Soy supplementation: Impact on gene expression in different tissues of ovariectomized rats and evaluation of the rat model to predict (post)menopausal health effect

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\textbf{ABSTRACT}

This toxicogenomic study was conducted to predict (post)menopausal human health effects of commercial soy supplementation using ovariectomized rats as a model. Different target tissues (i.e. breast, uterus and sternum) and non-target tissues (i.e. peripheral blood mononuclear cells (PBMC), adipose and liver) of ovariectomized F344 rats exposed to a commercially available soy supplement for eight weeks, were investigated. Changes in gene expression in these tissues were analysed using whole-genome microarray analysis. No correlation in changes in gene expression were observed among different tissues, indicating tissue specific effects of soy isoflavone supplementation. Out of 87 well-established estrogen responsive genes (ERGs), only 19 were found to be significantly regulated (p < 0.05) in different tissues, particularly in liver, adipose and uterus tissues. Surprisingly, no ERGs were significantly regulated in estrogen sensitive breast and sternum tissues. The changes in gene expression in PBMC and adipose tissue in rats were compared with those in (post)menopausal female volunteers who received the same supplement in a similar oral dose and exposure duration in human intervention studies. No correlation in changes in gene expression between rats and humans was observed. Although receiving a similar dose, in humans the plasma levels expressed as total free aglycones were several folds higher than in the rat. Therefore, the overall results in young ovariectomized female F344 rats indicated that using rat transcriptomic data does not provide a suitable model for human risk or benefit analysis of soy isoflavone supplementation.

\textbf{1. Introduction}

The advances in health care and the public initiative towards healthy living have increased the number of women in the (post)menopausal age group worldwide. The menopause transition results in declining ovarian functions and leads to significant hormonal changes in the female body, in particular in the reduced production of the female hormone estrogen. As a result women in this age group face several physical and mental difficulties including hot flashes, night sweats, vaginal atrophy, and an increased risk of developing osteoporosis. Menopause is inevitable and many women during their menopausal period take hormone replacement therapy (HRT) or use soy based dietary supplements. These soy based supplements contain phenolic plant compounds called isoflavones \cite{1,2} which have structural similarities with estradiol \cite{3}. Despite inconsistencies among the available data, there is growing evidence supporting the notion that soy isoflavones are effective in the reduction of menopausal symptoms \cite{4,5}. Therefore, the popularity and availability of soy supplements are increasing, although concern exists within governmental and public health related organizations \cite{6,7} regarding the safety of long-term exposure to self-administered high levels of soy isoflavones, which may exceed the levels that can be obtained from the diet.

The effects of soy isoflavones are thought to be induced via estrogen receptors (ERs) of which an alpha (ER\textsubscript{α}) and beta (ER\textsubscript{β}) form exist. soy

\textbf{Abbreviations:} ERGs, estrogen responsive genes; ER\textsubscript{α}, estrogen receptor alpha; ER\textsubscript{β}, estrogen receptor beta; PBMC, peripheral blood mononuclear cells

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isoﬂavones may regulate transcription of ER target genes via the estrogen-responsive elements (EREs) present within gene promoter regions in target tissues. It has been shown that ERα activation stimulates cell proliferation, whereas ERβ activation leads to inhibition of proliferation and stimulation of apoptosis [8–10]. It is known that the relative occurrence of ERα and ERβ is tissue dependent. For example, ERα is predominant in the mammary gland, the epididymis, testis, uterus, kidney and the pitiutary gland, whereas ERβ is more present in the prostate, bladder and lungs [11–14]. When (post)menopausal women produce small amounts of endogenous estradiol, it is possible that binding of SIF from food supplements to ERs may produce estrogenic effects especially in ERB-sensitive target tissues, because soy isoﬂavones have been shown to be particularly ERβ-active [10,11]

During the past decades, the application of microarray technology has opened up new opportunities to study the effects of food and food supplements in the control of cellular processes and related health effects upon exposure to different type of compounds [15–17]. By using transcriptomic techniques it is possible to directly compare changes in gene expression, not only in different tissues of the same species, but also in similar tissues among different species. We investigated whether a transcriptomic approach using ovariectomized rats would be an adequate model to predict (post)menopausal health risks and/or beneﬁts of soy isoﬂavone supplementation. Therefore, in the rat study reported here we have investigated the changes in gene expression in different target (i.e. breast, uterus and sternum tissues) and non-target (PBMC, adipose and liver tissues) tissues of ovariectomized female F344 rats exposed to a commercially available soy supplement, corresponding to a dose of 2 mg soy isoﬂavones/kg bw (1 gavage/day) in 1% DMSO solution for seven days per week, during 8 weeks. One day before sacriﬁce the animals received an extra dose in the evening instead of a dose early next morning. This was done to make the experiment comparable with the time schedule of the two human intervention studies (see below) carried out by the Division of Human Nutrition, Wageningen University in order to facilitate direct comparison of both rat and human data after exposure to the same supplement. The dose level used (i.e. 2 mg soy isoﬂavones/kg body weight per day) provides the equivalent dose for a female adult consuming the same soy based supplement. This dose was also selected to provide a direct comparison with the human intervention studies (mentioned in Section 2.5).

2.5. Related experiments

The effects on gene expression in PBMC and adipose tissue after soy isoﬂavones intake in the present rat study were compared with the gene expression data of a previously reported short term rat study, and of two human intervention studies (see below).

2.5.1. Short term rat study

A short-term (2 days) rat experiment was performed earlier at the same animal facility centre of Wageningen University, after approval by the ethical committee on animal experimentation of Wageningen University. This short-term study, in which rats from the same strain (i.e. ovariectomized F344) were exposed to a similar soy isoﬂavone supplement, and also PBMC were collected and studied by microarray analysis, was mainly designed for quantification of the bioavailability of soy isoﬂavone [18].

2.5.2. Human intervention studies with the same commercial soy supplement

Two human intervention studies were conducted at the Division of Human Nutrition of Wageningen University [4,19]. The studies were approved by the Medical Ethical Committee of this university and registered at clinicaltrials.gov under number NCT01232751 and NCT01556737. Both of these studies were double blind cross-over studies with the exposure time of two eight week intervention periods and an eight week washout period in between. A total of 30 (post)
menopausal, equol-producing women with an average age of 61.1 ± 5.8 years were recruited for the first human intervention study in which PBMCs were collected [4]. Twenty six (post)menopausal women were recruited for the second human intervention study with the same supplement as the first study and in which adipose tissue was collected [19]. All volunteers were recruited from Wageningen or the direct surrounding areas. All participants received the same soy supplement as used in the rat studies and 2 capsules in the morning and 2 in the evening after the meal resulting in a dose of about 1.5 mg soy isoflavones/kg bw per day and a placebo treatment.

2.6. Preparation of test solutions

The commercial soy supplement, Phytosoya, was obtained from Arkopharma (Carros, France). The supplement had a total soy isoflavones content of 70.3 mg/gram supplement [21]. The content of the three glucosides; genistin, daidzin and glycitin was 7.54, 40.03 and 22.72 mg/g supplement, respectively. An oral gavage dose of 2 mg soy isoflavones/kg bw which corresponds with 0.4 mg soy isoflavones/rat was applied per day. The stock solutions were freshly prepared daily, sonicated for 30 min and were placed on a multi-axle-rotating-mixture (CAT RM-40, Laufen, Germany) machine to avoid any aggregation during the period of gavage dosing. The control rats received a gavage dose of nanopure water containing 1% DMSO.

2.7. Sample collection and analysis

After the 8 weeks dosing the animals were sacrificed after anaesthesia with a mixture of isoflurane and oxygen followed by removal of blood from the dorsal aorta with a syringe containing 0.1 mL lithium heparin. The collected blood was immediately transferred into 10 mL tubes and kept on a multi-axle-rotating mixer (CAT RM-40, Laufen, Germany). The average amount of collected blood was 5–6 mL. Collected blood samples were divided into two parts, one part (3.5 mL) for isolating PBMC and another part (about 1–2 mL) for separating plasma. The first part of the collected blood samples was used for micro array analysis and the second part was used for measuring the plasma concentration of soy isoflavone. White adipose, breast, uterus, liver, and sternum tissues were also collected. After collection the tissue samples were frozen immediately in liquid nitrogen and stored at −80 °C until further analysis. The plasma samples were prepared by centrifuging the blood for 5 min at 10,000 rpm (Biofuge centrifuge, Heraeus Sepatech, UK) and were collected in Safe-lock 0.5 mL Eppendorf tubes (Eppendorf AG, Hamburg, Germany). These samples were placed on ice during the sample collection of the in vivo experiment and stored at −80 °C until analysis.

2.8. Extraction and analysis of soy isoflavones from rat plasma

The enzymatic hydrolysis, solid phase extraction and HPLC (High Performance Liquid Chromatography) analysis of soy isoflavone in plasma samples were performed as described earlier [19,21]. To avoid inter laboratory variations among the present in vivo rat study and the human intervention studies, analysis of soy isoflavones concentrations in plasma and micro-array analysis were performed at the same laboratories using similar protocols. Averages and standard deviations of plasma soy isoflavones concentrations of the dose group and the control were calculated using Microsoft Excel.

2.9. Isolation of rat PBMC, mRNA and running microarray analysis of different tissues

Packages from the Bioconductor project [22], integrated in an online software program [23], were used to analyse the array data. Various advanced-quality metrics, diagnostic plots, pseudo images, and classification methods were used as described by Heber and Sick [24], to determine the quality of the arrays before statistical analysis. The probes on the Rat Gene 1.1 ST array were redefined using current genome information [25]. In this study, probes were reorganized on the basis of the gene definitions available in the National Center for Biotechnology Information (NCBI) Rattus norvegicus Entrez Gene database, based on the rat genome build 5.1 (custom CDF v17). Normalized gene expression estimates were calculated with the Robust Multichip Average method [26]. Subsequently, the dataset was filtered to only include probe sets that were active (i.e. expressed) in at least 4 samples, using the universal expression code (UPC) approach (UPC score > 0.50) [27]. Differentially expressed probe sets were identified by using linear models, applying moderated t-statistics that implemented intensity-based empirical Bayes regularization of standard errors [28,29].

Probe sets with p < 0.05 were considered to be significantly regulated. Changes in gene expression were related to functional changes using gene set enrichment analysis (GSEA) performed according to Subramanian et al. [30]. GSEA focuses on groups of genes that share a common biological, biochemical or metabolic function. GSEA has the advantage that it is unbiased, because no gene selection step is used. Only gene sets consisting of more than 15 and less than 500 genes were taken into account. Gene sets were derived from the Kyoto Encyclopedia of Genes and Genomes (KEGG) [31]. Effects of soy isoflavones treatment versus control were compared using ranked lists based on significance (t-values), using methods described by Plassier et al. [32].

3. Results

Fig. 1 shows an overview of the results from the microarray analysis of ovariectomized female F344 rats. After normalization and filtering of a total of 19,311 probe sets, 39–50% probe sets were retained in the different tissues. The percentage of significantly regulated (p < 0.05) genes within this data set amounted to only 1–13.6% (i.e. 74 to 1305 genes out of 7556 to 9561 robust multichip average (RMA) normalized and UPC filtered genes), and was lowest for the breast tissue (i.e. only 1%), with increasing number of genes being regulated in sternum, PBMC, liver, adipose and uterus tissues (i.e. 2.3, 6.1, 10.4, 13.6 and 13.6%, respectively). Fig. 1 also shows the number of significantly up and down regulated (false discovery rate, FDR < 0.25) biological pathways. In line with the total number of significantly regulated genes, the lowest number of significantly changed biological pathways was found in breast tissue (i.e. 68 gene sets) and the highest number in uterus tissue (i.e. 309 gene sets). In the right column of Fig. 1 the human homologous genes in PBMC and adipose tissue which were also found in the rat are indicated. These homologous genes were retrieved from the human intervention studies mentioned above [4,19]. Out of 6413 and 7731 human homologous genes, only 10 and 88 genes were significantly (p < 0.05) regulated in rat PBMC and adipose tissue, respectively.

As the effects of soy isoflavones are considered to be mostly mediated via the estrogen receptors (ERs), we investigated the number of differentially regulated ERGs in the six selected rat tissues. These genes were selected based on their occurrence in the Dragon DRGs database (http://datam.i2r.a-star.edu.sg/ergdbV2/). Table 1 shows the list of ERGs and the direction of their regulation in the different rat tissues. It is shown that out of 87 ERGs available in the specified website only 19 were found to be significantly regulated in the different tissues. The highest number of ERGs that were significantly regulated was found in liver tissue (7 ERGs) followed by adipose tissue (6 ERGs), uterus tissue (5 ERGs) and PBMC (3 ERGs). Surprisingly no ERGs were significantly regulated in breast and sternum tissues. Moreover, it appeared that there was no similarity in the pattern of regulation of the various ERGs among the different tissues.

Because most of the ERGs were not or only slightly regulated by soy isoflavone administration in different tissues of ovariectomized rat, we investigated the genes that were affected most in the three target
tissues, namely breast, uterus and sternum, by using a 'fold change (FC)' threshold. Only significantly affected genes with an FC value > 2 were selected to be investigated for their involvement in different biological processes, and the higher the FC value the more the gene is affected. Table 2 shows the significantly regulated genes, with an FC > 2. In ST, no genes were regulated with an FC value > 2. In breast tissue 14 genes with an FC > 2 were identified (i.e. 19% of the significantly regulated genes) of which 13 were up regulated, and in uterus tissue 11 genes (i.e. 1% of the significantly regulated genes) of which 9 were up regulated.

According to the information in the NCBI database the up regulated genes are involved in metabolism such as fatty acid, lipid and glucose metabolism, in stress responses such as detection of oxidative stress, responses to abiotic stimuli, defence mechanisms and immune responses such as cellular response to tumour necrosis factor, antigen binding, and intercellular signal transduction. Down regulated genes are involved in DNA and protein binding and inflammatory response. Table 3 shows the significantly regulated (p < 0.05) biological pathways (BPs) in different tissues of the present rat study, and the related human disease, as indicated in the Kyoto Encyclopaedia of Genes and Genomes (KEGG) database. The total list of biological pathways that were significantly up- or down regulated in different tissues can be found in supplementary Table 2. A total of 33 diseases related KEGG based biological pathways were found to be significantly regulated in different tissues in the present rat study. The highest number of up regulated biological pathways was found in liver tissue (i.e. 19) followed by adipose tissue (10), PBMC (7), and uterus tissue (2). Although only 5 and 3 biological pathways were significantly regulated in breast and sternum tissue, respectively, they all were up regulated, whereas in liver and adipose all the BPs were down regulated. Interestingly, when a disease related pathway was significantly up regulated in the surrogate tissues, PBMC or adipose tissue, it was mostly down regulated in one or more target tissues, and vice versa. No direct correlation was found between effects in the surrogate tissues and the target tissues. Also in the gene set enrichment scatter graphs we made to compare up and down regulated BPs in different surrogate and target tissues (results not shown), not any correlation was observed.

In Fig. 2 the gene expression patterns in PBMC of the present rat study were compared to those obtained in the short-term rat study [18]. Only 145 genes were significantly regulated in both data sets (data not shown) of which fifteen marker genes were identified to be highly correlated (deviation value 0 to ± 10) and their gene expression was significantly changed in the same direction (increased or decreased) in both studies. As can be concluded from the information included in supplementary Table 3, most of the genes thus identified are involved in biological pathways related to increase in immune response and in cell proliferation.

In the human intervention studies mentioned above, van der Velpen et al. [4,19] reported significant regulation of ERGs in human PBMC and adipose tissue following soy isoflavone supplementation. Table 4 shows the reported significantly expressed ERGs in PBMC of human volunteers that were also significantly regulated in PBMC of the present

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**Table 2**

| Gene expression analysis: | Gene set enrichment analysis: |
|--------------------------|-------------------------------|
| 61, 253, 140, 98, 940 and 954 genes significantly up regulated in BT, LT, PBMC, ST, UT and AT, respectively. | 68, 198, 143, 184, 309 and 205 genes sets were significantly up regulated in BT, LT, PBMC, ST, UT and AT, respectively. |
| 13, 566, 330, 87, 365 and 293 genes significantly down regulated in BT, LT, PBMC, ST, UT and AT, respectively. | 41, 23, 134, 95, 296 and 15 gene sets significantly up regulated (FDR<0.25) in BT, LT, PBMC, ST, UT and AT, respectively. |
| 27, 175, 9, 89, 13 and 190 gene sets significantly down regulated (FDR <0.25) in BT, LT, PBMC, ST, UT and AT, respectively. | 3 and 67 genes significantly (p<0.05) up regulated in PBMC and AT, respectively. |
| 7 and 21 genes significantly (p<0.05) down regulated in PBMC and AT, respectively. | |

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**Fig. 1.** Flow chart of the microarray analysis indicating significantly changed genes and gene sets induced in different tissues of ovariectomized F344 rats after oral gavage dosing (2 mg soy isoflavones/kg bw per day for 8 weeks) of a commercial soy supplement. The number of corresponding human homologue genes found in rats is also indicated (right hand column). AT, adipose tissue; BT, breast tissue; LT, liver tissue; PBMC, peripheral blood mononuclear cells; ST, sternum tissue and UT, uterus tissue.
Table 1
List of significantly (p < 0.05) up and down regulated estrogen-responsive genes (ERGs) in different tissues of ovariecortized rats after 8 weeks oral dosing with the frequency of one gavage dose/day of a commercial supplement (2 mg soy isoflavones/kg bw) compared with control. Up and down regulated enrichments were indicated by up and down arrow marks. A list of all ERGs can be found in the Dragon ERG database (https://web.archive.org/web/20160118215946/http://data.imr.a-star.edu.sg/ergdbV2).

| Name of the gene | PBMC | AT | UT | ST | LT |
|------------------|------|----|----|----|----|
| AR               | ↓   | ns | ns | ns | ns | absent |
| ANXA4            | ↓ns | ↑ns| ↑ns| ↑ns| ↑ns| ↓   |
| DUSP1            | ↓ns | ↑ns| ↑ns| ↑ns| ↑ns| ↓   |
| IGF1             | absent | ↑ns| ↑ns| ↑ns| ↑ns| ↓   |
| IL6R             | ↓ns | ↑ns| ↑ns| ↑ns| ↑ns| ↑   |
| IGFBP5           | absent | ↑* | ↑ns| ↑ns| ↑ns| ↑   |
| IGFBP2           | absent | ↑* | ↑absent | ↑absent | ↑absent | ↑   |
| KIT              | absent | ↑* | ↑absent | ↑absent | ↑absent | ↑   |
| NR2F6            | absent | ↑* | absent | ↑absent | ↑absent | ↑   |
| PGR              | absent | ↑* | absent | ↑absent | ↑absent | ↑   |
| PNOC             | absent | ↑* | absent | ↑absent | ↑absent | ↑   |
| PAWR             | absent | ↑* | ↑absent | ↑absent | ↑absent | ↑   |
| RARA             | ↓* | ns | ns | ns | ns | ↓   |
| RB1              | ↑ns | ↑ns| ↑ns| ↑ns| ↑ns| ↓   |
| SCARB1           | ↑ns | ↑ns| ↑ns| ↑ns| ↑ns| ↓   |
| SCP2             | ↑* | ↑ns| ↑ns| ↑ns| ↑ns| ↑   |
| TGFβ1            | ↑ns | ↑ns| ↑ns| ↑ns| ↑ns| ↑   |
| TEP1             | ↓* | ↑ns| ↑ns| ↑ns| ↑ns| ↑   |
| TIMP1            | absent | ↑* | ↑ns| ↑ns| ↑ns| ↑   |

ns = not significant.

Table 2
List of the significantly expressed (fold change > 2 and p < 0.05) genes in different target tissues of rats (negative numbers indicate down regulation).

| Tissue | Gene symbol | Description | Functions/process | Fold change |
|--------|-------------|-------------|-------------------|-------------|
| Breast | Adipoq      | Adiponecin, C1Q and collagen domain containing | Fat cell differentiation, detection of oxidative stress, fatty acid oxidation and glucose metabolism. | 4.9 |
|        | Rnase2      | Ribonuclease, RNase A family, 2 (liver, eosinophil-derived neurotixin) | A protein-coding gene involved in nucleic acid binding. | 4.5 |
|        | Mcpt11      | Mast cell protease 1-like 1 | Involved in proteolysis (protein breakdown). | 2.9 |
|        | Cpx3        | Carboxypeptidase A3, mast cell | A protein-coding gene involved in zinc ion binding | 2.9 |
|        | Thrp        | Thyroid hormone responsive | Lipid metabolism biosynthesis process. | 2.8 |
|        | Cd163       | CD163 molecule | Receptor mediated endocytosis | 2.3 |
|        | Tusc5       | Tumour suppressor candidate 5 | Involved in the response to the biotic stimulus | 2.3 |
|        | Cdo1        | Cysteine dioxygenase type I | Metabolism of cysteine | 2.3 |
|        | Fabp4       | Fatty acid binding protein 4, adipocyte | Fat cell differentiation, cholesterol homeostasis, fatty acid metabolism and cytokine production | 2.2 |
|        | Prkar2B     | Protein kinase, CAMP dependent regulatory, type 2 | Fatty acid metabolism | 2.1 |
|        | Mpz         | Myelin protein zero | Cell-cell junction maintenance | 2.1 |
|        | Af3         | Activating transcription factor 3 | Involved in DNA and protein binding. | –2.1 |
| Uterus | LOC684146   | Ig kappa chain V-II region 26-10-like | No information provided by NCBI database | 10.2 |
|        | Igkv28      | Immunoglobulin kappa chain variable 28 | No information provided by NCBI database | 8.7 |
|        | LOC500181   | Ig kappa chain V-V region K2-like | No information provided by NCBI database | 3.4 |
|        | Igj         | Immunoglobulin joining chain | A protein-coding gene involved in antigen binding | 2.9 |
|        | Nos2        | Nitric Oxide Synthase 2, Inducible | Involved in aging, cellular response to tumour necrosis factor, intracellular signal transduction and nitric acid biosynthetic process | 2.8 |
|        | Mzb1        | Marginal zone B and B1 cell-specific protein | Involved in integrin activation, regulation of B cell proliferation and positive regulation of immunoglobulin biosynthetic process | 2.7 |
|        | LOC100364706| Lambda-chain C1-region-like | Involved in antigen binding | 2.5 |
|        | LOC262795   | Immunoglobulin G heavy chain | Involved in antigen binding | 2.2 |
|        | Fgf         | Fibrinogen gamma chain | Involved in inflammatory response, platelet activation and aggregation | –2.1 |
|        | Rup2        | Urinary protein 2 | No information provided by NCBI database | –3.9 |
Table 3

Significantly (p value < 0.05) regulated biological pathways after 8 weeks oral administration of a commercial soy supplement in ovariectomized rats. Only KEGG based biological pathways were included that were related to a distinct human tissue. Up and down regulation is indicated by up and down arrows.

| KEGG BASES BPs | PBMC | AT | LT | BT | ST | UT |
|----------------|------|----|----|----|----|----|
| Alzheimer's disease | ↑ | ↓ | ns | ns | ns | ns |
| Parkinson's disease | ↑ | ↓ | ns | ns | ns | ns |
| Huntington's disease | ↑ | ↓ | ns | ns | ns | ns |
| Prion diseases | ns | ↓ | ↓ | ns | ns | ns |
| Bacterial invasion of epithelial cells | ns | ↓ | ↓ | ↑ | ↑ | ↑ |
| Leishmaniasis | ↓ | ↓ | ↓ | ns | ns | ↑ |
| Chagas disease (American trypanosomiasis) | ns | ns | ns | ↑ | ns | ns |
| African trypanosomiasis | absent | absent | ns | ns | ns | |
| Malaria | ns | ns | ↓ | ↑ | ↑ | ↑ |
| Toxoplasmosis | ns | ns | ↓ | ns | ns | ns |
| Amoebiasis | ns | ns | ↓ | ns | ↑ | ↑ |
| Staphylococcus aureus infection | ↓ | ns | ↓ | ↑ | ns | ns |
| Hepatitis C | ns | ns | ns | ns | ns | ns |
| Pathways in cancer | ns | ns | ↓ | ns | ns | ns |
| Pancreatic cancer | ns | ns | ↓ | ns | ns | ns |
| Thyroid cancer | ns | ns | ↓ | ns | ns | ns |
| Melanoma | ns | ↓ | ns | ns | ns | ns |
| Bladder cancer | ns | ns | ↓ | ns | ns | ns |
| Chronic myeloid leukemia | ns | ns | ↓ | ns | ns | ↑ |
| Acute myeloid leukemia | ↓ | ns | ns | ns | ns | ns |
| Small cell lung cancer | ns | ns | ↓ | ns | ns | ns |
| Non-small cell lung cancer | ns | ns | ns | ns | ns | ns |
| Autocrine thyroid disease | ↓ | ns | ns | ns | ns | ns |
| Systemic lupus erythematosus | ns | ns | ↓ | ↑ | ↑ | ↑ |
| Rheumatoid arthritis | ns | ↓ | ↓ | ↑ | ↑ | ↑ |
| Allergen rejection | ↓ | absent | ns | ns | ns | ns |
| Graft-versus-host disease | ↓ | absent | ns | ns | ns | ns |
| Primary immunodeficiency | ↓ | absent | ns | ns | ns | ns |
| Hypertrophic cardiomyopathy (hcm) | ns | ↓ | ↓ | ns | ns | ns |
| Arrhythmogenic right ventricular cardiomyopathy (ARVC) | ns | ↓ | ↓ | ns | ns | ns |
| Dilated cardiomyopathy | ↓ | ns | ↓ | ns | ns | ns |
| Viral myocarditis | ns | ↓ | ↓ | ns | ns | ns |

ns = not significant.

BP, biological pathways; AT, adipose tissue; BT, breast tissue; LT, liver tissue; PBMC, peripheral blood mononuclear cells; ST, sternum tissue and UT, uterus tissue.

identified for adipose tissue (Fig. 3b). As can be seen in the supplementary table 6, these marker genes appeared to be involved in biological processes related to immune and inflammatory responses. In addition scattered plots were made using all homologous genes (i.e. 6413 homologous genes for human and rat PBMC and 7731 homologous genes for human and rat adipose tissue, see Fig. 1) without applying any significant cut-off value, but also in this situation no correlation between results in rats and humans was observed (results not shown).

4. Discussion

The results of this eight week study in ovariectomized rats show that the overall gene expression data after soy isoflavone supplementation are tissue specific for the regulation of estrogen responsive genes (ERGs) (see Table 1), and even more for genes not known to be ERGs. The different responses in gene expression in different tissues might be explained different ERα/ERβ ratios in these tissues [11–14]. This may complicate the use of surrogate tissues like PBMC or adipose tissue to predict the effects in target tissues such as breast, uterus or sternum tissue. In addition to differences in gene regulation upon soy isoflavone supplementation among different tissues, there also appeared to be a considerable difference in gene expression in the same tissue of the same species upon different exposure duration, as was evident by the absence of a correlation between the gene expression data in PBMC collected in the short- and the long-term rat study (see Fig. 2). We also did not observe a correlation between changes in gene expression in similar tissues (PBMC and adipose tissue) from rats and humans upon similar dosing and exposure duration (see Fig. 3; Table 4 and 5).

It was the aim of the present study to investigate whether it was possible to predict the effect of soy isoflavones supplementation in human target tissues, such as breast, uterus or sternum tissue. This was
Table 5
Significantly expressed human estrogen responsive genes (ERGs) in adipose tissue of human volunteers reported by van der Velpen et al. [4] and their expression in adipose tissue in the present long-term rat study. Up and down regulated gene expressions are indicated by up and down arrows.

| Sl. No. | Genes Description | Human | Rat |
|---------|-------------------|-------|-----|
| 1       | NR4A1 nuclear receptor subfamily 4, group A, member 1 | ↓     | ↓   |
| 2       | RET ret proto-oncogene | ↓     | a   |
| 3       | TK1 thymidine kinase 1, soluble | ↓     | a   |
| 4       | TGFBR3 transforming growth factor, beta 3 | ↓     | ↑ns |
| 5       | NOS3 nitric oxide synthase 3, endothelial cell | ↓     | ↓ns |
| 6       | GOT1 glutamic-oxaloacetic transaminase 1, soluble | ↓     | ↓ns |
| 7       | TNC tenasin C | a     |     |
| 8       | IGF2 insulin-like growth factor 2 | ↓     | ↓   |
| 9       | PPIF peptidylprolyl isomerase F | ↓     | ↑ns |
| 10      | ME1 malic enzyme 1, NADP(+)-dependent, cytosolic | ↓     | ↓ns |
| 11      | NME3 NME/NM23 nucleoside diphosphate kinase 1 | ↓     | a   |
| 12      | TUBG1 tubulin, gamma 1 | ↓     | ↑ns |
| 13      | THBD Thrombomodulin | ↓     | a   |
| 14      | NCAM2 neural cell adhesion molecule 2 | ↓     | a   |
| 15      | KITLG KIT ligand | ↓     | ↑   |
| 16      | ELOVL2 ELOVL fatty acid elongase 2 | ↓     | a   |
| 17      | CCND1 cyclin D1 | ↓     | ↑ns |
| 18      | CNKSR3 CNKSR family member 3 | ↓     | a   |
| 19      | CYCS cytochrome c, somatic | ↓     | a   |
| 20      | DHCR24 24-dehydrocholesterol reductase | ↓     | ↓ns |
| 21      | KIR3DL2 killer cell immunomodulin-like receptor, three domains, long cytoplasmic tail, 2 | ↓     | a   |
| 22      | PAR5 phosphoribosylaminomimidazole carboxylase, phosphoribosylaminomimidazolesuccinocarboxamim | ↓     | a   |
| 23      | TFF1 trefoil factor 1 | ↓     | a   |
| 24      | HSDP1 heat shock protein 1 (chaperonin) | ↓     | ↑ns |
| 25      | KPN2 karyopherin alpha 2 (RAG cohort 1, importin alpha 1) | ↓     | a   |
| 26      | ARMCK3 armadillo repeat containing, X-linked 3 | ↓     | ↓ns |
| 27      | ENO1 enolase 1, (alpha) | ↓     | ↑ns |
| 28      | SGCD sarcoglycan, delta (dystrophin-associated glycoprotein) | ↓     | a   |
| 29      | SEMA5B sema domain, seven thrombospondin repeats (type 1 and type 1-like), transmembrane domain | ↓     | a   |
| 30      | G6PD glucose-6-phosphate dehydrogenase | ↓     | ↓ns |
| 31      | RAMP3 receptor (G protein-coupled) activity modifying protein 3 | ↓     | a   |
| 32      | AURKB aurora kinase B | ↓     | a   |
| 33      | ESR2 estrogen receptor 2 (ER beta) | ↓     | a   |
| 34      | C1QBP complement component 1, q subcomponent binding protein | ↓     | ↑   |
| 35      | CENPA centromere protein A | ↓     | a   |
| 36      | AC02 aconitate 2, mitochondrial | ↓     | ↑ns |
| 37      | RUNX1 runx-related transcription factor 1 | ↓     | ↑   |
| 38      | MCM4 minichromosome maintenance complex component 4 | ↓     | a   |
| 39      | NR4A3 nuclear receptor subfamily 4, group A, number 3 | ↓     | a   |
| 40      | FOXF1 forkhead box F1 | ↓     | a   |
| 41      | ORMDL2 ORMD-like 2 (S. cerevisiae) | ↓     | ↑ns |
| 42      | MARCKS myristoylated alanine rich protein kinase C substrate | ↓     | ↑ns |
| 43      | SLC12A2 solute carrier family 12 (sodium/potassium/chloride transporter), member 2 | ↓     | ↓ns |
| 44      | SPRY1 sprout homolog 1, antagonist of FGF signaling (Drosophila) | ↓     | a   |
| 45      | GARS glycyl-tRNA synthetase | ↓     | ↓ns |
| 46      | RPA3 replication protein A3 | ↓     | ↓ns |
| 47      | STMN1 stathmin 1 | ↓     | a   |
| 48      | IRS1 islet-1/IRS synthetase | ↓     | ↑ns |
| 49      | ITGAV integrin, alpha V | ↓     | ↑ns |
| 50      | UMAP8 nucleoporin 88 | ↓     | ↑ns |
| 51      | TXNIP thioredoxin interacting protein | ↑     | ↑ns |
| 52      | NRF1 nuclear respiratory factor 1 | ↑     | ↑ns |
| 53      | ECE1 endothelin converting enzyme 1 | ↑     | ↑ns |
| 54      | EFEMP1 EGF-containing fibulin-like extracellular matrix protein 1 | ↑     | ↑   |
| 55      | MPL Myelo- proliferative leukemia virus oncogene | ↑     | a   |
| 56      | GSTO1 glutathione S-transferase omega 1 | ↑     | ↓ns |
| 57      | HIP1R huntingtin interacting protein 1 related | ↑     | a   |
| 58      | PAX8 paired box 8 | ↑     | a   |
| 59      | PTPN18 protein tyrosine phosphatase, non-receptor type 18 (brain-derived) | ↑     | ↓ns |
| 60      | WB1 WD repeat and SOCS box-containing 1 | ↑     | ↑ns |
| 61      | IGF1R insulin-like growth factor 1 receptor | ↑     | ↑ns |
| 62      | GNG7 guanine nucleotide binding protein (G protein), gamma 7 | ↑     | a   |
| 63      | SFRP1 secreted frizzled-related protein 1 | ↑     | ↓ns |
| 64      | MCM7 minichromosome maintenance complex component 7 | ↑     | ↓ns |
| 65      | SATB1 SATB homeobox 1 | ↑     | ↓ns |
| 66      | INPP4B inositol polyphosphate-4-phosphatase, type II | ↑     | ↓ns |
| 67      | THBS2 thrombospondin 2 | ↑     | ↓ns |
| 68      | PTGER2 prostaglandin E receptor 2 (subtype EP2), 53kDa | ↑     | a   |
| 69      | WISP2 WNT1 inducible signaling pathway protein 2 | ↑     | ↑   |
| 70      | BCL2L11 BCL2-like 11 (apoptosis facilitator) | ↑     | a   |
| 71      | ABCB1 ATP-binding cassette, sub-family B (MDR/TAP), member 1 | ↑     | a   |

(continued on next page)
done by evaluating the similarities in the gene expression pattern of the surrogate tissues PBMC and adipose tissue in rats and humans after administration of the similar oral dose of a commercially available soy isoflavone containing supplement. Because the results of the surrogate tissues (PBMC and adipose tissue) in rats differed from those in humans, and because there was no correlation between changes in gene expression between surrogate and target tissues in rats, we were unfortunately not able to predict possible health effects in humans. Although transcriptomic techniques are a powerful tool to predict early biological responses related to different breast cancer cell lines (i.e. human breast cancer cell line MCF-7, human embryonic kidney cells 293, murine calvarias osteoblasts MC3T3E1, and murine

**Table 5** (continued)

| Sl. No. | Genes          | Description                  | Regulation | Human | Rat |
|--------|----------------|------------------------------|------------|-------|-----|
| 72     | TSC22D3        | TSC22 domain family, member 3| ↑          | a     |     |
| 73     | NFKBIA         | nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, alpha| ↑↑         | ns    |     |
| 74     | ANKRD44        | ankyrin repeat domain 44     | ↑↑         | ns    |     |
| 75     | CTNND1         | catenin (cadherin associated protein), delta 1| ↑↑         | ns    |     |
| 76     | BCL2L1         | BCL2-like 1                  | ↑↑         | ns    |     |
| 77     | TNFSF8         | tumour necrosis factor (ligand) superfamily, member 8| ↑↑         | a     |     |
| 78     | CCNG2          | cyclin G2                    | ↑↑         | a     |     |
| 79     | FKBP6          | FK506 binding protein 8      | ↑↑         | a     |     |
| 80     | ZNF362         | zinc finger protein 36, C3H type-like2| ↑↑         | ns    |     |
| 81     | SLA            | Sec like adaptor             | ↑↑         | a     |     |
| 82     | S100P          | S100 calcium binding protein P| ↑↑         | a     |     |

a = absent.
ns = not significantly different.

**Fig. 3.** Rank-rank scattered plot for significantly changed common PBMC genes in rat and (post)menopausal women (3a), and significantly changed common genes in adipose tissue of rats and (post)menopausal women (3b). Each dot represents the t-value of a single gene and the highlighted dots (♦) indicate the correlated genes. Corresponding human homologous genes were retrieved from the study with (post)menopausal women taking the same supplement, in a similar dose (about 1.5 mg soy isoflavone/kg bw per day) and time duration (i.e. 8 weeks) [4,19]. Genes significantly changed in the same direction in both treatments are in Cartesian quadrants I and III, while genes significantly changed in opposite directions are in quadrants II and IV.
monocytic cells RAW 264.7) transfected with the estrogen-responsive reporter gene construct 3×ERE-TATA-Luc found that the transcriptional potency of equol for the ERα receptor was 1.1 fold higher and for ERβ receptor 1.2 fold higher than that of daidzein. Kalita and Milligan [37] using Ishikawa and yeast cells, expressed the relative potencies of different estrogenic compounds compared to estradiol (E2) and reported that the relative potency of equol is about 2–8 fold higher than that of daidzein. Comparable values, ranging from 1–4.7, have been reported in two review papers [38,39]. In contrast to these results a much higher potency factor of 100 was reported by Sathymoorthy and Wang [40] based on proliferation and mRNA expression of the oestrogen-responsive pS2 gene in MCF7 cells. Because the reported relative potencies of equol compared to daidzein, based on different test systems, differ considerably, no distinct overall potency factor can be established. But based on the available data we assumed a reasonable estimate of a factor of 10 for the relative potency of equol compared to daidzein. Thus the concentration of 19 nM as mentioned above could be converted into about 120 nM daidzein aglycone equivalents. As can be seen by the dotted lines in Fig. 4 the conclusion drawn above could not be induced an ERα related response, but could induce a moderate ERβ related response, is still valid.

For comparison with the rat study we also calculated the total free aglycone concentrations in plasma of human volunteers in the human intervention study where in addition to daidzein and equol, also genistein and glycitein were detected [19]. After converting the μg/mL concentrations reported by these authors to nM and using again a value of 3% for the amount of soy isoflavones that can be present in the circulation in the free form (see above), the total free aglycone concentration in these human volunteers could be estimated to be about 130 nM, on average. This is about 6.8 fold higher than the free aglycones concentration in rat plasma. Considering that the daidzein concentration in human plasma is highest (0.47 μg/mL) followed by equol (0.35 μg/mL), and that lower concentrations were found for genistein (0.18 μg) and glycitein (0.12 μg/mL), one could speculate that, even considering the higher potency of equol compared to daidze E2, the concentrations of free aglycones in human plasma were also not able to induce a significant ERα related response, but that a significant ERβ related response could be possible (Fig. 4). This might explain the beneficial health effects that have been reported in several human intervention studies or randomised control trials, such as alleviation of menopausal effects [4,5,40–42]. But activation of ERβ is also associated with anti-proliferative and anti-carcinogenic effects in hormone sensitive female breast cancer patients [33,43].

The observed difference in plasma concentration between rats and humans after administration of the same soy isoflavone supplement at a similar dose level (2 mg soy isoflavone/kg bw) and for the same duration (i.e. 8 weeks) can be due to species variation in ADME characteristics and/or a difference in the dosing regimen. In the present study the rats were dosed once per day in the morning and in the human intervention study the volunteers took 2 capsules in the morning and 2 in the evening after the meal. Gu et al. [35] also observed considerable species differences in plasma soy isoflavone concentrations between female adult Sprague-Dawley rats and premenopausal woman volunteers 24 h after a similar single dose; the concentration of total daidzein was 4 fold higher in human plasma compared to that of the rat. This is in line with the results of the present study. Setchell et al. [40] also found significant species differences in the circulating concentrations of aglycones between rodents and humans. Based on differences in the proportion of unconjugated soy isoflavone in plasma of humans and that of rodents, particularly in certain strains of mice, they also questioned the value of rodent models for the assessment of effects of soy isoflavone in humans. However, Setchell et al. [40] used different isoflavone sources and administration protocols to treat animals and human volunteers, and animals were treated with much higher dose levels compared to humans, leading to higher aglycone concentrations in the plasma of rats compared to humans. In addition, the adult humans span a wide range of ages (i.e. from 21 to 65 years).
We also investigated a number of pathways in the rat that might have been affected by soy isoflavone treatment. In the present study the PPAR signalling pathway was up regulated in breast uterus and liver tissues (see supplementary Table 2) indicating a positive effect of soy isoflavone treatment. However, this pathway was significantly down regulated in human PBMC following administration of the same supplement. Other studies have shown that both ERs and PPARs can influence each other and thus might lead to different effects of soy isoflavone [45,46]. It has been reported that soy isoflavone are capable to bind and activate all three isoflavins namely, PPARα, PPARβ and PPARγ, of the peroxisome proliferator-activated receptors (PPARs) [44]. These PPARs are a group of transcription factors, which play an essential role in the regulation of cellular differentiation, carbohydrate, lipid and protein metabolism and tumorigenesis [44]. It was found that the p53 pathway was significantly up regulated in PBMC and breast tissue of rats (see supplementary Table 2). P-53 is an important and one of the most studied stress response pathways conserving stability of DNA, and protecting cells from DNA damage [47]. Under normal conditions this tumour suppressor gene is constitutively expressed, but it is negatively regulated by the pathway sensor called Mdm2, and then degraded. However, a variety of stress events in the cells, especially those related to DNA damage, activate a series of events that stabilize the p53 protein by inhibiting its degradation. Once activated p53 regulates divergent groups of target genes related to cell cycle arrest [48], DNA repair mechanism [49,50] and induction of apoptosis [51]. Hence activation of this p53 gene has been reported to be associated with prevention of cancer. Therefore up regulation of the p53 pathway, as observed in the present rat study, could be considered as a possible beneficial effect of exposure to soy isoflavones.

Given the fact that the correlations in gene expression in PBMC and adipose tissue between the present rat study and the human intervention study, both using the same soy isoflavones supplement, were limited, it is of importance to consider the possible limitations of the current rat study. In the rat study the animals were dosed once per day by gavage, whereas the human volunteers took the dose in two portions per day. This difference in dose regimen, together with possible effects of the circadian cycle could have influenced the gene expression. Furthermore, the comparison of the effects of soy isoflavones in rats and humans was based on the use of a similar external dose, and no consideration was given to the internal dose. As indicated above, higher plasma concentrations of soy isoflavones were found in humans compared to the rat. We previously reported that rats can metabolize soy isoflavone faster than humans [21]. So providing the same external dose on a kg/bw basis appeared to result in somewhat dissimilar plasma concentration in rats and humans, and this could have contributed to the differences in gene expression between rats and humans, as observed in the present study.

In our study we have used young ovariectomized rats and considered them as a suitable model for postmenopausal women. Although it is a well-established model, it also has its limitations. Brinton [52] mentioned that ovariectomy is a widespread model of menopause and is typically done by removing ovaries from young reproductively competent healthy animals. The limitations he mentioned about the ovariectomy model of menopause are that in this model it is assumed that ovaries are the only organs capable of producing ovarian hormones and therefore, plasma levels of ovarian hormones are indicative of ovarian hormone concentrations in organs. However, this assumption was challenged by Caruso et al. [53], who found that changes in plasma levels of neuroactive steroid levels after ovariectomy do not necessarily reflect the steroid levels in either the peripheral or central nervous system. Secondly, in our experiments outcomes obtained from analyses of short-term ovariectomy are generalized to long-term ovarian hormone deprivation. However, this assumption ignores the adaptive responses that organs can undergo to compensate for the loss of ovarian hormones. Although removal of the ovaries in young animals is a model that broadly generalizes to human menopause. This assumption can also be challenged because adaptations can occur in the estrogen-regulated metabolic pathways during aging. In humans, ovarian hormone deprivation is chronic, in case no hormone therapy is initiated. According to the review of Brinton [52] chronic exposure in animal models is entirely feasible of human menopause. It can be thus concluded that fully developed, aged female rats may have better physiological similarities with postmenopausal women and may therefore be a more appropriate animal model.

5. Conclusion

In the present study there are considerable differences in gene expression between various surrogate and target tissues. In addition, no cross-species early biomarkers for health effects of soy isoflavone could be identified. Based on these results it can be concluded that the use of transcriptomic data in young ovariectomized rats is challenging, but does not provide a suitable model for human risk or benefit analysis for soy isoflavone supplementation.

Conflict of interest

The authors have declared no conflict of interest.

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