**	6.2	15.9	26.7**	24.9
OXPHOS	58	4.02	6.73	6.3	13.5	26.1	16.0
Interleukin	16	4.27	3.73	3.3	20.2**	18.2**	36.2
Mitotic cell cycle	30	4.35	4.00*	5.2	11.5*	10.6*	19.6

[1] Group of genes involved in biological pathways (estrogen-responsive, energy metabolism, inflammation and cell cycle) with significant changed expression in two human intervention studies or differentially expressed human homologs in the rat experiment.
[2] Based on two human crossover intervention trials, with 27 subjects for PBMCs, 24 subjects for WAT.
[3] Two groups with 5 rats each, all providing both PBMCs and WAT.
[4] Summary effect size (SES) in % calculated as $\exp((\tau*\mathrm{sqrt}(\beta 1_{\text{st axis}}^2 + \beta_{\text{2nd axis}}^2)/SD)-1)*100)$ and interpreted as follows: if the exposure changes from placebo to supplement, gene expression for this selected group of genes changes with SES %.
** Significance determined by the separate models for each tissue with $P < 0.05$ or * for $P < 0.10$.
PBMC, peripheral blood mononuclear cell; WAT, white adipose tissue; ER, estrogen responsive; OXPHOS, oxidative phosphorylation.

The interspecies correlation within the biological pathways was only significant for WAT with regard to the estrogen-responsive genes ($r = 0.31$) and for PBMCs for the OXPHOS genes ($r = 0.33$). The intertissue correlation between PBMCs and WAT was statistically

significant for estrogen-responsive genes with r = 0.23 in humans and r = 0.22 in rats, and for cell cycle genes with r = 0.39 in humans and r = 0.47 in rats (Table 5.5). For the other biological pathways, gene expression effects between the tissues and the species were not significantly correlated.

Table 5.5 Intertissue and interspecies correlation coefficients for effect estimates from separate multivariate models for each tissue for estrogen-responsive genes and genes related to energy metabolism, inflammation and cell cycle

Genes in biological pathway[1]	Intertissue correlation between PBMC and WAT[2]				Interspecies correlation between human and rat[2]			
	Human		Rat		PBMC		WAT	
	N_{genes}	R (95% CI)	N_{genes}	R (95% CI)	N_{genes}	R (95% CI)	N_{genes}	R (95% CI)
ER	136	0.23 (0.06-0.38)	146	0.22 (0.05-0.36)	129	0.05 (-0.13-0.22)	166	0.31 (0.16-0.44)
OXPHOS	57	0.05 (-0.21-0.30)	57	0.17 (-0.09-0.41)	56	0.33 (0.07-0.55)	58	0.08 (-0.18-0.33)
Interleukin	15	-0.33 (-0.72-0.23)	15	0.29 (-0.27-0.70)	15	0.32 (-0.24-0.71)	15	0.11 (-0.43-0.59)
Mitotic cell cycle	30	0.39 (0.03-0.67)	30	0.47 (0.13-0.71)	30	0.03 (-0.33-0.39)	30	0.14 (-0.23-0.48)

[1] Group of genes involved in biological pathways (estrogen-responsive, energy metabolism, inflammation and cell cycle) with significant changed expression in two human intervention studies or differentially expressed human homologs in the rat experiment.
[2] Intertissue and interspecies correlation calculated from separate models by correlating regression coefficients for the genes for both species.
PBMC, peripheral blood mononuclear cell; WAT, white adipose tissue; ER, estrogen responsive; OXPHOS, oxidative phosphorylation.

DISCUSSION

In this study, gene expression effects after isoflavone supplementation vs. placebo were quantified and compared in PBMCs and WAT of postmenopausal women and OVX rats. In PBMCs and WAT of humans, the gene expression after isoflavone supplementation changed on average with ~8% and this effect size was 3-4 fold stronger in rats (24% change in expression in PBMCs and 31% in WAT). Similar effect sizes were found for ER-related genes in WAT for both species and the gene expression effects correlated significantly between species for WAT (r = 0.31), and between the two tissues (r ~ 0.23) for the two species. For OXPHOS, none of the SES was significant, but the interspecies correlation (r = 0.33) pointed towards similar effects in human and rat PBMCs. For interleukin, significant SES were

observed in rats only, but these effects did not correlate between tissues or species. For mitotic cell cycle, the SES were marginally significant for WAT in humans and both tissues in rat. For this geneset, the intertissue correlation was high ($r = 0.39$ for human and $r = 0.47$ for rat).

In this study, a multivariate regression model was used to quantify gene expression changes in PBMCs and WAT of human and rat after exposure to isoflavones. This quantification can potentially be used in addition to the usual qualitative evaluation of gene expression effects with an up- or downregulation in pathways or gene sets, because the multivariate model summarizes the effect size (SES) of a group of genes. A 3-4 fold larger effect size and larger explained variance of the model was observed in rats compared to humans (Table 5.2). Although the dose and duration of exposure to isoflavones was similar, this difference is in line with previously observed gene expression effects in genetically homogeneous inbred rat strains and genetically independent women (22).

GSEA revealed more similarities in effects between rats and humans on gene sets like energy metabolism, inflammation and cell cycle (Table 5.3) than direct comparison of gene expression of all individual genes (Figure 5.2). Effect sizes of significant gene expression in a selection of these biological pathways were successfully quantified with the multivariate regression model, but data were not always consistent between tissues or species (Table 5.4). For the estrogen-responsive genes, the effect size was significant in WAT in both species and significant correlations between the two tissues and the two species were observed. Adipose tissue is known as an estrogen-responsive tissue and despite the fact that PBMCs display ERs they are not particularly known as estrogen responsive (23, 24). The similarity of the intertissue correlation coefficients in human ($r = 0.23$) and rat ($r = 0.22$) for these effects is interesting, given that for humans PBMCs and WAT were not collected from the same individuals, which was the case for the rats. The estrogens are known to induce gene expression via the ERs and this gene set consists of particular ER target genes, which might explain part of the observed effects and correlations. However, it must be noted that the percentage of estrogen-responsive genes compared to the other genes with significantly changed expression is low (< 7%) in PBMCs and WAT.

To our knowledge, this is the first study that compared gene expression data from human intervention trials with a rat experiment in which the dose (~1.5 mg/kg bw vs 2.1 mg/kg bw), duration (8 weeks) and target group (postmenopausal women vs OVX rats) were aligned and the same methods in the same lab were used for measurement of gene expression. Despite this, effect sizes and correlations may be influenced by choices made

in aligning the human studies and rat experiment; with regard to duration, scaling for life expectancy could have been used and with regard to dose, scaling for metabolic rate could have been used. These estimates are also highly influenced by the selection of the groups of genes as dependent variables and by the loss of almost 20% of the rat genes because of recoding of the rat genes to human homologs. With regard to the model, the difference in study design for the human and rat experiment with regard to data pairing complicated the modelling, but by choosing different covariates, unbiased estimates of the supplement effects were obtained. Another important issue is that rats are known to be equol producers (25), while this is only the case for 20-30% of Western populations (12) and that differences are observed in gene expression between the equol-producers and non-producers in human intervention studies (14, 26). The gene expression data of human WAT was available for only a small number of equol producers (n = 7) compared to non-producers (n = 17). However, equol-producing phenotype was not used as a covariate in the multivariate regression model to maintain the similarity of the human and rat model. Thus, if effects would be stronger and specific for equol producers, the inclusion of non-producers may have weakened our effect estimates in humans.

For risk assessment, it is important to quantitatively compare effects within and between species to reduce uncertainty in extrapolation from animal to human. While two previous studies qualitatively compared gene expression effects across species using the parallelogram approach (6, 7), our research showed the use of a multivariate model for quantification of gene expression effects and subsequent quantitative comparison of these effects between tissues and species. Despite this, the estimated intertissue and interspecies correlations cannot be directly used for risk assessment, because this would also require data on gene expression in target tissue, i.e. breast and uterus, and its relation to health endpoints. Nevertheless, this model provides an important tool to further explore intertissue and interspecies similarities and might contribute to advancing use of transcriptomics in the parallelogram approach for risk assessment.

ACKNOWLEDGEMENTS

We thank Mark Boekschoten and Guido Hooiveld for their excellent assistance in the micro array analysis and derivation of human homologs from rat data and Shohreh Keshtar for her technical assistance.

The authors' responsibilities were as follows-VvdV, AG, LAA, PCH, FXRvL, MAI, EGS, PvtV,: designed the research; VvdV: conducted the human intervention studies; MAI: conducted the rat experiment; VvdV and CJFtB: analyzed the data; VvdV: wrote the manuscript; and AG, MAI, CJFtB, FXRvL, LAA, PCH, EGS, and PvtV: critically reviewed the manuscript.

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Supplemental Table 5.1 Overlap of differentially expressed genes in rats after the isoflavone supplement compared to placebo with significantly changed genes in humans with the same treatment compared to placebo

	PBMC		WAT	
	Human	Rat	Human	Rat
N significantly changed genes (human) / N differentially expressed human homologs (rat)	339	540	1169	1237
N upregulated	165	110	577	840
N downregulated	174	430	592	397
	PBMC		WAT	
N overlapping genes	12		86	
N upregulated	2		38	
N downregulated	7		15	
Upregulated overlapping genes (gene name, EntrezID)	CABP5, 56344 SUMO1, 7341		CYBRD1, 79901 VCPIP1, 80124 MYOF, 26509 CASP8AP2, 9994 FAM180A, 389558 RGS22, 26166 GALNT15, 117248 TC2N, 123036 FHL2, 2274 IL6R, 3570 HMCN1, 83872 PRPF40A, 55660 DENND2D, 79961 C10orf137, 26098 CD200R1, 131450 PSPC1, 55269 ATOH8, 84913 FAM210B, 116151 KDM5A, 5927	SPOCK2, 9806 POLI, 11201 GALNT12, 79695 PPL, 5493 OSBP2, 23762 DSG2, 1829 EFEMP1, 2202 MBNL3, 55796 FMO2, 2327 PLAC8, 51316 PHF20, 51230 WISP2, 8839 MEIS1, 4211 PNISR, 25957 ZBTB26, 57684 ARFIP1, 27236 ATXN3, 4287 TMEM178A, 130733 AMZ1, 155185
Downregulated overlapping genes (gene name, EntrezID)	STYXL1, 51657 LRG1, 116844 SIGLEC10, 89790 FGD6, 55785 TP53I11, 9537 MTMR11, 10903 SNX30, 401548		DPP9, 91039 BCKDK, 10295 NR4A1, 3164 FAM171A2, 284069 ACSL3, 2181 S1PR3, 1903 PRKACA, 5566 HIPK4, 147746	sep-11, 55752 TRPM4, 54795 CLASRP, 11129 PRDX5, 25824 RUNX1, 861 PGP, 283871 PKM, 5315

Supplemental Table 5.1 continues on next page

Supplemental Table 5.1 *Continued*

	PBMC		WAT	
	Human	Rat	Human	Rat
Other overlapping (gene name, EntrezID)	ISG20L2, 81875 CACYBP, 27101 GNG5, 2787		MRPL33, 9553 EFHD1, 80303 SLC16A1, 6566 LSR, 51599 KITLG, 4254 LDLRAP1, 26119 ANPEP, 290 KNSTRN, 90417 PTH1R, 5745 WDR66, 144406 ALDH1A2, 8854 RASSF5, 83593 SSC5D, 284297 LMOD1, 25802 BPGM, 669 C10orf35, 219738 SELPLG, 6404	CD3E, 916 MYO18A, 399687 WNT5B, 81029 C12orf45, 121053 RAPGEF5, 9771 RARS2, 57038 XPNPEP2, 7512 NDUFA4, 4697 RPP25, 54913 MYO5B, 4645 SLC25A32, 81034 SLC25A14, 9016 HP, 3240 SLCO3A1, 28232 DECR1, 1666 RAMP3, 10268

The gene expression data for rats is recoded into human homologs; for PBMCs 670 differentially expressed, but 540 have human homologs and for WAT 1486 differentially expressed, but 1236 have human homologs. WAT human data are significantly changed genes for equol producers receiving the isoflavone supplement, n = 7. PBMC, peripheral blood mononuclear cells; WAT white adipose tissue.

Supplemental Table 5.2 Estrogen responsive genes with significantly changed expression and differentially expressed human homologs of PBMC and WAT in humans and rats. Data compared to ERGDB database.

Gene name	Description	Human			Rat	
		PBMC	WAT EP	WAT NP	PBMC	WAT
CTNND1	catenin (cadherin-associated protein), delta 1	-1.13	1.19			
NME2	NME/NM23 nucleoside diphosphate kinase 2	-1.12		-1.13		
SPRED1	sprouty-related, EVH1 domain containing 1	-1.11				
NRP1	neuropilin 1	-1.10				
STAB1	stabilin 1	-1.09				
HSPA1A	heat shock 70kDa protein 1A	-1.08				
PTPRO	protein tyrosine phosphatase, receptor type, O	-1.07				
TIMELESS	timeless circadian clock	-1.07				
SLC25A5	solute carrier family 25 (mitochondrial carrier /// adenine nucleotide translocator), member 5	-1.05		-1.08		
CACYBP	calcyclin binding protein	-1.05			1.40	
PSMD8	proteasome (prosome, macropain) 26S subunit, non-ATPase, 8	-1.04				
EDEM1	ER degradation enhancer, mannosidase alpha-like 1	1.05				
FOXP1	forkhead box P1	1.05				
MYB	v-myb myeloblastosis viral oncogene homolog (avian)	1.05				
BCL2L1	BCL2-like 1	1.06	1.19			
STXBP1	syntaxin binding protein 1	1.07				
MUC1	mucin 1, cell surface associated	1.07		-1.12		
FKBP5	FK506 binding protein 5	1.08				
ERBB2	v-erb-b2 erythroblastic leukemia viral oncogene homolog 2, neuro/glioblastoma derived oncogene homolog (avian)	1.08				
TNC	tenascin C		-1.36			
IGF2	insulin-like growth factor 2 (somatomedin A)		-1.35			
PPIF	peptidylprolyl isomerase F		-1.28			
ME1	malic enzyme 1, NADP(+)-dependent, cytosolic		-1.27			
NR4A1	nuclear receptor subfamily 4, group A, member 1		-1.27	-1.16	-1.25	-2.44
NME1	NME/NM23 nucleoside diphosphate kinase 1		-1.27			
TGFB3	transforming growth factor, beta 3		-1.26	-1.11		
TUBG1	tubulin, gamma 1		-1.24			
THBD	thrombomodulin		-1.24			
NCAM2	neural cell adhesion molecule 2		-1.23			
KITLG	KIT ligand		-1.20			1.18
ELOVL2	ELOVL fatty acid elongase 2		-1.20			
CCND1	cyclin D1		-1.20			
NOS3	nitric oxide synthase 3 (endothelial cell)		-1.19	-1.09		
CNKSR3	CNKSR family member 3		-1.19			
CYCS	cytochrome c, somatic		-1.18			
DHCR24	24-dehydrocholesterol reductase		-1.18			
KIR3DL2	killer cell immunoglobulin-like receptor, three domains, long cytoplasmic tail, 2		-1.18			
PAICS	phosphoribosylaminoimidazole carboxylase, phosphoribosylaminoimidazole succinocarboxamide synthetase		-1.18			
TFF1	trefoil factor 1		-1.18			
HSPD1	heat shock 60kDa protein 1 (chaperonin)		-1.17			

Supplemental Table 5.2 continues on next page

Supplemental Table 5.2 *Continued*

Gene name	Description	Human			Rat	
		PBMC	WAT EP	WAT NP	PBMC	WAT
KPNA2	karyopherin alpha 2 (RAG cohort 1, importin alpha 1)		-1.16			
ARMCX3	armadillo repeat containing, X-linked 3		-1.16			
ENO1	enolase 1, (alpha)		-1.16			
TK1	thymidine kinase 1, soluble		-1.15	-1.11		
RET	ret proto-oncogene		-1.15	-1.13		
SGCD	sarcoglycan, delta (35kDa dystrophin-associated glycoprotein)		-1.15			
SEMA5B	sema domain, seven thrombospondin repeats (type 1 and type 1-like), transmembrane domain (TM) and short cytoplasmic domain, (semaphorin) 5B		-1.15			
G6PD	glucose-6-phosphate dehydrogenase		-1.15			
RAMP3	receptor (G protein-coupled) activity modifying protein 3		-1.14			1.74
AURKB	aurora kinase B		-1.14			
ESR2	estrogen receptor 2 (ER beta)		-1.13			
C1QBP	complement component 1, q subcomponent binding protein		-1.13			
CENPA	centromere protein A		-1.13			
ACO2	aconitase 2, mitochondrial		-1.13			
RUNX1	runt-related transcription factor 1		-1.12			-1.15
MCM4	minichromosome maintenance complex component 4		-1.12			
NR4A3	nuclear receptor subfamily 4, group A, member 3		-1.12			
FOXF1	forkhead box F1		-1.12			
GOT1	glutamic-oxaloacetic transaminase 1, soluble (aspartate aminotransferase 1)		-1.11	-1.08		
ORMDL2	ORM1-like 2 (S. cerevisiae)		-1.11			
MARCKS	myristoylated alanine-rich protein kinase C substrate		-1.11			
SLC12A2	solute carrier family 12 (sodium/potassium/chloride transporters), member 2		-1.11			
SPRY1	sprouty homolog 1, antagonist of FGF signaling (Drosophila)		-1.11			
GARS	glycyl-tRNA synthetase		-1.11			
RPA3	replication protein A3, 14kDa		-1.10			
STMN1	stathmin 1		-1.10			
IARS	isoleucyl-tRNA synthetase		-1.10			
ITGAV	integrin, alpha V		-1.09			
NUP88	nucleoporin 88kDa		-1.08			
TXNIP	thioredoxin interacting protein		1.08			
NRF1	nuclear respiratory factor 1		1.09			
ECE1	endothelin converting enzyme 1		1.09		-1.13	
EFEMP1	EGF containing fibulin-like extracellular matrix protein 1		1.10			1.30
MPL	myeloproliferative leukemia virus oncogene		1.10			
GSTO1	glutathione S-transferase omega 1		1.11			
HIP1R	huntingtin interacting protein 1 related		1.11			
PAX8	paired box 8		1.12			
PTPN18	protein tyrosine phosphatase, non-receptor type 18 (brain-derived)		1.12			

Supplemental Table 5.2 *Continued*

Gene name	Description	Human			Rat	
		PBMC	WAT EP	WAT NP	PBMC	WAT
WSB1	WD repeat and SOCS box containing 1		1.12			
IGF1R	insulin-like growth factor 1 receptor		1.13			
GNG7	guanine nucleotide binding protein (G protein), gamma 7		1.13			
SFRP1	secreted frizzled-related protein 1		1.13			
MCM7	minichromosome maintenance complex component 7		1.13			
SATB1	SATB homeobox 1		1.14			
INPP4B	inositol polyphosphate-4-phosphatase, type II, 105kDa		1.15			
THBS2	thrombospondin 2		1.15			
PTGER2	prostaglandin E receptor 2 (subtype EP2), 53kDa		1.15			
WISP2	WNT1 inducible signaling pathway protein 2		1.16			1.81
BCL2L11	BCL2-like 11 (apoptosis facilitator)		1.17			
ABCB1	ATP-binding cassette, sub-family B (MDR/TAP), member 1		1.17			
TSC22D3	TSC22 domain family, member 3		1.18			
NFKBIA	nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, alpha		1.18			
ANKRD44	ankyrin repeat domain 44		1.19			
TNFSF8	tumor necrosis factor (ligand) superfamily, member 8		1.19			
CCNG2	cyclin G2		1.20			
FKBP8	FK506 binding protein 8, 38kDa		1.20			
ZFP36L2	ZFP36 ring finger protein-like 2		1.21			
SLA	Src-like-adaptor		1.34			
S100P	S100 calcium binding protein P		1.42			
KRT6B	keratin 6B			-1.19		
TPX2	TPX2, microtubule-associated, homolog (Xenopus laevis)			-1.17		
ADRB1	adrenoceptor beta 1			-1.15		
ZWINT	ZW10 interactor, kinetochore protein			-1.14		
EGR3	early growth response 3			-1.12		-1.21
CORO2B	coronin, actin binding protein, 2B			-1.11		
SSNA1	Sjogren syndrome nuclear autoantigen 1			-1.09		
BIK	BCL2-interacting killer (apoptosis-inducing)			-1.09		
FGF18	fibroblast growth factor 18			-1.08		
CTBP1	C-terminal binding protein 1			-1.07		
CELSR2	cadherin, EGF LAG seven-pass G-type receptor 2			-1.06		
TMED2	transmembrane emp24 domain trafficking protein 2			1.04		
sep-02	septin 2			1.05		
MAN1A1	mannosidase, alpha, class 1A, member 1			1.05		
RIF1	RAP1 interacting factor homolog (yeast)			1.05		
WDR43	WD repeat domain 43			1.06		1.16
NR2C1	nuclear receptor subfamily 2, group C, member 1			1.06		
LRRFIP2	leucine rich repeat (in FLII) interacting protein 2			1.06		
KIFAP3	kinesin-associated protein 3			1.07		
OLFM1	olfactomedin 1			1.07		

Supplemental Table 5.2 continues on next page

Supplemental Table 5.2 *Continued*

Gene name	Description	Human			Rat	
		PBMC	WAT EP	WAT NP	PBMC	WAT
APOA2	apolipoprotein A-II			1.08		1.34
RPL15	ribosomal protein L15			1.08	1.22	
EIF5	eukaryotic translation initiation factor 5			1.09		
LRRC49	leucine rich repeat containing 49			1.09		1.36
TMED7	transmembrane emp24 protein transport domain containing 7			1.09		
ABCE1	ATP-binding cassette, sub-family E (OABP), member 1			1.09		
RBBP8	retinoblastoma binding protein 8			1.09		
ZNF230	zinc finger protein 230			1.12		
FXYD2	FXYD domain containing ion transport regulator 2			1.15		
GPRC5A	G protein-coupled receptor, family C, group 5, member A			1.16		1.58
BLNK	B-cell linker				-1.47	
IL6	interleukin 6 (interferon, beta 2)				-1.42	
KYNU	kynureninase				-1.39	
P2RY6	pyrimidinergic receptor P2Y, G-protein coupled, 6				-1.39	
FAIM3	Fas apoptotic inhibitory molecule 3				-1.37	
KRT8	keratin 8				-1.30	
MYO1E	myosin IE				-1.29	
NRP2	neuropilin 2				-1.28	
HLA-DRB1	major histocompatibility complex, class II, DR beta 1				-1.28	
PRKCE	protein kinase C, epsilon				-1.25	-1.23
SLC16A7	solute carrier family 16, member 7 (monocarboxylic acid transporter 2)				-1.25	
SH3BP5	SH3-domain binding protein 5 (BTK-associated)				-1.24	
ZFP36	ZFP36 ring finger protein				-1.22	
GDF15	growth differentiation factor 15				-1.21	
PKIB	protein kinase (cAMP-dependent, catalytic) inhibitor beta				-1.18	
PSEN2	presenilin 2 (Alzheimer disease 4)				-1.18	
GPR18	G protein-coupled receptor 18				-1.18	
GADD45B	growth arrest and DNA-damage-inducible, beta				-1.16	
TRPS1	trichorhinophalangeal syndrome I				-1.16	
AIM1	absent in melanoma 1				-1.14	1.26
ULK1	unc-51-like kinase 1 (C. elegans)				-1.11	
EIF2S1	eukaryotic translation initiation factor 2, subunit 1 alpha, 35kDa				1.16	
EIF1AY	eukaryotic translation initiation factor 1A, Y-linked				1.16	
TRIP13	thyroid hormone receptor interactor				1.16	
PLAA	phospholipase A2-activating protein				1.17	
PDIA4	protein disulfide isomerase family A, member 4				1.17	
ATAD2	ATPase family, AAA domain containing 2				1.17	1.20
PLEKHF2	pleckstrin homology domain containing, family F (with FYVE domain) member 2				1.20	
SSR3	signal sequence receptor, gamma (translocon-associated protein gamma)				1.21	1.16
SFPQ	splicing factor proline/glutamine-rich				1.23	

Supplemental Table 5.2 *Continued*

Gene name	Description	Human			Rat	
		PBMC	WAT EP	WAT NP	PBMC	WAT
CRP	C-reactive protein, pentraxin-related				1.25	
FOS	FBJ murine osteosarcoma viral oncogene homolog					-2.53
JUNB	jun B proto-oncogene					-1.45
SERPINA3	serpin peptidase inhibitor, clade A (alpha-1 antiproteinase, antitrypsin), member 3					-1.32
MMP16	matrix metallopeptidase 16 (membrane-inserted)					-1.29
PTGES	prostaglandin E synthase					-1.28
IGFBP5	insulin-like growth factor binding protein 5					-1.26
FLT4	fms-related tyrosine kinase 4					-1.26
PGM5	phosphoglucomutase 5					-1.24
DNASE1	deoxyribonuclease I					-1.24
GRID2	glutamate receptor, ionotropic, delta 2					-1.23
TOB1	transducer of ERBB2, 1					-1.23
SOCS3	suppressor of cytokine signaling 3					-1.22
IER2	immediate early response 2					-1.21
BTG2	BTG family, member 2					-1.21
ZNF703	zinc finger protein 703					-1.20
WNT10B	wingless-type MMTV integration site family, member 10B					-1.19
PREX1	phosphatidylinositol-3,4,5-trisphosphate-dependent Rac exchange factor 1					-1.19
POLA2	polymerase (DNA directed), alpha 2, accessory subunit					-1.18
IRS1	insulin receptor substrate 1					-1.18
MYBL2	v-myb myeloblastosis viral oncogene homolog (avian)-like 2					-1.17
JUN	jun proto-oncogene					-1.17
HES1	hairy and enhancer of split 1, (Drosophila)					-1.16
L1CAM	L1 cell adhesion molecule					-1.15
HMGCR	3-hydroxy-3-methylglutaryl-CoA reductase					-1.15
SEMA4A	sema domain, immunoglobulin domain (Ig), transmembrane domain (TM) and short cytoplasmic domain, (semaphorin) 4A					-1.13
RXRA	retinoid X receptor, alpha					-1.11
SLC7A5	solute carrier family 7 (amino acid transporter light chain, L system), member 5					-1.10
GNB1	guanine nucleotide binding protein (G protein), beta polypeptide 1					1.14
SLC1A2	solute carrier family 1 (glial high affinity glutamate transporter), member 2					1.16
IGFBP4	insulin-like growth factor binding protein 4					1.16
NDRG1	N-myc downstream regulated 1					1.18
TPBG	trophoblast glycoprotein					1.18
SLC38A1	solute carrier family 38, member 1					1.19
RAB18	RAB18, member RAS oncogene family					1.19
HUNK	hormonally up-regulated Neu-associated kinase					1.19
AR	androgen receptor					1.20
TRA2A	transformer 2 alpha homolog (Drosophila)					1.20
HAT1	histone acetyltransferase 1					1.22
SLC7A1	solute carrier family 7 (cationic amino acid transporter, y+ system), member 1					1.23

Supplemental Table 5.2 continues on next page

Supplemental Table 5.2 *Continued*

Gene name	Description	Human			Rat	
		PBMC	WAT EP	WAT NP	PBMC	WAT
SF3A3	splicing factor 3a, subunit 3, 60kDa					1.23
IL1R1	interleukin 1 receptor, type I					1.23
NUCB2	nucleobindin 2					1.23
SEMA3C	sema domain, immunoglobulin domain (Ig), short basic domain, secreted, (semaphorin) 3C					1.25
TSPAN5	tetraspanin 5					1.27
ITGBL1	integrin, beta-like 1 (with EGF-like repeat domains)					1.27
GALNT4	UDP-N-acetyl-alpha-D-galactosamine:polypeptide N-acetylgalactosaminyltransferase 4 (GalNAc-T4)					1.27
PTPRU	protein tyrosine phosphatase, receptor type, U					1.29
LHFP	lipoma HMGIC fusion partner					1.33
TGFA	transforming growth factor, alpha					1.33
ANXA3	annexin A3					1.38
PRSS23	protease, serine, 23					1.42
PTGS2	prostaglandin-endoperoxide synthase 2 (prostaglandin G/H synthase and cyclooxygenase)					1.43
PARD6B	par-6 partitioning defective 6 homolog beta (C. elegans)					1.44
ALB	albumin					1.47
BICD1	bicaudal D homolog 1 (Drosophila)					1.48
OPRK1	opioid receptor, kappa 1					1.49
SLC39A8	solute carrier family 39 (zinc transporter), member 8					1.53
OLR1	oxidized low density lipoprotein (lectin-like) receptor 1					1.58
KRT7	keratin 7					1.58
KRT19	keratin 19					1.59
PKP2	plakophilin 2					1.60
MSX2	msh homeobox 2					1.60
PTGS1	prostaglandin-endoperoxide synthase 1 (prostaglandin G/H synthase and cyclooxygenase)					1.69
PLOD2	procollagen-lysine, 2-oxoglutarate 5-dioxygenase 2					1.84
ARNT2	aryl-hydrocarbon receptor nuclear translocator 2					1.98
SLC26A3	solute carrier family 26 (anion exchanger), member 3					2.01
CDH11	cadherin 11, type 2, OB-cadherin (osteoblast)					2.61
CXADR	coxsackie virus and adenovirus receptor					2.69
AHSG	alpha-2-HS-glycoprotein					4.85
# sign changed ER genes		19	82	39	35	84
# sign changed genes		339	1169	883	540	1237
% estrogen responsive		5.60	7.01	4.42	6.48	6.79

PBMC peripheral blood mononuclear cell, WAT white adipose tissue, EP equol producer, NP non producer.

Chapter 6

General discussion

In this thesis, it was hypothesized that alignment and standardization of exposure and early effect markers in nutritional epidemiology and toxicological research would enable an integrated approach for improved risk assessment (Figure 6.1). Following this framework, epidemiology could contribute importantly to defining dietary intake and biomarkers for exposure and early effects in the population. Toxicology could complement this by measuring similar markers and by defining disease pathways and health endpoints resulting from exposure.

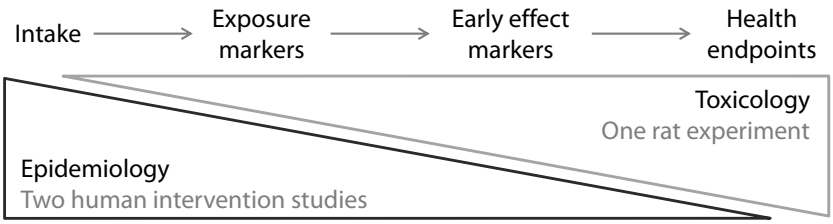

Figure 6.1 The concept of integrating toxicological and epidemiological approaches to improve risk assessment.

The practical application of this concept was further developed using isoflavones as a case study. This topic was chosen because the safety of isoflavone consumption at higher doses is still under debate (1-3) and the European Food Safety Authority (EFSA) has expressed concerns on effects of isoflavones on tumour promotion and thyroid function (4).

Two human intervention studies were conducted to assess effects of isoflavone supplementation on gene expression in peripheral blood mononuclear cells (PBMCs, chapter 2) and white adipose tissue (WAT, chapter 3) of postmenopausal women. For improvement of risk assessment, data from human intervention studies were used to model the relation between intake and exposure markers (chapter 4) and the two intervention studies were aligned with a rat experiment to model the relation between supplemental isoflavone intake and early effect markers in both species (chapter 5).

In this discussion, the results of the two intervention studies with isoflavone supplements in postmenopausal women will be addressed first. Subsequently, the parallelogram approach for aligning studies in epidemiology and toxicology will be used as a framework for evaluating the contribution of our results to improvement of risk assessment (Figure 6.2).

MOLECULAR EFFECTS OF ISOFLAVONES

Rationale for the human intervention studies

Because isoflavone intake is low in the general Western population (5), observational studies that can be used to assess the association between isoflavone intake and disease endpoints are scarce (6, 7). Therefore, human health effects of isoflavone supplementation in the Western population are often determined using intervention trials. The generalization of the results of these intervention studies is complicated because the group of isoflavones consists of 4 different important members; genistein, daidzein, glycitein and the daidzein-metabolite equol. The first three are often supplied as supplements or in food products from which the isoflavone content and dose are not always properly defined (8). In the two human intervention studies in this thesis, isoflavone composition and dose were systematically determined by HPLC analysis. Both intervention studies focused on whole-genome gene expression effects of isoflavone supplementation of ~100 mg/day; in the ISO study with a "low genistein, high daidzein" (LG) supplement, and in the ISO II study with the same LG supplement in one sub study and a "high genistein, high daidzein" (HG) supplement in the other paralleled sub study. Both studies had a similar placebo-controlled crossover design with two intervention periods of eight weeks and an eight week washout in between.

In our two studies, postmenopausal women were selected as potential high risk group, because they produce only little endogenous estradiol to compete with isoflavones for the estrogen receptors (ERs). Furthermore, the equol-producing phenotype can be an important modifier of effects, because equol has higher binding affinity on ERs than its precursor daidzein (9, 10). Only 20-30% of the Western population is equol producer (10) and this phenotype is often not adequately characterized in intervention studies (8). For the ISO study, only equol-producing postmenopausal women were recruited, because it was hypothesized that they might be a specific high risk group. The ISO II study focused on the effect of two isoflavone supplements in a general postmenopausal population, which made it possible to compare results between equol producers and non-producers.

In the ISO study, gene expression effects were studied in peripheral blood mononuclear cells (PBMCs) and in the ISO II study these effects were studied in adipose tissue. PBMCs are easily accessible and previous studies have shown that those cells can be used to study nutrition-induced effects on gene expression (11). Because the ISO study indicated a lack of estrogen-responsive effects in PBMCs, the ISO II study was conducted with the more estrogen-responsive and also accessible abdominal adipose tissue (12, 13). Use of

whole-genome gene expression as early effect marker measured by microarrays enabled the exploration of the whole spectrum of possible effects of isoflavone supplementation, including the estrogen-responsive effects of isoflavones, instead of focusing on a few intermediate markers of specific health effects. The exposure duration of 8 weeks represented a moderate to long-term period at which plasma isoflavone concentrations have reached steady state.

Effects of isoflavones on gene expression in PBMCs and adipose tissue

In the ISO study, expression of a total of 357 genes was significantly changed in PBMCs of equol-producing postmenopausal women after exposure to the LG supplement and these effects were related to oxidative phosphorylation (OXPHOS), inflammation and cell cycle. In the ISO II study, expression of 883 and 1169 genes was significantly changed in adipose tissue of non-producers and equol producers, respectively, after intake of the LG supplement. For the HG supplement, expression of 547 and 631 genes was significantly changed in adipose tissue of non-producers and equol producers, respectively, and effects of both isoflavone supplements were found on energy metabolism and inflammation. In this paragraph the observed effects in the two intervention studies on the biological pathways of energy metabolism, inflammation and cell cycle will be discussed.

Energy metabolism

In PBMCs of equol-producing postmenopausal women in the ISO study, downregulation of OXPHOS was observed after intake of the LG supplement. This was mainly driven by downregulation of several NADH dehydrogenase ubiquinone flavoprotein (NDUF) genes, which may result in reduced reactive oxygen species (ROS) production and oxidative stress. As these processes have the potential to induce DNA damage as a precursor for cancer (37), beneficial effects might be expected from downregulation of OXPHOS. In adipose tissue of both equol-producing and non-producing postmenopausal women in the ISO II study, expression of OXPHOS was also downregulated in addition to other energy metabolism-related genes after intake of the LG supplement, while these genes were marginally upregulated after intake of the HG supplement. The effects of the LG supplement on gene expression related to energy metabolism may be beneficial because of their resemblance with gene expression effects of caloric restriction, which is known to be beneficial for longevity (14). The downregulation in expression of energy metabolism-related genes by the LG supplement, might have induced phenotypical effects, for instance on adipocyte cell size, body fat distribution or body weight. Effects on body weight and

adipocyte size in subcuteaneous adipose tissue were not observed in our study, while effects on body fat distribution were not assessed. However, our study size and duration were not designed to evaluate effects on these phenotypic markers. This could also be the reason why isoflavone-induced decrease in gene expression of RBP4 and in expression of genes related to cholesterol metabolism in adipose tissue were not reflected by changes in serum RBP4 and cholesterol (total, HDL and triglycerides) concentrations. It should be noted, however, that for these markers adipose tissue is not the main organ for production, as they are very well known to be produced in the liver.

Inflammation

In both PBMCs and adipose tissue, gene expression effects of isoflavone supplementation on inflammation were found. More specifically, in PBMCs of equol-producing postmenopausal women downregulation of gene sets related to toll-like receptor (TLR) signaling and complement and coagulation were observed. In adipose tissue, the effects were dependent on supplement-type (LG or HG) as well as on equol producer status. After intake of the LG supplement, expression of genes in inflammation-related gene sets were significantly upregulated in equol producers. The inflammation-related effects in PBMCs and adipose tissue seem opposite, which could be explained by the fact that inflammation plays a different role in these tissues; PBMCs are a group of cells that mediate immune responses (15), while inflammation in adipose tissue only starts playing a role when fat mass is increasing (16-18). Analysis of the specific genes involved, suggested that the gene expression changes in PBMCs and in adipose tissue after isoflavone supplementation may be beneficial with regard to inflammation, although this might only be the case for equol producers.

Cell cycle

With regard to cell cycle effects, expression of histones and histone-like genes was downregulated in PBMCs of equol-producing postmenopausal women after intake of the LG supplement. These effects may contribute to the alleged effects of isoflavones on cell cycle arrest, which might provide time for DNA damage repair or induction of apoptosis and could consequently prevent replication of cells with DNA damage and formation of tumors. Effects on cell cycle were also observed with gene set enrichment analysis (GSEA) in adipose tissue of postmenopausal women after intake of both supplements, but further analysis revealed that only a few genes were significantly changed.

Final considerations and conclusion

A final question is whether the observed effects on gene expression are direct effects of isoflavones on these tissues, or whether they are secondary to effects on another tissue, for instance the liver. There is no current literature on the uptake of isoflavones in PBMCs, but isoflavones are known to end up in both the glandular and the adipose fraction of breast tissue (19) and therefore they might also be present in subcutaneous adipose tissue.

In conclusion, effects of isoflavone supplementation on gene expression might point towards beneficial effects on energy metabolism and inflammation in PBMCs and adipose tissue, and on cell cycle in PBMCs. Other important findings were the observed differences in effects for the two different isoflavone supplements and the two equol-producing phenotypes in the ISO II study, and the apparent limited estrogen-responsive gene expression in both studies. The latter finding will be further discussed below.

Estrogen-related and other potential pathways induced by isoflavones

In our two intervention studies it was observed that the hypothesized estrogen-responsive effects were marginal in both PBMCs and adipose tissue as they were not identified by GSEA. Also, in a separate comparison of significantly changed genes with the estrogen-responsive gene database (ERGDB) not more than 7% of the genes with significantly changed expression after isoflavone supplementation were identified as estrogen-responsive genes in both tissues. The prevailing hypothesis with regard to ER-related effects is that isoflavones preferentially bind to the ERβ in the following order of binding affinity: genistein is more potent than or comparable to equol, and these two are more potent than daidzein. Glycitein binds the least strong to the ERβ (24-26). The limited estrogen-responsive effects observed in our studies can be explained by multiple reasons. Firstly, PBMCs are not particularly known as estrogen-responsive, while adipose tissue is, albeit not as estrogen-responsive as breast and uterus tissue (12, 13). Secondly, expression levels of the ERs are suggested to be different in postmenopausal compared to premenopausal women (20-22), because postmenopausal women produce little endogenous estradiol (23). Expression might potentially change by epigenetic control. To check this, expression of ERα and ERβ was verified in both PBMCs and WAT of post- and premenopausal women. Although the number of available samples in each group was low (n = 26 in total, 4-8 per group), there was no indication of a difference in ER expression between pre- and postmenopausal women and between PBMCs and WAT (unpublished results). Thirdly, isoflavones might not predominantly induce estrogen-responsive effects, but mainly affect other molecular pathways. This might be in line with

the extensive phase II metabolism that isoflavones undergo and by which they are mainly bound to glucuronide or sulfate esters in the systemic circulation (27). This means that only 1-2% of the isoflavones is present as aglycones which are able to structurally bind to the ERs (19, 28). Therefore, the question remains which other molecular pathways are induced by isoflavones and could potentially explain the effects in the other 93% of the affected genes, observed in our studies. Animal and in vitro experiments showed that next to the ERs, expression of the progesterone receptor (PR), androgen receptor (AR), Vitamin D receptor (VDR) and Retinoic acid receptor (RAR) was changed after exposure to isoflavones (29). These effects were mostly related to estrogen-responsive effects, such as presence of estrogen-responsive elements (ERE) in promotor sequences of PRs and VDR, or through ER-dependent mechanisms, which is the case for the AR. With regard to human health effects, both peroxisome proliferator-activated receptors alpha (PPARα) and PPARγ are known to be induced by isoflavones (30, 31). PPARs can induce mRNA transcription via heterodimerization with the retinoid X receptor and involvement of the liver X receptor (LXR) after exposure to isoflavones. Expression of the PPARα and LXR genes were significantly reduced in PBMCs of equol-producing women and in adipose tissue of non-producers after LG isoflavone supplementation. According to GSEA, PPARα target genes were significantly reduced in these groups and also in adipose tissue of equol producers taking the LG supplement. So, activation of PPAR by isoflavones might explain part of the observed effects on both energy metabolism and inflammation in our studies. Lastly, also the aryl hydrocarbon receptor (AhR) is known to be induced after exposure to isoflavones which might explain the observed effect of isoflavones on cell cycle (30). The exact involvement of other nuclear receptors, molecular pathways and signalling routes cannot be elucidated from the results of the two intervention studies, but might explain the differences in effects between the two isoflavone supplements and between the equol-producing phenotypes. Finally, other factors might influence molecular effects of isoflavones, like production of other gut-metabolites of daidzein such as O-desmethylangolensin (O-DMA, (32)), potential ER polymorphisms (33, 34) and DNA methylation of certain genes (35).

Our intervention studies vs. literature

To our knowledge, in two parallel intervention studies the effects of isoflavones on whole-genome gene expression in PBMCs of healthy postmenopausal women were earlier examined (9, 36), while this has not been published for adipose tissue. Niculescu *et al*. clearly observed a difference in effects after intake of a high dose (~900 mg/day) genistein-rich supplement for 84 days between equol producers (2 in placebo and 5 in supplement group)

and non-producers (10 in placebo and 11 in supplement group). These effects were observed on cell differentiation, cAMP signaling, G-protein-coupled metabolism and steroid hormone activity (9). The study of Wang *et al*. observed marginal effects after an 8 week intervention with 50 mg isoflavones (composition not defined) in 20 participants vs. 18 participants in the control group on genes related to nicotinamide phosphoribosyltransferase (NAMPT), Fc γ R-mediated phagocytosis and cytokine interactions (36). In comparison, our studies were crossover studies in which equol producing phenotype of the participants ($n > 24$) and isoflavone content of the supplements were well characterized. Furthermore, in our studies gene chips with ~20,000 genes were used to study the effects on gene expression. Although dose levels and study size are very different, some of the effects observed in these studies resemble effects observed in our studies, like effects on cell differentiation and cytokine interactions.

Beneficial or adverse molecular effects?

The suggested beneficial effects on gene expression related to energy metabolism and inflammation in both PBMCs and adipose tissue, and cell cycle in PBMCs in our studies, are in line with results from human intervention studies. In literature, marginal beneficial effects are generally suggested on osteoporosis, cardiovascular disease, adipose tissue distribution and cancer risk (see introduction). Our studies showed that at the higher end of a physiological relevant dose of ~100 mg isoflavones/day, no indications were observed for adverse effects on gene expression. It should be noted that gene expression effects as such cannot predict beneficial or adverse effects on physiological or clinical health outcomes. This would require that the link between gene expression effects and health effects should be clearly established. To this end, studies with longer duration are needed, such as the 5-year study by Unfer *et al*. (37), in which both health endpoints and gene expression need to be studied.

RISK ASSESSMENT

The relation between isoflavone intake and exposure markers in humans

In chapter 4, plasma concentrations of daidzein, equol, genistein and total isoflavones after isoflavone intake were assessed by a log-linear regression model in which equol-producing phenotype was used as a covariate. For this model, data were used from 103 postmenopausal women, who participated in one of the two intervention studies or in

a similar study with soy foods. The results indicated a large degree of inter-individual variation which was in line with literature. This could originate from different sources, such as differences in uptake efficiency and metabolism of isoflavones. Also, microbiota or genetic variation in transporter genes might play a role in this. Other studies hypothesized that fat, carbohydrate and fiber intake could explain inter-individual variation, but taking into account background dietary intake in our model did not confirm this hypothesis.

The relation between isoflavone intake and gene expression effects in human and rats

In chapter 5, gene expression effects in PBMCs and adipose tissue of postmenopausal women and ovariectomized (OVX) F344 rats after isoflavone supplementation vs. placebo were quantified and compared. The effect size of gene expression was quantified using multivariate log-linear regression and showed a 3-4 fold larger effect size in rats than in humans. The quantification of estrogen-responsive effects and effects in biological pathways of energy metabolism, inflammation and cell cycle enabled quantification of intertissue and interspecies comparability of gene expression effects.

The parallelogram approach for improvement of risk assessment

Part of the hypothesis of the current thesis was that improvement of risk assessment can be achieved by standardization of markers of exposure and early effects across aligned human intervention studies and animal experiments (Figure 6.2). Exposure markers for the human and rat studies were measured in a standardized way using HPLC with electrochemical detection in the same laboratory with the same quality control measures. For the early effect markers, gene expression measured by whole-genome microarrays was also performed in the same laboratory and using similar data platforms.

In this thesis, isoflavone plasma concentrations were successfully modelled by intake for postmenopausal women as well as gene expression effects after isoflavone intake for both humans and rats (black arrows in Figure 6.2). Results from the latter model were used to quantify effect sizes for gene expression in PBMCs and WAT of postmenopausal women and OVX rats; moreover, intertissue and interspecies correlations were obtained for the selected gene sets. These correlations were not directly generalizable and usable for risk assessment since they were derived for two surrogate tissues and only for the case of isoflavones. However, the results suggest that this can be an important tool to further explore intertissue and interspecies similarities and might contribute to advancing use of

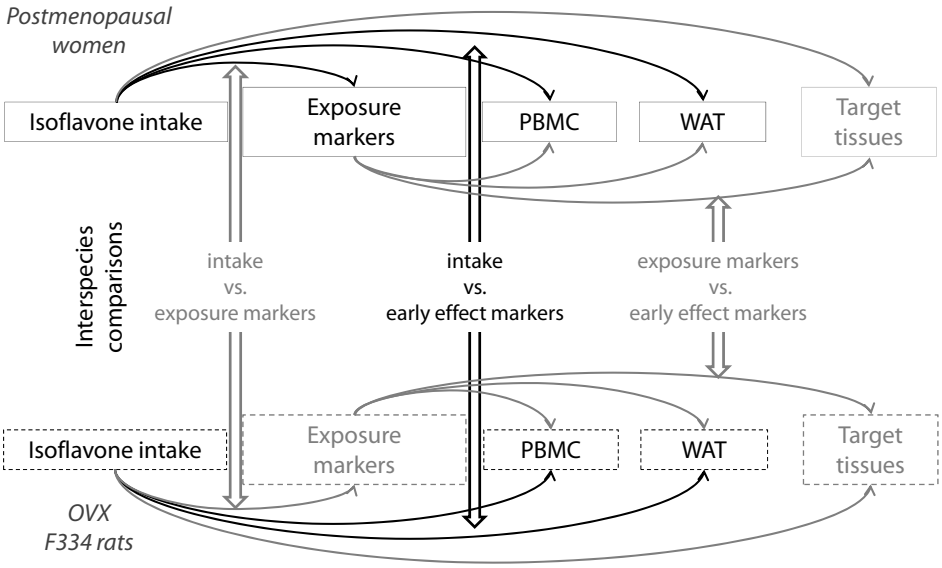

Figure 6.2 Parallelogram approach using transcriptomics for gaining insight in intertissue and interspecies differences in which the black parts represent the achieved comparisons and the grey parts represent comparisons for future research. OVX, ovariectomized; PBMC, peripheral blood mononuclear cell; WAT, white adipose tissue.

transcriptomics in the parallelogram approach for risk assessment. In addition, the log-linear regression model for explaining concentrations of exposure markers from isoflavone intake, can be used to compare this relation between species and also advance the parallelogram approach for risk assessment.

Although the standardized dose is a strength of our studies, an important disadvantage is that only one dose (~2 mg/kg bw) and one exposure time (eight weeks) were taken into account and that dose-response effects on gene expression were not considered. Furthermore, in this thesis the full parallelogram could not be completed due to data gaps, which are insufficient exposure marker data for rats and missing data on target tissues for both species (grey arrows in Figure 6.2). Exposure marker data was only available for 6 rats for one dose, which implied that models for intake vs. exposure markers could not be evaluated for rats. As known from literature, the pharmacokinetics of isoflavones in the two species are very different (28); therefore modeling of this relation for rats and the relation between exposure markers vs. early effect markers for both humans and rats would be valuable for risk assessment.

Because of the hypothesized estrogen-responsive effects, likely target tissues for isoflavone supplementation would be breast and uterus. Quantification of effects on gene expression in these tissues and the subsequent quantitative intertissue and interspecies comparison would also advance the parallelogram approach. Unfortunately, target tissues from the animal experiment were not available for our data analysis and comparable samples could not be collected in a human intervention setting from healthy women or in representative clinical settings.

Integration of toxicological and epidemiological data for risk assessment

Next to standardization and alignment of exposure and early effect markers for improvement of risk assessment, the hypothesis was that epidemiology could contribute in defining dietary intake in the population and toxicology could complement by defining disease and health endpoints (Figure 6.1). In this thesis, two human intervention studies were conducted. In addition to these studies, data from an intervention study with soy products with an intake of ~50 mg isoflavones/day was used, which enabled linear regression modelling of intake and exposure markers. However, insight in soy and isoflavone supplement consumption patterns across the population is still lacking.

Disease endpoints were not studied in the current rat experiment because for instance cancer development is hardly expected after eight weeks of exposure to isoflavones. Nevertheless, incorporating hard endpoints in the parallelogram approach is essential for improvement of risk assessment, especially since the link between the early effect markers and the hard endpoints is needed for extrapolation of effects to the human situation. Incorporation of hard endpoints could be achieved by longer animal experiments or human observational studies with sufficient participants, in a population with a large intake range and long follow-up time.

CONCLUSION

Isoflavone safety: case closed?

The two human intervention studies presented in this thesis suggested that the effects of isoflavone supplementation on gene expression in PBMCs of equol-producing and WAT equol-producing and non-producing postmenopausal women might be beneficial. The ISO II study showed that the molecular effects were highly influenced by supplement

composition and equol-producing phenotype, but both studies showed no distinct estrogen-responsive gene expression effects were present in either PBMCs or adipose tissue.

If the negative effects via the ER, observed in in vitro and animal experiments, are less relevant for the human *in vivo* situation than previously assumed and if the positive effects generally found in human intervention studies and in this thesis are taken into account, we might conclude that doses up to 100 mg/day among postmenopausal women do not raise safety concerns. However, isoflavones might still dominantly induce estrogen-responsive effects in target tissues, like breast and uterus. Moreover, in our studies conclusions could only be drawn on molecular effects and not on phenotypical, functional or disease-related endpoints. Thus, although the evidence obtained in this study does not increase safety concerns of isoflavones, the case cannot yet be closed.

Integrated risk assessment: a step forward?

With regard to integrating toxicological and epidemiological approaches for risk assessment, we showed the use of two models; one for quantifying the relation between intake and exposure markers in humans and one for quantifying the relation between intake and early effect markers as determined by whole-genome gene expression in humans and rats. The latter model also enables quantification of intertissue and interspecies similarities. Together these models and comparisons can be applied in the parallelogram approach to advance risk assessment.

Future directions

Although our studies did not support an urgent safety issue with isoflavones, long term studies to understand their beneficial or even adverse health effects are still lacking. Also, further research into the effects of isoflavone content and composition and equol producer status on the involved molecular pathways and signaling routes is recommended, especially with regard to estrogenic target tissue.

The quantitative models proposed in this thesis need to be validated for the case of isoflavones by filling the data gaps on exposure markers and target tissue in rats (Figure 6.2). Furthermore, early effect marker data in human estrogenic target tissue, such as breast and uterus, can potentially be obtained in intervention studies in a clinical setting, to further validate the quantitative model and intertissue and interspecies comparisons in the

parallelogram approach. Lastly, for the actual use of these models in risk assessment, more dose-response data is needed and determining the quantitative relation of transcriptomics as early effect markers with disease endpoints is crucial.

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Nederlandse samenvatting

Dit proefschrift beschrijft een project dat tot doel had de risicobeoordeling te verbeteren door de kennis en expertise van toxicologie en epidemiologie beter te combineren. Deze samenwerking werd in de praktijk gebracht door de veiligheidsaspecten van de consumptie van supplementen met isoflavonen te bestuderen.

Isoflavonen zijn ook wel bekend als planten-estrogenen en de belangrijkste isoflavonen zijn daidzeïne, genisteïne en glyciteïne. Deze isoflavonen komen vooral voor in soja en sojaproducten en zijn bekend vanwege hun gunstige effecten op de gezondheid zoals gevonden in epidemiologische studies in Azië waar veel soja geconsumeerd wordt. Deze gezondheidseffecten zijn onder andere minder overgangsklachten, osteoporose, hart- en vaatziekten en bepaalde soorten kanker. De hypothese achter het mechanisme van de gezondheidseffecten van isoflavonen is dat ze dezelfde structuur als estradiol hebben en daardoor aan de estrogeenreceptoren binden. Via binding aan deze receptoren worden genen tot expressie gebracht en via regulatie van genexpressie kunnen vervolgens metabole en signalering routes in het lichaam worden aan- of uitgezet. Als dit op langere termijn regelmatig gebeurt zou dit verschillende gezondheidseffecten kunnen induceren.

In westerse landen consumeert men niet zoveel sojaproducten, maar kunnen vrouwen die in de overgang zijn wel supplementen met isoflavonen gebruiken tegen overgangsklachten. Deze vrouwen consumeren dagelijks een relatief hoge dosis en de vraag is of deze supplementen bij een dergelijke hoge dosis nog steeds gunstige gezondheidseffecten hebben of dat eventuele schadelijke effecten de overhand krijgen. Vooral personen die darmbacteriën hebben die daidzeïne om kunnen zetten in het metaboliet equol, wat sterker werkt op de estrogeen receptoren dan daidzeïne, kunnen gevoeliger zijn voor de positieve maar ook de negatieve effecten van isoflavonen.

In de traditionele risicobeoordeling worden effecten uit dierstudies vaak geëxtrapoleerd naar de humane situatie, wat verschillende onzekerheidsfactoren met zich meebrengt. Dit kan verbeterd worden door de samenwerking tussen epidemiologie (humane data) en toxicologie (dierexperimentele data) te versterken. Dit kan bijvoorbeeld gedaan worden door het meten van vroege veranderingen in het metabolisme door blootstelling aan een bepaalde component, een techniek die gebruikt wordt in zowel de epidemiologie als de toxicologie. Een techniek die staat vaker gebruikt wordt om gevoelig te meten en zo vroege veranderingen te detecteren is transcriptomics, oftewel het meten van genexpressie. Dit zou de mogelijkheid kunnen geven tot beter inzicht in de onzekerheidsfactoren binnen de risicobeoordeling.

In dit proefschift worden eerst de gezondheidseffecten van isoflavonen onderzocht in twee interventiestudies. Dit wordt gevolgd door de toepassing van deze data voor de verbetering van de risicobeoordeling.

In **hoofdstuk 2** wordt een humane interventiestudie beschreven waarin 30 postmenopausale vrouwen een isoflavonensupplement hebben geconsumeerd. Dit supplement bevatte relatief veel daidzeïne en weinig genisteïne (LG-supplement). Deze vrouwen waren zo geselecteerd dat ze allemaal equol konden aanmaken uit daidzeïne. De genexpressie profielen in witte bloedcellen (PBMCs) lietem effecten van het supplement zien op inflammatie, oxidatieve phosphorylering en de celcyclus. In deze witte bloedcellen werden geen effecten gevonden op genen die gereguleerd worden door de oestrogeenreceptor en ook niet op andere groepen genen (gensets van de 'gene set enrichment analysis' of GSEA) geassocieerd met deze effecten. De genexpressie-effecten die we in deze studie vonden zouden kunnen wijzen op potentiële positieve effecten van isoflavonen op het ontstaan van kanker en hart- en vaatziekten, maar deze studie betrof genexpressie-effecten op witte bloedcellen, dus effecten op andere organen moeten nog beter bestudeerd worden.

In **hoofdstuk 3** wordt een andere humane interventiestudie beschreven waarin 58 postmenopauzale vrouwen een supplement met relatief veel daidzeïne en weinig genisteïne (LG-supplement) of een supplement met evenveel daidzeïne als genisteïne (HG-supplement) slikten. De gezondheidseffecten van deze supplementen werden onderzocht door te kijken naar genexpressieveranderingen in vetweefsel. De resultaten lieten zien dat beide supplementen effecten hebben op energiemetabolisme en inflammatie, maar dat zowel het effect als de richting van het effect erg afhankelijk zijn van de samenstelling van het supplement en van de vraag of de deelnemers equol konden aanmaken. Consumptie van het LG-supplement resulteerde in effecten op het energiemetabolisme die erg leken op de effecten van calorische restrictie. Daarnaast veroorzaakten beide supplementen veroorzaakten anti-inflammatoire effecten in het vetweefsel van postmenopauzale vrouwen die equol konden aanmaken.

In **hoofdstuk 4** wordt er een start gemaakt met de verbetering van de risicobeoordeling. In dit hoofdstuk worden de plasmawaarden na isoflavonenconsumptie van de twee hierboven beschreven studies gecombineerd met vergelijkbare data van een studie met sojaproducten. Deze plasmawaarden en de isoflavoneninname werden gemodelleerd volgens een loglineare curve. Uit deze modellering werd duidelijk dat er grote verschillen zijn in de plasmawaarden tussen de verschillende personen. Bovendien bleef deze variatie bestaan als we het model corrigeerden voor de voedingsinname van de deelnemers.

In **hoofdstuk 5** worden de genexpressiedata van de twee interventiestudies gecombineerd met genexpressiedata van een dierstudie. Meer specifiek betrof het data van PBMCs en vetweefsel van postmenopauzale vrouwen en PBMCs en vetweefsel van ratten zonder eierstokken (zogenaamde geovarectomiseerde ratten, ook postmenopauzaal) na consumptie

van hetzelfde supplement, namelijk het supplemen dat rijk was aan daidzeïne en arm in genisteïne (LG-supplement). Deze data werd met elkaar vergeleken door 'gene set enrichment analysis', oftewel GSEA, waarin bestaande gensets worden vergeleken met de genexpressie door isoflavonen. Daarnaast werd de grootte van de genexpressie-effecten gekwantificeerd in een multivariaat model. In beide weefsels, van zowel de vrouwen als de ratten, werden genexpressie-effecten gevonden op energiemetabolisme, inflammatie en celcyclus. Voor deze effecten werd ook een kwantitatieve vergelijking van genexpressie-effecten tussen PBMCs en vetweefsel en tussen vrouwen en ratten gedaan. Daardoor is dit model een belangrijk hulpmiddel in het gebruik van transcriptomics voor de risicobeoordeling.

Beide humane interventiestudies resulteerden in aanwijzingen voor positieve gezondheidseffecten van het gebruik van supplementen met isoflavonen. Deze effecten worden beïnvloed door de samenstelling van het supplement en door het al dan niet kunnen omzetten van daidzeïne in equol. In beide studies werd gevonden dat de effecten via de estrogeenreceptor minder sterk waren dan verwacht op basis van binding aan de estrogen receptor en de daarop volgende gentranscriptie activatie. Op basis hiervan zou geconcludeerd kunnen worden dat er tot een dosis van 100 mg/dag geen negatieve gezondheidseffecten te verwachten zijn van het gebruik van deze isoflavonensupplementen. Wel dient opgemerkt te worden dat we alleen moleculaire effecten op twee weefsels bestudeerd hebben en dat het mogelijk is dat effecten van isoflavonen op andere weefsels anders en misschien zelfs ongunstig uitpakken. In de literatuur ontbreken studies die de effecten van isoflavonen op langere termijn (> 5 jaar) bestuderen. Daardoor is het, ook al vinden wij geen aanwijzingen voor negatieve effecten van isoflavonen, niet mogelijk om een definitief oordeel te geven over de veiligheid van isoflavonen op basis van onze studies.

Met betrekking tot de risicobeoordeling geeft dit proefschrift richting aan de samenwerking tussen toxicologie en epidemiologie door het gebruik van modellen om genexpressiedata te integreren. Deze benadering kan in de toekomst worden gebruikt om genexpressiedata te kunnen vergelijken en te gebruiken voor de risicobeoordeling. Ook met betrekking tot isoflavonen, kan deze benadering nog verder worden uitgebreid naar zogenaamde doelwitorganen van estrogene activiteit, zoals borst- en baarmoederweefsel. Dit weefsel kan zowel bij mensen, in een klinische setting, als bij dieren verzameld worden na blootstelling aan isoflavonen. Voor uiteindelijk gebruik van deze modellen voor de risicobeoordeling is het nodig om meer dosiseffectdata te verzamelen en is het van belang dat de genexpressie-effecten worden vertaald in daadwerkelijke gezondheidseindpunten.

Dankwoord
(Acknowledgements)

Nou, dit was het dan, 4 jaar onderzoek in 160 pagina's. Zelf geschreven, maar zeker niet alleen gedaan.

Ik wil beginnen met het bedanken van mijn begeleiders, die het brein waren achter dit ambitieuze project.

Pieter, bedankt voor je visie op de integrated risk assessment en je geduld in het uitleggen van alle epidemiologische modellen. Ook je gevoel voor mensen was een hele waardevolle toevoeging aan het project.

Evert, het was heel fijn dat je ook na je pensioen tijd vond om bij dit project betrokken te blijven. Je bijdrage kwam altijd goed van pas en het was prettig om met je samen te werken.

Anouk, bedankt voor je hulp met het opzetten van de studies en je goede bijdrage aan mijn papers, dankzij jou zijn ze een stuk leesbaarder geworden.

Lydia, super leuk dat je op het einde toch nog mijn promotor bent geworden. Bedankt voor de positieve benadering die je altijd met je meebracht.

Naast mijn begeleiders waren er een aantal anderen dicht betrokken bij het project. Peter, bedankt voor je inhoudelijke betrokkenheid en het ter beschikking stellen van de HPLC. Dini, bedankt voor alle technische ondersteuning daarbij. Ik vond het erg fijn om met jullie samen te werken! Cajo, het was goed om een expert aan boord te hebben om mee te sparren over de multivariate modellen, bedankt voor de goede samenwerking.

Rolaf and Arif, thank you for the close collaboration on this project. It was a real challenge to actually improve of risk assessment, but together we made a good start.

Jacqueline, bedankt voor je betrokkenheid bij dit project vanuit de NVWA. Ik hoop dat jullie met deze thesis een stapje verder zijn gekomen naar de verbetering van de risicobeoordeling.

Dan zijn er de opponenten die mijn thesis hebben gelezen en beoordeeld.

Wendy, although we could not proceed with the grant proposal we were working on, I'm really happy that you took the time to read my thesis and to come to the Netherlands to be one of the opponents.

Jaap Keijer, Yvonne van der Schouw en Hubert Noteborn, bedankt voor het doorlezen en beoordelen van mijn thesis en fijn dat jullie aanwezig willen zijn om te opponeren.

Er zijn nog een hele hoop anderen die betrokken zijn geweest bij de humane interventies en andere zaken rond deze thesis. Allereerst wil ik Anita en Jantien bedanken voor hun betrokkenheid bij beide interventiestudies. Ook mijn studenten hebben hier een grote

bijdrage aan geleverd. Sylvia, Jentina, Lidiya, Sabina, Xenia, thank you so much for the work you did during the two intervention studies. Ineke, bedankt voor je werk aan de HPLC method en leuk dat je daarna nog eens terug wilde komen voor labwerk in de studie. Mechteld en Shohreh, bedankt voor al jullie hulp in het lab bij NMG en met alle studenten, maar het was natuurlijk vooral heel gezellig! Karen, Jacqueline en Gabriëlle, bedankt voor alle dingen die jullie hebben geregeld en de gezelligheid op het secretariaat op de derde. Dr. Guido en Dr. Mark, bedankt voor jullie hulp met de arrays, het omcoderen van genen en natuurlijk alle andere zaken die erbij kwamen kijken ;).

Dan niet te vergeten mijn collega's. Linde, Eef, Martinette, Janne en Cornelia, bedankt voor de gezelligheid op de derde en vierde, de (blinde) lunchwandelingen, kaas, eeh, visfondues en Sinterklaas in Londen. Renate, het was fijn om je als kamergenoot te hebben, en de gezellige gezamelijke etentjes met Anne en Elske waren altijd leuk! Annemarie, de loopjes waren erg verfrissend en Franzel en Dieuwertje, bedankt voor al jullie carrière-technische adviezen.

Ik was ook regelmatig bij NMG te vinden, waar ik altijd een warm welkom kreeg. Milène, bedankt voor je eeuwige vrolijkheid en ik zal onze -20°C ervaring in Kuopio nooit vergeten. Diederik, bedankt voor het sparren, squashen en het voor eens en altijd duidelijk maken dat ik niet moet drinken met grote mannen. Rinke, bedankt voor je hulp met de vet samples. Oh, eeh, trouwens… Wieberen!

Michiel, het was altijd dolle boel tijdens de practica van Food en Pharma. Noortje, je bent alweer een tijdje weg, maar we hebben je erg gemist als katalysator.

En dan probeerde ik er ook nog een sociaal leven op na te houden, waaraan ook een hoop mensen een bijdrage hebben geleverd. Mijn bestuur, met hun niet-aflatende steun, enerverende en relativerende teksten tijdens borrelavonden of weekendjes weg. De miepjes, die overal in Nederland lekker carrière aan het maken zijn, maar altijd bereid zijn om te reizen voor gezelligheid. De W10 oud-huisgenootjes, die ook allemaal lekker zijn gaan PhD-en, het was superfijn om jullie regelmatig te zien in Wageningen of in de UK. Mijn hardloopbuddies, voor de dinsdagavonden, weekenden en halve marathons.

Tom, supergaaf dat jij de uitdaging aan wilde om onze welbekende formule om te draaien, ik voor de schermen, jij achter de schermen, ik ben benieuwd hoe we het ervan afbrengen. Inger, jij bent vier jaar lang een luisterend oor geweest voor alle ups en downs met betrekking tot mijn thesis en honderdduizend andere dingen. Supertof dat jullie mijn paranimfen zijn!

Pap en mam, wie had 29 jaar geleden gedacht dat één van jullie kinderen een proefschrift zou schrijven! Bedankt voor alle steun, weekendjes uitrusten en de schrijfdagen thuis, waarbij jullie lekker de lunch verzorgden! Inge, groot zusje, vier jaar na het begin van onze weddenschap, toch écht allebei weer een mooi diploma op zak. Nu zijn we allebei Dr. ☺

Antoine, thank you for supporting me through my PhD and even more for calming me down from time to time when I was taking it too seriously. The cover looks great, thanks for designing it for me! I hope that now we live in the same country, we can finally start enjoying our life together. Je t'aime!

About the author

CURRICULUM VITAE

Vera van der Velpen was born in Zwolle on the 21st of August 1985. After she finished high school (gymnasium) in 2003, she went to Wageningen to start her BSc studies in Nutrition and Health. She proceeded with a 2 year master in Food Safety, during which she was a fulltime member of the board of the Wageningen Rowing Club Argo for 1 year. After her MSc internship at Nestlé in Lausanne, she started her PhD at the department of Nutrition and Health, which resulted in the current thesis. In 2013, she won the Foppe ten Hoor award during the Dutch Nutritional Science Days and a Young Investigator award during the Polyphenols for Health Conference in Buenos Aires. In March 2014, she started her new job as a Senior Research Associate at the Nutrition Department at the University of East Anglia in the United Kingdom.

PUBLICATIONS

Vera van der Velpen, Anouk Geelen, Peter C. Hollman, Evert G. Schouten, Pieter van 't Veer and Lydia A. Afman. *Isoflavone supplement composition and equol producer status affect gene expression in adipose tissue: A randomized controlled trial in postmenopausal women*. Submitted - 2014.

Vera van der Velpen, Peter C. Hollman, Monique van Nielen, Evert G. Schouten, Marco Mensink, Pieter van 't Veer and Anouk Geelen. *Large inter-individual variation in isoflavone plasma concentration limits use of isoflavone intake data for risk assessment*. Accepted for publication, European Journal of Clinical Nutrition - 2014.

Vera van der Velpen, Anouk Geelen, Evert G. Schouten, Peter C. Hollman, Lydia A. Afman and Pieter van 't Veer. *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*. Journal of Nutrition - 2013.

Lídia J.R. Lima, Vera van der Velpen, Judith Wolkers-Rooijackers, Henri J. Kamphuis, Marcel H. Zwietering and M.J. Rob Nout. *Microbiota dynamics and diversity at different stages of industrial processing of cocoa beans into cocoa powder*. Applied and Environmental Microbiology - 2012.

PUBLICATIONS IN PREPARATION

Vera van der Velpen, Anouk Geelen, Mohammed A. Islam, Cajo J.F. ter Braak, F.X. Rolaf van Leeuwen, Lydia A. Afman, Peter C. Hollman, Evert G. Schouten and Pieter van 't Veer. *Quantitative comparison of gene expression profiles of humans and rats after isoflavone supplementation*.

Mohammed A. Islam, Guido J.E.J. Hooiveld, J.H. Johannes van den Berg, Mark V. Boekschoten, Vera van der Velpen, Albertinka J. Murk, Ivonne M.C.M. Rietjens, F.X. Rolaf van Leeuwen. *Dietary isoflavones: bioavailability and gene expression profiling in female ovariectomized rats*.

EDUCATIONAL ACTIVITIES

Year	Course	Location
2010	Applied Data Analysis	Wageningen
	Concepts and Methods in Epidemiology	Wageningen
	Exposure Assessment	Wageningen
	Master class Statistics	Wageningen
	Nutritional Science Days	Deurne
	PhD Week	Baarlo
	Preparing PhD research proposal	Wageningen
2011	NUGO week	Wageningen
	Nutrigenomics in Clinical Interventions	Kuopio
	Nutrition and Lifestyle Epidemiology	Wageningen
	Nutritional Science Days	Deurne
	PhD tour	Mexico and US
	Scientific Writing	Wageningen
	Teaching and Supervising thesis students	Wageningen
2012	Interdisciplinary communication	Wageningen
	NUGO week	Helsinki
	Nutritional Science Days	Deurne
	Risk Assessment and Management	Utrecht
2013	Career perspectives	Wageningen
	International Conference on Polyphenols and Health	Buenos Aires
	NUGO week	Munich
	Nutritional Science Days	Deurne

The studies presented in this thesis were performed with financial support from the Netherlands Food and Consumer Product Safety Authority (NVWA).

Financial support from Wageningen University for printing this thesis is gratefully acknowledged.

Cover design Antoine Goupil de Bouillé
Layout Renate Siebes, Proefschrift.nu
Printed by Ridderprint, Ridderkerk