**A risk assessment-driven quantitative comparison of gene expression profiles in PBMCs and white adipose tissue of humans and rats after isoflavone supplementation**

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List of abbreviations
ER – estrogen receptor
ERGDB – estrogen responsive gene database
GSEA – gene set enrichment analysis
OVX – ovariectomized
PBMC – peripheral blood mononuclear cells
PCA – principal component analysis
RDA – redundancy analysis
RMSE - root mean square effect
SES - summary effect size
WAT – white adipose tissue

**Research Highlights:**

- Gene expression data from one animal experiment and two human intervention studies were quantitatively compared
- A multivariate model was used to estimate effect sizes of gene expression in PBMCs and WAT of both species
- Intertissue correlations between PBMCs and WAT were significant for estrogen-related genes in both humans and rats
- Interspecies correlations were significant for oxidative phosphorylation in PBMCs and estrogen-related genes in WAT
- Estimation of gene expression effect size and correlations between tissues and species can be useful for risk assessment

## Abstract

Quantitative insight into species differences in risk assessment is expected to reduce uncertainty and variability related to extrapolation from animals to humans. This paper explores quantification and comparison of gene expression data between tissues and species from intervention studies with isoflavones.

Gene expression data from peripheral blood mononuclear cells (PBMCs) and white adipose tissue (WAT) after 8wk isoflavone interventions in postmenopausal women and ovariectomized F344 rats were used. A multivariate model was applied to quantify gene expression effects, which showed 3 to 5-fold larger effect sizes in rats compared to humans. For estrogen responsive genes, a 5-fold greater effect size was found in rats than in humans. For these genes, intertissue correlations (r=0.23 in humans, r=0.22 in rats) and interspecies correlation in WAT (r=0.31) were statistically significant. Effect sizes, intertissue and interspecies correlations for some groups of genes within energy metabolism, inflammation and cell cycle processes were significant, but weak.

Quantification of gene expression data reveals differences between rats and women in effect magnitude after isoflavone supplementation. For risk assessment, quantification of gene expression data and subsequent calculation of intertissue and interspecies correlations within biological pathways will further strengthen knowledge on comparability between tissues and species.



**Key words:** Risk assessment, gene expression, species and tissue differences, quantitative evaluation, isoflavones, multivariate model

## 1. Introduction

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 soy consumption was associated with lower incidence of several types of cancer, osteoporosis and cardiovascular disease (Messina, 2010). However, results from in vitro and animal studies still raise doubts about their safety (Andres et al., 2011; Assessment, 2007; Wuttke et al., 2007), especially because of the putative activation of estrogen receptors (ER). Recently, we showed that the ER might not be the main transcription factor responsible for the induced gene expression effects of isoflavones in human peripheral blood mononuclear cells (PBMCs) and adipose tissue (van der Velpen et al., 2013; van der Velpen et al., 2014). Also, it has been shown that the proportions of the more active free isoflavones in the circulation were markedly higher (20-150 times) in rodents than in humans (Setchell et al., 2011). Furthermore, over the past few years human studies have not confirmed the adverse effects found in *in vitro* and in animal studies, except for a disputable adverse effect related to endometrial thickness (Unfer et al., 2004), which was not confirmed in more recent studies (Alekel et al., 2014; D'Anna et al., 2007; Palacios et al., 2007; Palacios et al., 2010). Altogether, this further raises the question of the applicability of animal models to predict effects of isoflavones in humans.

In risk assessment, potential adverse health effects in humans are generally extrapolated from animal experiments, which causes one of several sources of variability and uncertainty in risk assessment (Abt et al., 2010). Application of new techniques, like quantitative structure-activity relationship modelling, physiologically based biokinetic modelling and transcriptomics, might enable better quantification of interspecies differences (Pettit et al., 2010). Especially the use of transcriptomics is promising, as this data can be derived in a similar manner from animal experiments and human studies, enabling direct comparison. Furthermore,

transcriptomics can be considered a powerful tool for detection of early effect markers, especially when changes in gene expression are considered within biological pathways (Elliott et al., 2007) with e.g. gene set enrichment analysis (GSEA (Subramanian et al., 2005)). Several studies have demonstrated the use of gene expression in the risk assessment of e.g. dibutyl phtalate (Euling et al., 2013a; Euling et al., 2013b; Makris et al., 2013), coumarin (Kienhuis et al., 2009a), acetaminophen (Kienhuis et al., 2009c) and benzo(a)pyrene (Moffat et al., 2015). These often regard qualitative and semi-quantitative approaches (Bourdon-Lacombe et al., 2015), while quantitative approaches could enable better comparison between tissues and species and advance the use of gene expression in risk assessment (Burgess-Herbert & Euling, 2013; Chepelev et al., 2015) .

In this paper, we aim to quantitatively evaluate and compare gene expression data from two tissues (PBMCs and white adipose tissue (WAT)) after isoflavone interventions in women and rats using a multivariate model.

## 2. Materials and Methods

Data from two human intervention studies and one rat experiment were used, aligned for dose, duration and target group, and obtained with standardized gene expression methods.

### *2.1 Human intervention studies*

The two human studies, ISO and ISO II, were conducted at the Division of Human Nutrition of Wageningen University, approved by the Medical Ethical Committee of this university and described earlier by Van der Velpen et al. (van der Velpen et al., 2013; van der Velpen et al., 2014). Both studies, were double-blind placebo-controlled crossover studies with eight week intervention periods and eight week washout periods in between.

The ISO study determined the effects on PBMC gene expression of an isoflavone supplement in 27 equol-producing postmenopausal women. In the ISO II study, the effects of the same supplement on WAT gene expression were determined in 24 postmenopausal women, both equol producers (n=7) and non-producers (n=17). In- and exclusion criteria for both studies and the screening procedure for equol producers have previously been described (van der Velpen et al., 2013; van der Velpen et al., 2014). In brief, exclusion criteria were use of soy products or isoflavone supplements, hormone related medication, anti-inflammatory medicines, or antibiotics in the past 3 months. In addition, women with severe heart conditions, thyroid conditions, removed ovaries or prior diagnosis of cancer were excluded, as well as known alcohol and drug abuse, smoking habits, a BMI above 35 kg/m2 and self-reported allergy to soy. These studies were registered at clinicaltrials.gov under NCT01232751 and NCT01556737.

### *2.2 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. This experiment was a parallel study with eight-week-old female inbred ovariectomized (OVX) F344 rats, all equol producers, with an 8-week dosing period. For our analysis, data from 5 treated rats and 5 control rats were used. Further detailed experimental conditions are described by Islam et al. (M. A. Islam et al., 2016). Oral gavage stock of the supplement was daily and freshly prepared in 10ml water containing 1% DMSO. After eight weeks, the animals were anesthetized with a mixture of isoflurane and oxygen and blood was removed from the dorsal aorta.

*2.3 Supplements and doses*

All 3 studies were performed with the same isoflavone supplements. In the ISO study a batch bought in October 2010 was used, which contained 60% daidzein, 13% genistein and 27% glycitein. In the ISO II study and the rat experiment a batch bought in November 2011 was used, which had a similar isoflavone profile and contained 56% daidzein, 16% genistein and 28% glycitein (Table 1).

The postmenopausal women in the two studies ingested ~100 mg isoflavones/day (aglycone equivalents), similar to daily intakes of over-the-counter isoflavone supplements. This dose equivalent to 1.34 (range 0.88-1.70) and 1.42 mg/kg bw/day (range 0.94-1.81). For the animal study, the intake was scaled to body weight and was 2.0 mg/kg bw/day.

*Table 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[a] | | ISO II study[a] | | Rat experiment[b] |
|---|---|---|---|---|---|
| | mg/day | mg/ kg bw/day | mg/day | mg/ kg bw/day | mg/ kg bw/day |
| Total isoflavone | 93.9 | 1.34 | 100.1 | 1.42 | 2.0 |
| Daidzin | 53.2 | 0.76 | 52.0 | 0.74 | 1.0 |
| Daidzein | 3.2 | 0.05 | 3.7 | 0.05 | 0.09 |
| Genistin | 11.4 | 0.16 | 16.5 | 0.23 | 0.33 |
| Genistein | 0.4 | 0.001 | 0 | 0 | 0.00 |
| Glycitin | 23.8 | 0.34 | 26.3 | 0.37 | 0.52 |
| Glycitein | 2.0 | 0.03 | 1.5 | 0.02 | 0.05 |

*a) In the human intervention studies, the absolute amount per day was the same for every participant; the dose per kg bw was calculated on the basis of individual bw of the participants.*

*b) In the rat experiment, the dose per kg bw was similar for each rat*

*2.4 Transcriptomics*

In the human studies, gene expression after both supplement and placebo treatment was measured in PBMCs (n=27, all equol producers) and WAT (n=24; 7 equol producers and 17 non-producers) by Affymetrix human gene 1.1 ST arrays (van der Velpen et al., 2013; van der Velpen et al., 2014). In the rat experiment, the gene expression in PBMCs and WAT (n=5 for the control and n=5 for the treated group, all equol producers) was measured by Affymetrix rat gene 1.1 ST arrays.

*2.5 Data analysis*

Data analysis of the Affymetrix chips was described before for the human studies (van der Velpen et al., 2013; van der Velpen et al., 2014) and the animal study (M. A. Islam et al., 2016). In brief, expression values of the data were calculated using the robust multichip average (RMA) method and normalised using quantile normalisation in MADMAX (Bolstad et al., 2003; Irizarry et al., 2003; Lin et al., 2011).

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.

Due to the design differences in the human and the rat study, changes in gene expression in humans should be correctly indicated as 'significantly changed expression of genes', meaning the difference in response between start and end of the intervention. In rats, the difference in response between treated and control rats would be correctly named as 'significantly different expressed human homologs'. However, for readability of this article, we will refer to all of these as 'significantly changed genes'.

To study whether effects on gene expression in humans were similar to rats, significantly changed genes in PBMCs (n=27) and WAT (n=7) from equol-producing postmenopausal

women were compared to the significantly changed genes in PBMCs (n=5) and WAT (n=5) of OVX F344 rats. 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 (false discovery rate (FDR) < 0.25) were visualised using Cytoscape.

To study alleged estrogen-responsive effects of isoflavone supplementation, the significantly changed human genes (separate for equol producers and non-producers in human WAT) and significantly changed rat genes were compared to the human estrogen-responsive genes registered in the estrogen-responsive gene database (ERGDB, data downloaded September 2013, (Tang et al., 2004)).

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 human PBMC and WAT dataset were matched in advance to account for missing genes, which resulted in removal of some genes from each dataset. This process was also performed for the rat PBMC and WAT dataset. The multivariate analysis was performed using redundancy analysis (RDA), also known as least-squares reduced-rank regression (Davies & Tso, 1982; Legendre & Legendre, 2012). Like principal component analysis (PCA), RDA can handle any number of response variables (ter Braak & Šmilauer, 2012), 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 different tissues (PBMCs and WAT). 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 (Wilkinson & Rogers, 1973):

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

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

Supplement and tissue have two levels each; supplement 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 the control:

[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 (ter Braak & Šmilauer, 2012), 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 significantly changed genes were used as dependent variables in the multivariate analysis; all significantly changed genes in 1) human PBMCs, 2) human WAT, 3) rat PBMCs and 4) rat WAT. Another four gene sets relating to different biological pathways were used as well; 5) significantly changed genes from the ERGDB in both species, 6) oxidative phosphorylation (OXPHOS) genes present in the biological pathway of energy metabolism, 7) interleukin genes from inflammation pathways, 8) mitotic cell cycle genes from cell cycle-related pathways.

## 3 Results

The overlap between gene expression effects in PBMCs of equol-producing postmenopausal women (339 genes) and PBMCs of rats (540 genes), was only 12 genes. Between gene expression effects in WAT of equol-producing postmenopausal women (1169 genes) and rats (1237 genes), the overlap was 86 genes (Figure 1 and Supplemental Table 1).

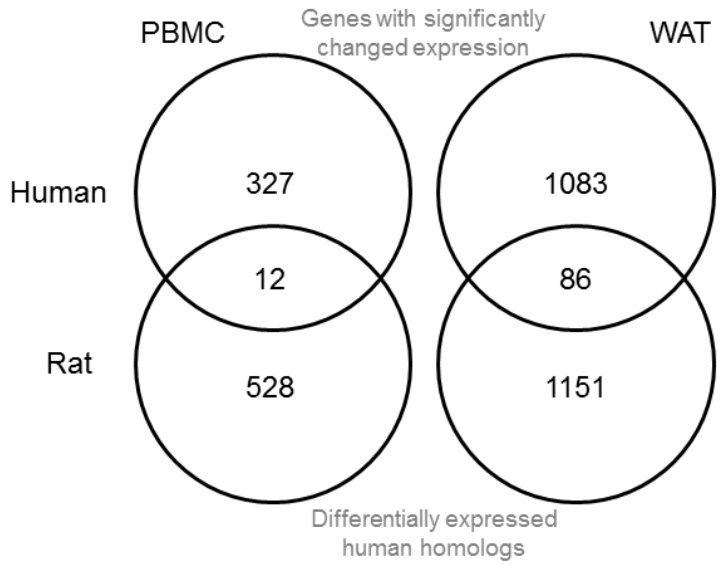


*Figure 1. Overlap between significantly changed genes in peripheral blood mononuclear cells (PBMCs, n=27) of postmenopausal equol-producing women and PBMCs of OVX F344 rat (n=10) and the overlap between these genes in white adipose tissue (WAT) of equol-producing women (n=7) and rat (n=10).*

GSEA on the gene expression data 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 2). Gene sets in both tissues which were significantly enriched, but could not be categorized in one of the previous pathways, were

specified as ‘other’. Although the gene sets in the group of ‘nuclear receptors’ are regulated in the same direction for both species and tissues, the other groups of gene sets have at least one deviating species/tissue. For example, rat PBMCs upregulated for ‘energy metabolism’, whereas all other groups were downregulated; human WAT upregulated for ‘inflammation’, but downregulated in all other groups; rat PBMCs upregulated for ‘cell cycle’, but downregulated in all other groups.

*Table 2. 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[a] | | Rat[b] | |
|---|---|---|---|---|---|
| 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 | | | *↑ | |

[a)] *GSEA on human data is performed for PBMCs with n = 27 and for WAT with n = 7, all equol producers.*

[b)] *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.*

To check the ER hypothesis, 1069 estrogen-responsive genes from the ERGDB were compared to our data, which showed that 19 of the 339 genes (5.6%) in human PBMCs were estrogen-responsive (Supplemental Table 2). In WAT of equol producers, 82 out of 1169 genes (7.0%)

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 genes (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 genes were overlapping.

Next to the previous qualitative evaluation of isoflavone effects, the overall gene expression effect was quantified. When genes with significantly changed expression in human PBMCs were used as dependent variables, the SES indicated 8.1% change of gene expression ($P<0.05$) in these PBMCs (Table 3). Similarly, a significant SES was obtained for human WAT (7.0%), which was also observed for significantly changed genes in the rat model for both PBMCs (23.9%) and WAT (34.0%). Smaller, non-significant, SES' were observed, when these analyses were performed for the tissue from which the genes were not selected (e.g. significant PBMC genes vs. WAT).

*Table 3. 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*

| Tissue | Human intervention studies[a] (Within-subject changes) | | | | Rat experiment[b] (Between-group differences) | | | |
|---|---|---|---|---|---|---|---|---|
| | $N_{genes}$[c] | PBMC[d] (%) | 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 |
| $WAT_{all\ subjects}$ | 1158 | 3.16 | 7.01* | 6.9 | 1231 | 10.9 | 34.0* | 35.1 |
| $WAT_{non\text{-}prod}$[e] | 1158 | 3.15 | 5.02 | 4.9 | | | | |

*a) Based on two human crossover intervention trials, with 27 equol producing women for PBMCs, and 24 women (7 equol producers) for WAT.*

*[b] Two groups (control and supplement) of 5 rats each, PBMCs and WAT collected from all rats.*

*[c] Number of significantly changed genes in tissues, rat genes decoded into human homologs. Number deviates from figure 1, due to matching of the datasets as described in the methods.*

*[d] 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 %.*

*[e] Analysis in 24 non-producers only for WAT*

*[*] Significance determined by the separate models for each tissue (P<0.05).*

*PBMC, peripheral blood mononuclear cell; WAT, white adipose tissue.*

The same analysis was performed on a selection of ER-related genes and the genes from the biological pathways identified using GSEA, which generated pathway-specific effect sizes of gene expression after isoflavone supplements (Table 4). This also resulted in larger effect sizes in the biological pathways of rats than of humans. For the estrogen-responsive effects, the SES was 5.5% (P<0.05) in WAT of postmenopausal women and 26.7% (P<0.05) for OVX rats. 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 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*

| Biological pathway | $N_{genes}$[a] | Human intervention studies[b] (Within-subject changes) | | | Rat experiment[c] (Between-group differences) | | |
|---|---|---|---|---|---|---|---|
| | | PBMC[d] (%) | 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 |

[a] *Group of significantly changed genes involved in biological pathways (estrogen-responsive (ER), energy metabolism (OXPHOS), inflammation (Interleukin) and cell cycle (Mitotic cell cycle))*

[b] *Based on two human crossover intervention trials, with 27 subjects for PBMCs, 24 subjects for WAT.*

[c] *Two groups of 5 rats each, all providing both PBMCs and WAT.*

[d] *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 with* $P < 0.05$ *or* * *for* $P < 0.10$

*PBMC, peripheral blood mononuclear cell; WAT, white adipose tissue; ER, estrogen responsive; OXPHOS, oxidative phosphorylation.*

Additionally, model outputs were used to calculate correlations between the regression coefficients of the individual genes of the two tissues and species, which resulted in significant interspecies correlations for ER genes in WAT (r=0.31) and for OXPHOS genes in PBMCs (r=0.33, Table 5). Significant intertissue correlations between PBMCs and WAT were observed for ER genes in humans (r=0.23) and in rats (r=0.22), and for cell cycle genes in humans (r=0.39) and in rats (r=0.47, Table 5). For the other biological pathways, gene expression effects between the tissues and the species were not significantly correlated.

*Table 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*

| Biological pathway[a] | Intertissue correlation PBMC vs WAT[b] | | | | Interspecies correlation human vs rat[b] | | | |
|---|---|---|---|---|---|---|---|---|
| | 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) |

*[a] Group of significantly changed genes involved in biological pathways (estrogen-responsive (ER)), energy metabolism (OXPHOS), inflammation (Interleukin) and cell cycle (Mitotic cell cycle))*

*[b] 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.*

## 4 Discussion

In this study, effects on gene expression after isoflavone supplementation vs. placebo were qualitatively evaluated, further quantified and compared between PBMCs and WAT of postmenopausal women and OVX rats. Qualitative analysis revealed overlapping effects in energy metabolism, inflammation and cell cycle processes. In rats, the magnitude of gene expression effects after isoflavone supplementation 3 to 5-fold larger than in humans after the same intervention. A similar difference in magnitude (5-fold) was also observed when only ER genes were analysed, with significant intertissue correlations in both species (~22%) and an interspecies correlation in WAT (30%). Some significant effect sizes, intertissue and interspecies correlations were also found in biological pathways relating to OXPHOS, inflammation and cell cycle.

Qualitative evaluation with GSEA revealed more similarities in effects between rats and humans (Table 2) than direct comparison of gene expression of individual genes (Figure 1, supplemental table 1), especially on gene sets related to energy metabolism, inflammation and cell cycle. Quantification of gene expression effects was successfully performed using a multivariate model, calculating summary effect sizes (SES) for expression changes in general (Table 3) and within biological pathways (Table 4). The observed 3-5 fold larger effect size for rats compared to humans (Table 3) is in line with previously observed effects on gene expression in genetically homogeneous inbred rat strains and in genetically independent women (Sparks et al., 2005). Moreover, a previous study reported an 18-fold difference in gene expression effects between rat and human primary hepatocytes (Black et al., 2012). In our study, the observed differences could be explained by the higher proportion of free isoflavones (aglycones) in rodents than in humans (Setchell et al., 2011) as it is hypothesized that aglycone forms are more bioactive than conjugated forms (M. A. Islam et al., 2015).

The model was applied to a set of ER genes to further evaluate the hypothesis that isoflavones predominantly induce their effects via the ER, which resulted in significant effect sizes in WAT of both species. These effects were expected as WAT is known as estrogen-responsive, while PBMCs are not, despite literature suggests that they display ERs (Dahlman-Wright et al., 2006; Wend et al., 2012). Again, for ER genes the effect size in rats (26.7%) was ~5 times greater than in humans (5%). In addition, it must be noted that the percentage of estrogen-responsive genes compared to the whole set of significantly changed genes is low (<7%) in PBMCs and WAT of both species (Supplemental table 2). As the early isoflavone work started with animal experiments (Folman & Pope, 1969; Messina, 2010), these differences in effect size could explain the origin of the ER hypothesis and that we were unable to confirm ER-related effects in human PBMCs and WAT in our two intervention studies (van der Velpen et al., 2013; van der Velpen et al., 2014). The additional correlation analysis in the current study suggests a 30% correlation between ER genes in WAT of both species. Furthermore, a ~22% correlation of the gene expression effects between PBMCs and WAT was found for both species. This similarity in both species is interesting, because of the difference in study design between the rat experiment and the human trials, in which the rat PBMCs and WAT samples were collected from the same animals, while the human PBMCs and WAT samples originated from different studies.

With regard to the effect sizes and correlations of the other evaluated biological pathways, the results were not consistent. OXPHOS genes, used as a model for energy metabolism-related effects, showed no significant SES for any tissue, but did show a significant interspecies correlation for PBMCs. Interleukin genes, on the other hand, as a model for inflammation related effects, showed significant effect sizes in both rat tissues but not in human tissues, but no intertissue and interspecies correlations. Mitotic cell cycle genes showed borderline (<0.1) significant effect sizes in human WAT and rat PBMCs and WAT, and significant intertissue

correlations in both humans (r=0.39) and rats (r=0.47). This apparent ambiguity in outcomes could be explained by the selection of genes used in the analyses, but also by the roles of these genes in the different tissues; i.e. PBMCs play a key role in inflammation regulation of the body, while WAT is responsible for the regulation of energy metabolism in WAT and cell cycle is a general process in any tissue. The calculated correlations compare to the general conservation of gene expression between species, which is reported to be ~40% between human and mouse depending on tissue (Zheng-Bradley et al., 2010), and r~0.35 between rat and human (Prasad et al., 2013). In the current study, other factors could play a role in the observed species correlations, such as polymorphisms of the estrogen receptor (Hall et al., 2006; Y. Qin et al., 2014) or effects of isoflavones on DNA methylation (W. Y. Qin et al., 2009) or microRNAs (Xia et al., 2014).

To our knowledge, this is the first study to compare gene expression data from human trials with a rat experiment in which the dose, duration and target group 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 the rat experiment. Specifically, scaling for life expectancy was not applied, resulting in a similar duration for both studies (8wks). In addition, scaling for metabolic rate was not applied to calculate the dose, but for both species this was of a similar order of magnitude per kg bw (total range 0.88-1.81mg/kg bw for humans and 2mg/kg bw for rats). The difference in final dose could have contributed to, but cannot completely explain, the observed 3-5 fold difference in effect size between the two species. The presented estimates are also expected to be highly influenced by the selection of the groups of genes as representatives for the biological pathways and by removing 20% of the rat genes by recoding into human homologs. Modelling was complicated by the difference in study design for the human and rat experiment with regard to data pairing, but by choosing different covariates unbiased estimates of the effect of the

isoflavone supplement were obtained. In more detail, the calculated effect sizes relate to the baseline in each species and not to the total or residual variation, therefore the effect sizes are measured on the same scale, which enables comparison of effect size. In the current study, p-values were used to select significantly changed genes for the direct comparison between species and tissues (Figure 1), the comparison to ER genes, as well as for the model, which could have resulted in an overestimation of the similarities between the species and tissues due to the presence of false positives. Another important issue is that rats are known to be equol producers (Setchell et al., 2005), while this is only the case for 20-30% of Western populations (Setchell & Clerici, 2010). In previous studies, differences in gene expression were observed between equol-producers and non-producers (Niculescu et al., 2007; van der Velpen et al., 2014), which also applies to estrogen responsive genes (7.0% in producers, 4.4% in non-producers). However, this difference is not always addressed in extrapolation of findings from isoflavone animal studies to humans. With regard to our model this means that if effects would be stronger and specific for equol producers, the inclusion of non-producers in the WAT data may have attenuated the effect estimates in humans. Indeed, the effect size was not significant in WAT when calculated for non-producers only (n=24, Table 3) suggesting that the reported effect for the whole population (n=31) might be driven by the effect in equol producers. We could not perform this analysis for equol producers only, because of the small number in our study population.

## 5 Conclusions

In summary, our quantitative evaluation of gene expression data after isoflavone supplementation showed that the effect size is greater in rats than in humans. Furthermore, the correlations of the isoflavone supplementation effects between these species are weak, although they are statistically significant in a number of biological pathways, including the estrogen receptor pathway, known as the predominant route of biological activity of isoflavones. Our model provided quantitative data for the effect size in two tissues of two species and the correlation between these effect sizes that could be used in existing toxicological models, such as the parallelogram approach (Kienhuis et al., 2009a; Kienhuis et al., 2009c). However, to complete and verify this parallelogram approach and to draw a conclusion regarding possible health effects of isoflavones, data on gene expression in target tissue, i.e. breast and uterus, and dose-response data would be needed. While the use of gene expression data in risk assessment is increasing, and discussions are still ongoing regarding the most appropriate use of this data (Bourdon-Lacombe et al., 2015; Burgess-Herbert & Euling, 2013), further quantitative evaluation of similarities and differences between tissues and species could be a step forward towards reducing uncertainty and variability in risk assessment practices.

In conclusion, our model enabled quantitative comparison of gene expression data that could be used to further explore effect correlations between tissues and between species for risk assessment, in addition to the currently applied qualitative evaluation of gene expression effects within pathways or gene sets.

## Author contributions

The authors' responsibilities were as follows-VvdV, AG, LAA, PCH, FXRvL, MAI, EGS, and 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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## Conflict of interest

None of the authors declared a conflict of interest.

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