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Plasma bioavailability and changes in PBMC gene expression after treatment of ovariectomized rats with a commercial soy supplement

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ABSTRACT

The health effects of soy supplementation in (post)menopausal women are still a controversial issue. The aim of the present study was to establish the effect of the soy isoflavones (SIF) present in a commercially available supplement on ovariectomized rats and to investigate whether these rats would provide an adequate model to predict effects of SIF in (post)menopausal women. Two dose levels (i.e. 2 and 20 mg/kg b.w.) were used to characterize plasma bioavailability, urinary and fecal concentrations of SIF and changes in gene expression in peripheral blood mononuclear cells (PBMC). Animals were dosed at 0 and 48 h and sacrificed 4 h after the last dose. A clear dose dependent increase of SIF concentrations in plasma, urine and feces was observed, together with a strong correlation in changes in gene expression between the two dose groups. All estrogen responsive genes and related biological pathways (BPs) that were affected by the SIF treatment were regulated in both dose groups in the same direction and indicate beneficial effects. However, in general no correlation was found between the changes in gene expression in rat PBMC with those in PBMC of (post)menopausal women exposed to a comparable dose of the same supplement. The outcome of this short-term study in rats indicates that the rat might not be a suitable model to predict effects of SIF in humans. Although the relative exposure period in this rat study is comparable with that of the human study, longer repetitive administration of rats to SIF may be required to draw a final conclusion on the suitability of the rat a model to predict effects of SIF in humans.

Abbreviations: BPs, biological pathways; DMSO, dimethyl sulfoxide; E_2, estradiol; ECM, extracellular matrix; EREs, estrogen-responsive elements; ERs, estrogen receptors; GSEA, gene set enrichment analysis; HD, high dose; HPLC, high performance liquid chromatography; KEGG, kyoto encyclopedia of genes and genomes; LD, low dose; MDS, multidimensional scaling; NCBI, National Center for Biotechnology Information; PBMC, peripheral blood mononuclear cells; SIF, soy isoflavones; UPC, universal expression code.

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1. Introduction

Dietary isoflavones are phenolic plant compounds mostly found in soy [1–3]. They are biologically active and, depending on the concentration, they have estrogen agonist and antagonist effects due to structural similarity with estradiol [4]. Therefore, they are also known as phytoestrogens [4,5]. Soy isoflavones (SIF) have gained increasing interest in recent years because of their potential health benefits such as improvement of cardiovascular functions, bone mineral density, alleviation of menopausal symptoms and reduction of the occurrence of certain cancers like breast and prostate cancers [6–8]. The use of soy products in the USA increased significantly following their approval by U.S. Food and Drug Administration [9] and the decision [10] to allow food manufacturers to make cardiovascular health claims for soy protein products. Moreover a number of SIF-rich supplements have become available for over the counter sale in Western countries during the last decade [11]. In contrast, adverse health effects also have been reported [12–14], and recently the European Food Safety Authority (EFSA) rejected some of the major health claims of SIF, such as maintenance of bone mineral density and reduction of vasomotor symptoms associated with menopause [15]. According to EFSA [15], the data are not sufficient to establish a cause–effect relationship for these endpoints. Therefore, concern remains about the potentially harmful effects from self-administered dosages of these SIF rich products, which are freely available in pharmacies, health food stores and via online shopping on Internet [6,11,12].

SIF are present in soy based foods and food supplements, predominantly in their glucoside forms namely genistin, daidzin and glycitin. Their corresponding primary metabolites are genistein, daidzein and glycitein. These metabolites are produced initially mainly by β-glucosidase present in the duodenum and upper proximal jejunum [16,17] and further down in the intestinal tract by gut microflora [11,18]. These primary metabolites are known as aglycones and considered to be the biologically active forms of SIF [13,19]. However in the systemic circulation, glucuronides (i.e. the secondary metabolites) are found to be the predominant metabolites [20,21]. Therefore, enzymatic hydrolysis is commonly used to measure the total aglycone content in plasma [21–23]. Daidzin is one of the major soy glucosides present in the soy supplements. Approximately 20–30% of the Western population is able to metabolize the aglycone form of daidzin (diadzein), into the more potent metabolite equol, which is more bioavailable and has a higher binding affinity for the estrogen receptors [24–26]. This means that especially in (post)menopausal equol-producing women SIF supplementation will result in a considerable exposure related contribution to endogenous estrogen levels [27–29].

The effects of SIF are thought to be induced via estrogen receptors (ERs) [30,31] of which an alpha and beta form exist. SIF can regulate transcription of ER target genes via the estrogen-responsive elements (EREs) present within gene promoter regions in target tissues such as breast and uterus. It is known that activation of ERα stimulates cell proliferation, whereas ERβ is involved in inhibition of proliferation and stimulation of apoptosis [20,32]. Estradiol (E2) is ERα selective, whereas SIF are ERβ selective [15,32]. Because (post)menopausal women produce low levels of endogenous estradiol, it is expected that the intake of SIF by equol-producing (post)menopausal women will result in beneficial health effects of SIF supplementation [3,6,19,33].

During the past decade, 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. Understanding of the bioavailability, metabolism and induction of gene expression related to different biological processes is key to understand the health effects of SIF supplementation. There is, however, a lack of information whether data obtained from in vivo studies in experimental animals are suitable to predict human risks or benefits. Hence the aim of the present study was to establish the effect of SIF on plasma bioavailability and gene expression changes in ovariectomized rats and to investigate whether these rats would be an adequate model to predict effects of SIF supplementation in (post)menopausal women. The ovariectomized rats, mimicking the hormonal condition of (post)menopausal women, were dosed with a commercial SIF containing supplement. The bioavailability and the excretion of SIF were studied by measuring the concentration of SIF and the main metabolite equol in plasma, urine and feces. Changes in gene expression of peripheral blood mononuclear cells (PBMC) were characterized, because this could be a basis for rat to human comparison, since PBMC are easy to collect as marker tissue in studies with human volunteers, and are often used to predict changes in target tissue [34]. In addition, the effects on gene expression in rat PBMC were compared with the effect on gene expression in PBMC of (post)menopausal equol-producing women, who received the same commercial SIF supplement in a parallel study [23].

2. Materials and methods

2.1. Chemicals

Pure SIF standards (both glucosides and aglycones) and equol were purchased from LC laboratories (Woburn, MA, USA). Dimethyl sulfoxide (DMSO) was obtained from Acros Organics (Geel, Belgium), and enterodiol, β-glucuronidase H-5 (*Helix Pomatia* contains 60,620 unit/g solid), Optiprep™ and Tricine from Sigma–Aldrich (Steinheim, Germany). NaCl was from VWR International (Darmstadt, Germany) and acetonitrile and methanol from Biosolve BV (Valkenswaard, The Netherlands). Oasis HLB 1cc, solid phase extraction cartridges from Waters (Milford, MA, USA).

2.2. Animals

Fifteen ovariectomized F344 inbred rats, 9–10 weeks of age, were purchased from Harlan, (Horst, The Netherlands). The ovariectomy was performed at the age of 7–8 weeks by the supplier. After 2 weeks of acclimatization the animals were delivered at the animal facility, the Centre for Laboratory Animals (CKP), Wageningen University, Wageningen, The Netherlands. F344 rats were chosen for two reasons.

Table 1
Composition of isoflavone-free RMH-B diet.

Analysis	%	Amino acids	g/kg
Crude protein	23.5	Lysine	10.5
Crude fat	5.0	Methionine	3.6
Crude fiber	4.3	Methionine + cystine	6.6
Starch	38.3	Cystine	3.0
Sugar	4.0	Threonine	7.4
Linoleic acid	1.5	Tryptofan	2.0
Ash	5.5	Isoleucine	7.5
Dry matter	89.8	Arginine	14.7
		Phenylalanine	8.7
Energy (kJ/kg)	16,100	Histidine	4.6
		Leucine	11.2
Minerals	*%*	Tyrosine	4.1
Ca	0.92	Valine	10.3
P	0.63		
K	0.90	*Vitamins*	*IU/kg*
Mg	0.12	Vit. A	20,500
Na	0.42	Vit. D3	2000
Cl	0.74		*mg/kg*
		Choline	1000.0
		Vit. E	60.0
Trace elements	*mg/kg*	Vit. K3	2.4
Fe	105.0	Vit. B1 thiamine	13.0
Mn	70.0	Vit. B2 riboflavin	10.7
Zn	55.0	Vit. B3 niacin	32.0
Cu	17.5	Vit. B5 pentothenic acid	11.9
Co	0.2	Vit. B6 pyridoxine	12.5
I	0.4	Vit. B9 pyridoxine	3.8
		Vit. B12 pyridoxine	0.1
		Betaine	127.0
		Biotine	200.0
		Vit.C	95.0

ABDIETS (www.abdiets.com).

Firstly, according to US EPA [35], the inbred isogenic strain F344 rats are sensitive to estrogenic compounds and thus particularly suitable to study the effects of SIF. Secondly, the use of an inbred strain will minimize the background noise in micro-array analysis.

2.3. Housing and nutrition

After arrival at the animal facility, rats were housed in groups (3 animals/cage) and fed an SIF free RMH-B standard diet (ABDiets, Woerden, The Netherlands) (Table 1) ad libitum. Standard housing, day-light hours and humidity were maintained during the acclimatization period of 12 days. The animals were 11–12 weeks of age during the experiment, and had a bodyweight of 162–196 g. Animals were placed individually in metabolic cages, 24 h before receiving the first gavage dose.

2.4. Animal experiment

All aspects of the experimental protocol were reviewed and approved by the Animal Welfare Committee of Wageningen University (Wageningen, The Netherlands). Animals (5 rats per dose group) received one gavage dose for kinetic part, followed by a second gavage dose after 48 h for microarray analysis. The animals were sacrificed 4 h after the second dose and blood was collected for isolation of PBMC. Based on the life duration of rat and human (i.e. 2 years and 70 years, respectively) the relative exposure time for rats (i.e. two doses in 2 days, resulting in exposure during about 0.27% of their lifespan) is roughly comparable with that of the human volunteers in the parallel study (i.e. 56 days, resulting in exposure during about 0.22% of their lifespan). In addition, this study design allowed us to collect samples for both kinetic and gene expression studies using same animals, which is in accordance with the principles of the three Rs (Replacement, Reduction, and Refinement). Different dose groups were 0 (solvent control), 2 or 20 mg SIF/kg b.w., where the SIF was administered in the form of a commercial soy supplement (see Section 2.5). The low dose (LD) of 2 mg SIF/kg b.w. is comparable with the maximum daily human consumption (i.e. >100 mg/day; [3]) although the usual dose taken by women using soy supplementation lies between 20 and 80 mg/day [36]. However, to get a clear effect of SIF in this rat study, a 10 times higher dose (HD) (i.e. 20 mg SIF/kg b.w.) was used as well.

2.5. Preparation of test solutions

The commercial soy supplement, Phytosoya, was obtained from Arkopharma (Carros, France). The total content of the SIF present in the supplement and their composition were determined by HPLC and previously reported [17]. The supplement had a total SIF content of 70.3 mg/g supplement. The content of the three glucosides; genistin, daidzin and glycitin was 7.54, 40.03 and 22.72 mg/g supplement, respectively. The SIF content in this supplement was in accordance with the content indicated by the supplier on the label. On the basis of an average bodyweight of rats of 200 g, an oral gavage dose of 2 mg/kg b.w. corresponds with 0.4 mg SIF per rat. Therefore a dose of 5.69 mg supplement per rat was applied. Similarly, a 10 times higher amount was used for the 20 mg/kg dose group. For the administration of a bodyweight dependent gavage dose (i.e. maximum of 1 ml gavage/200 g b.w.), a 10 ml stock solution (i.e. 0.5 ml DMSO and 9.5 ml nanopure water) containing 56.9 mg supplement was prepared for the low dose group. Similarly, a stock solution with a 10 times higher concentration was prepared for the high dose groups. The stock solutions were freshly prepared and were kept at room temperature overnight. They were shaken thoroughly before use and placed on a multi-axle-rotating-mixturemachine (CAT RM-40, Slaufen, Germany) during the period of gavage dosing. The control rats received a gavage dose of nanopure water containing 5% DMSO.

2.6. Sample collection

Blood samples (~250 μl) were taken from the tail vein at 0, 10, 30 min and 1, 2, 8, 24, 48 h after dosing and collected in Microvette CB-300 tubes (Sarstedt AG & Co, Nümbrecht, Germany) containing lithium heparin. Directly after collecting the blood samples, plasma samples were prepared by centrifuging the blood samples for 5 min at 10,000 rpm (Biofuge centrifuge, HeraeusSepatech, UK). Plasma samples were collected in Safe-lock 0.5 ml Eppendorf tubes (Eppendorf AG, Hamburg, Germany) which were placed on ice to preserve degradation of the collected samples during the experiment. Urine samples (24 h) were collected

in 50 ml graduated sterile polypropylene tubes and finally stored in 14 ml round bottom tubes; the tubes were purchased from Greiner bio-one (Frickenhausen, Germany). Feces samples (24 h) were collected in 30 ml polystyrene tubes from Greiner bio-one (Gloucestershire, UK). All the urine and feces samples were stored at −80 °C until further analysis. After collection of the last blood sample (48 h after dosing), a second dose was administered to the animals to study the effects on gene expression. Then 4 h later the animals were sacrificed after anesthesia with a mixture of isoflurane and oxygen followed by removal of blood from the dorsal aorta. The collected blood was immediately transferred into 10 ml tubes and kept on a multi-axle-rotating-mixture machine (CAT RM-40, Staufen, Germany) to avoid clotting. This blood was used for the isolation of PBMC. For the isolation procedure see below.

2.7. Extraction of SIF from plasma, urine and feces

Extraction of SIF from plasma was performed with 1 cc Waters Oasis HLB cartridges (Milford, MA, USA). The plasma extraction method included enzymatic hydrolysis and was performed according to Saracino and Raggi [37] and modified because of the small volume of the plasma samples. In short, 50 μL of plasma sample was mixed with 17 μg (17 μl of a stock solution of 1 mg/ml) of enterodiol (internal standard) and 275 μg (22 μl of a stock solution of 12.5 mg/ml) of *H. Pomatia* mixture. The final volume was made 170 μl by adding sodium-acetate (pH 5, 0.5 M). The samples were then incubated for 18 h at 37 °C. After incubation, 830 μl sodium-acetate was added, followed by centrifugation for 10 min at 10,000 × *g*, at 4 °C. The collected supernatants were used for solid phase extraction followed by evaporation of the extraction medium. Finally, the dried extracts were re-dissolved in 75 μl eluent B (i.e. 25 mM sodium phosphate, pH 2.4) and injected in the HPLC system.

Extraction of SIF from urine samples was carried out following enzymatic hydrolysis. In short, 70 μL of urine sample was mixed with 375 μg (30 μl from stock 12.5 mg/ml) of *H. Pomatia* mixture. The samples were then incubated for 2 h at 37 °C. After incubation, 200 μl pure acetonitrile and 100 μl of 20% H_3PO_4 were added. The mixture was vortexed and centrifuged for 10 min at 10,000 × *g* at 4 °C, and the supernatants (100 μl) were collected in HPLC vials.

Before extraction, feces samples were lyophilized and powdered using pestle and mortar. Duplicate samples of 0.1 g were taken from each feces sample and mixed with 500 μl absolute ethanol followed by homogenization using Precellys 24 (Bertin) at 6500 rpm for 30 s with a short interval after 15 s. Samples were centrifuged for 10 min at 13,500 × g at 4 °C. Finally, 200 μl of the supernatants were collected in HPLC vials for analysis. No enzymatic hydrolysis was applied because from other reports it is known that feces only contains aglycons, as the gut microbiota are able to completely deconjugate all conjugated SIF, i.e. glucosides, glucuronides or sulphates [18,38].

2.8. HPLC analysis

The SIF content of the supplements was determined according to the method of Peñalvo et al. [39] by HPLC analysis with electrochemical detection. HPLC analyses were carried out on LachromHitachi equipment (Varian, Sugarland, TX, USA) with a L2100 masterpump, a L2100 auto-sampler and a CoulArray electrochemical detector equipped with a high sensitivity analytical cell (model 6210, 4-sensor cell, ESA Inc., Chelmsford, MA, USA). A data collection system Varian Star 6.2 software was used for controlling the instrument and collecting the data from the electrochemical detector. A Symmetry Shield C18 column (150 mm × 4.6 mm, 5 μm) from Waters with a Brownlee New guard precolumn (7 μm) (Shelton, USA) was used for the analysis of the samples. The SIF were measured on 4 channels; 300, 500, 550, and 600 mV and quantified at 500 mV. The limit of detection for all SIF was 0.01 μg/ml, whereas the limit of quantification was 0.02 μg/ml. Solutions of 10%, 55% and 30% acetonitrile in 25 mM sodium phosphate (pH 2.4) were used as eluent A, B and C, respectively. The injection volume for HPLC analysis was 10 μl and the flow rate was 1 ml/min. The total running time was 27 min. Elution was started with 30% of solvent B followed by a change of solvent B as 30, 100, 100, 30 and 30% in 0.0–0.1, 0.1–17, 17–19, 19–20 and 20–27 min, respectively. The retention times for genistein, daidzein, glycitein, equol and enterodiol were 17.5 ± 0.2, 12.8 ± 0.1, 12.1 ± 0.1, 16.3 ± 01 and 10.2 ± 0.2 min, respectively. The differences in SIF concentrations between the dose groups and the control were analyzed by student *t*-tests using Microsoft Excel.

2.9. Isolation of PBMC, mRNA and running microarray analysis

PBMC were isolated immediately after blood collection using OptiPrep™ (60% w/v iodixanol) as a density gradient medium following the online protocol by Axis-Shield (www.axis-shield-density-gradient-media.com > methodology > cells > C43). After isolation, all samples were dissolved in RLT buffer of Qiagen (Venlo, The Netherlands) for lysis of cells to isolate RNA, and stored at −20 °C until further analysis. RNA extraction was carried out by using Qiagen RNeasy Mini Kit (Qiagen, Venlo, The Netherlands). The extracted amount of RNA was quantified using a Nanodrop ND 1000 spectrophotometer (Nanodrop Technologies) and integrity (i.e. quality) was measured (ng/μl) using an Agilent 2100 Bioanalyzer with RNA 6000 Nanochips (Agilent Technologies, Amstelveen, The Netherlands). Finally, samples were selected for gene expression analysis on an AffymetrixGeneChip Rat Gene 1.1 ST plate (Affymetrix, Santa Clara, CA). One hundred nanogram of total RNA was used for whole transcript cDNA synthesis with the Ambion WT expression kit [catalog number 4411974] (Applied Biosystems/Life Technologies, Nieuwekerka/dIJssel, The Netherlands). Hybridization, washing and scanning of the Rat Gene 1.1 ST peg arrays was performed on a GeneTitan Instrument (Affymetrix, Santa Clara, CA) according to the manufacturer's recommendations.

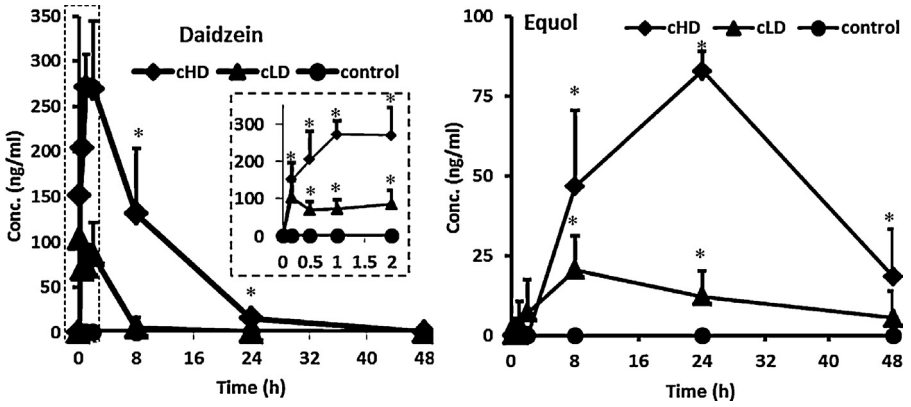

Fig. 1. Plasma curves of isoflavones in F344 ovariectomized rats after a single oral gavage dose (2 and 20 mg/kg b.w.) of a commercial soy supplement. Only daidzein and equol could be detected (the limit of detection for all SIF was 0.01 μg/ml, and the limit of quantification was 0.02 μg/ml). Data are expressed as mean ± SD. Sample size 5 rats/group. LD (▲) and HD (♦) stand for low and high dose group, respectively. A significant effect in LD and HD at specific time points is denoted by * ($p < 0.01$). The inserted graph shows the plasma curve of daidzein during the first 2 h.

Packages from the Bioconductor project [40], integrated in an online pipeline [41], were used to analyze the array data. Various advanced-quality metrics, diagnostic plots, pseudo images, and classification methods were used as described by Heber and Sick [42], to determine the quality of the arrays before statistical analysis. Three controls and one HD sample did not pass the quality control criteria, and were removed from the dataset. The final dataset thus obtained consisted of 11 arrays. The probes on the Rat Gene 1.1 ST array were redefined using current genome information [43]. 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 [44]. Subsequently, the dataset was filtered to only include probesets (genes) that were active (i.e. expressed) in at least 4 samples using the universal expression code (UPC) approach (UPC score > 0.50) [45]. This resulted in the inclusion of 7650 of the 19,311 (40%) probe sets present on the array. Differentially expressed probe sets were identified by using linear models, applying moderated t-statistics that implemented intensity-based empirical Bayes regularization of standard errors [46,47]. Probe sets that satisfied the criterion of $p < 0.05$, 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. [48]. 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) database [49]. Effects of the low and high dose of SIF versus control were compared using ranked lists based on significance (*t*-values), using methods described by Plaisier et al. [50].

3. Results

3.1. Kinetics

The commercial supplement used in the present study contains predominantly daidzin (~57%), and smaller amounts of glycitin (~32%) and genistin (~11%). See also Section 2.5. Fig. 1 shows the plasma concentrations of daidzein and equol following a single oral administration of a low dose of 2 mg/kg b.w. (LD) and a high dose of 20 mg/kg b.w. (HD). As is shown in Fig. 1, daidzein was rapidly absorbed with peak plasma concentrations within 1 h after dosing. The corresponding pharmacokinetic data are presented in Table 2. The maximum observed highest plasma concentration (Cmax) for daidzein was 3.2 fold higher and the area under the curve (AUC) was 6.6 fold larger in the HD group compared to the LD group. For equol there was an apparent lag time of about 1 h in both dose groups before its appearance in plasma. Both the Cmax and AUC for equol was 4.2 fold higher in the HD group than in the LD group. Furthermore it can be seen from Fig. 1 that equol is not completely cleared from the plasma within a period of 48 h after dosing.

Fig. 2 shows the urinary and fecal excretion of SIF and of equol, in samples collected over a 24 h period. In urine of the LD group, the highest concentration was found for daidzein, followed by equol and genistein, while glycitein could not be quantified because of an interfering peak in the chromatogram. In the HD group the highest concentration was found for daidzein, followed by glycitein, equol and genistein (see Fig. 2a and Table 3). The concentrations of SIF in different dose groups were significantly different compared to the control group and also between the different dose groups. In feces highest concentrations were found for equol, followed by daidzein and genistein (see Fig. 2b and Table 3) and we did not find any conjugated SIF (i.e. no glucosides, glucuronides or sulphates). Again due to interference of an unidentified peak in the chromatogram at the same retention time, it was not possible to quantify

Table 2
Plasma pharmacokinetic parameters of daidzein and equol in female ovariectomized F344 rats that received a single oral gavage dose (2 or 20 mg/kg b.w.) of SIF from a commercial SIF supplement.

Isoflavone dose (mg/kg b. w.)	Daidzein				Equol		
	C_{max} (ng/ml)	T_{max} (h)	t ½ (h)	AUC (μg h/ml)	C_{max} (ng/ml)	T_{max} (h)	AUC (μg h/ml)
2	85 ± 37	0.17	2.5	0.46	20 ± 11	8	0.56
20	272 ± 36	1.0	6	3.02	83 ± 6	24	2.39

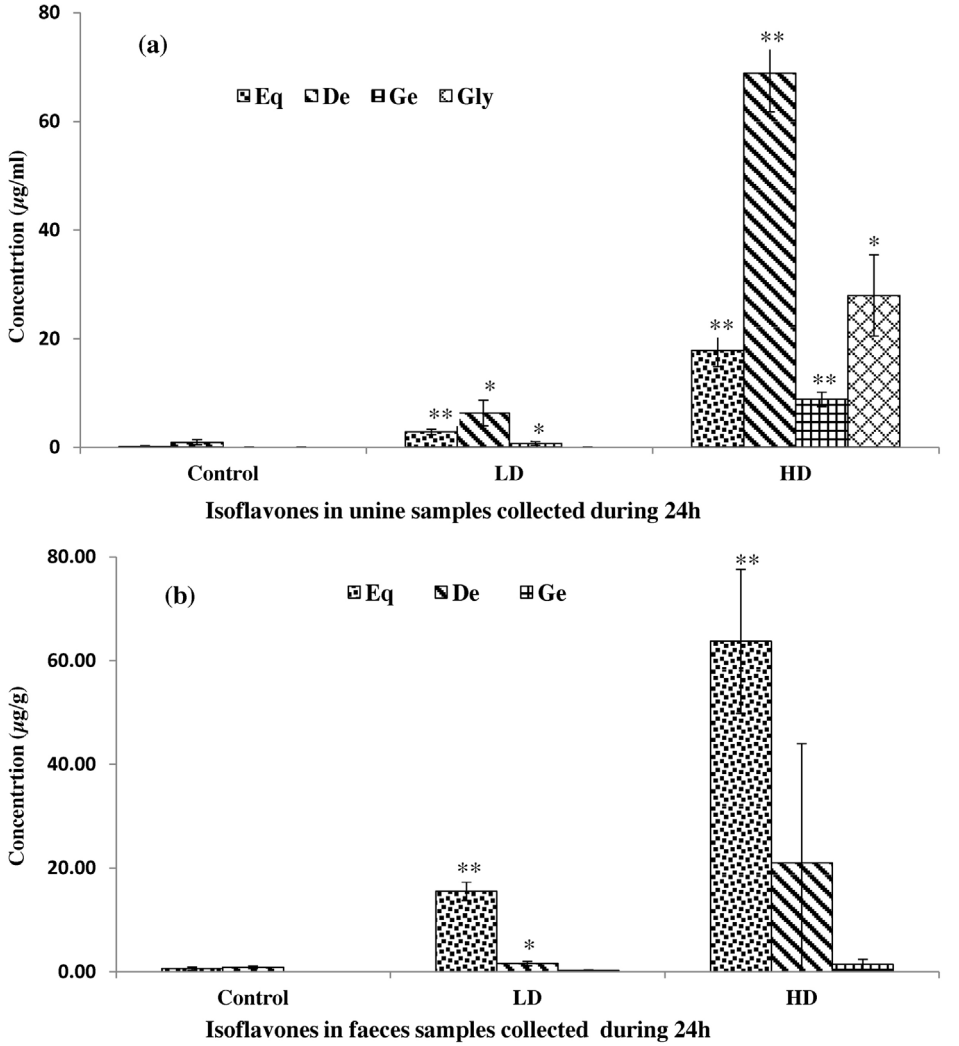

Fig. 2. Amount of isoflavones in urine (a) and feces (b) of F344 female ovariectomized rats over a 24 h time period after administration of a single oral dose (2 and 20 mg/kg bw SIF) of a commercial soy supplement. Data are expressed as mean ± SD. Sample size 5 rats/group. LD and HD stand for low and high dose, respectively. Significant effects between LD and HD groups compared to control at specific time points are denoted * ($p < 0.01$) and ** ($p < 0.001$).

Table 3
Amount of SIF in urine and feces (μg/rat) after 24 h in F344 ovariectomized rats ($n = 2$ for control and 5 for treatment groups). LD stands for low (i.e. 2 mg/kg b.w.) and HD for high (i.e. 20 mg/kg b.w.) dose. Significant differences with the control are denoted by *$p < 0.05$ or **$p < 0.01$.

Group	Daidzein			Equol			Glycitein			Genistein		
	Urine	Feces	[1] (%)	Urine	Feces	[2] (%)	Urine	Feces	[1] (%)	Urine	Feces	[1] (%)
LD	48 ± 23**	2.1 ± 1*	36	20 ± 6**	19 ± 4**	29	ND	ND	–	5.5 ± 3.0**	0.32 ± 0.1**	22
HD	434 ± 73**	19 ± 17	33	114 ± 38**	74 ± 30**	14	178 ± 56**	ND	22	56.0 ± 12.7**	1.40 ± 0.7*	22

[1] The relative amounts of the various isoflavones (sum in urine and feces), expressed as percentage of the administrated dose (%), was calculated based on "aglycone equivalents" by correcting for the difference in molecular weight between the respective glucoside and its aglycon.
[2] For equol, the amount was first converted into diadzein (aglycone) equivalents and then expressed as percentage of the administrated dose of daidzin based on "aglycone equivalents".

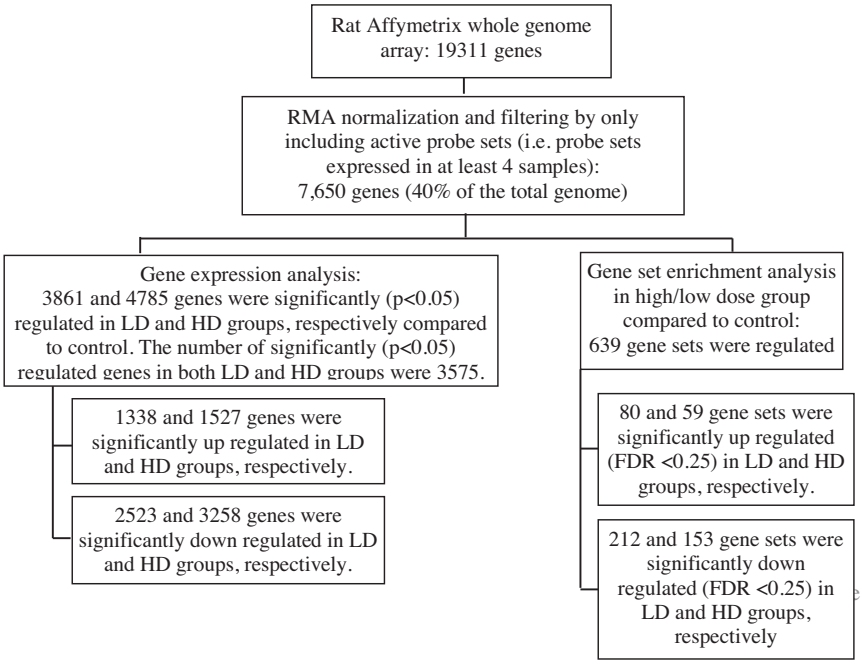

Fig. 3. Flow chart of microarray analysis.

glycitein in the feces of both LD- and HD-group rats. To estimate the relative amount (%) recovered in urine and feces, the administrated dose of the respective SIF as glucoside, was converted in 'aglycon equivalents' using their corresponding aglycone/glucoside molecular weight ratio [1]. For equol, the amount in feces and urine was first converted into 'daidzein equivalent' based on the respective molecular weights and then compared with the administrated dose of daidzin expressed as 'daidzein equivalents'.

The relative amount of daidzein recovered in urine plus feces collected over a 24 h period was 36% and 33% of the administered dose of daidzin (expressed as aglycone equivalents) for the LD and HD group, respectively (Table 3). When also equol was taken into account a total of 65% (36 + 29%) and 47% (33 + 14%) of the administrated dose of daidzin was recovered in the LD and HD group, respectively. For genistein the relative amount recovered in urine plus feces was 22% for both the LD and the HD group. Glycitin could not be detected in the urine and faces of LD rats, and only in the feces of HD rats, at a relative amount of 22% of the administered glycetin dose (Table 3).

3.2. Microarrays

Fig. 3 shows an overview of the microarray analysis. Out of 7650 filtered genes a total of 3861 and 4785 genes were significantly changed ($p < 0.05$) in the LD and the HD group compared to control, respectively. Of those genes 1338 were up regulated in the LD group and 1527 in the HD group, and 2523 genes were down regulated in the LD group and 3258 in the HD group. Fig. 4 shows a multidimensional scaling (MDS) plot that was created using the top 500 most affected genes. Separation of the samples on the first dimension (i.e. *X*-axis), explaining most variation,

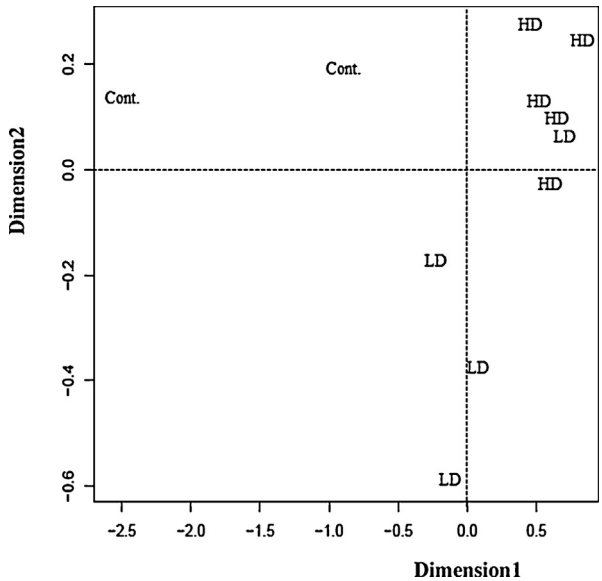

Fig. 4. Multidimensional scaling (MDS) plot off all arrays. The plot was generated using the top 500 most affected genes. The distances in both dimensions are arbitrary units where LH, HD and cont. stands for low dose, high dose and control rats, respectively.

suggests a dose response. In the 2nd dimension (i.e. *Y*-axis) a smaller variation and a separation of the LD group from the HD and control groups is observed.

In Fig. 5 the log (base 2) fold change plot of all the 7650 filtered genes, without using any cut off value, in the two different treatment groups compared to the control is presented. The upper right quadrant shows up regulation of genes in both dose groups and the lower left quadrant

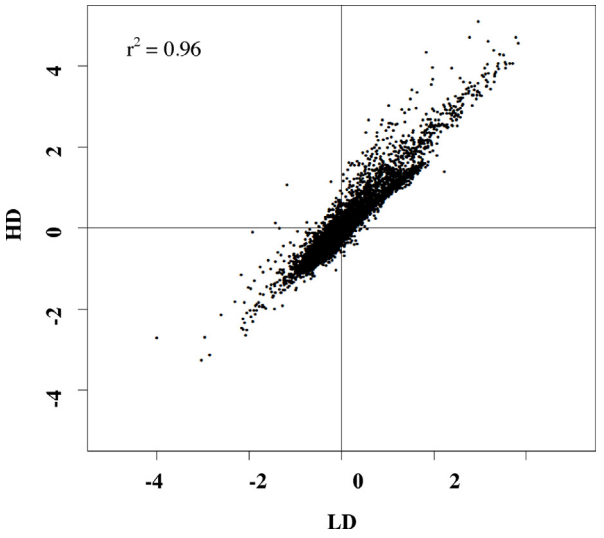

Fig. 5. Log (base 2) fold change (FC) plot of LD vs. HD groups compared to controls. The log2 FC plots were prepared using data of all 7650 genes.

shows down regulation of genes in both groups. It can be seen that administration of the SIF supplement produced a maximum 4-fold change in gene expression where more genes were positively regulated. Moreover, there is a positive correlation ($r^2 = 0.96$) in effects on gene expression between the LD and the HD group.

As the effect of SIF are expected to be induced via estrogen receptors (ERs), we have selected all the estrogen-responsive genes (ERGs) for the rat available in Dragon estrogen-responsive database (http://datam.i2r.a-star.edu.sg/ergdbV2/) which is based on the publication of Tang et al. [51]. Table 4 shows the significantly ($p < 0.05$) up and down regulated estrogen-responsive genes (ERGs) in the LD and HD groups. Out of 87 available rat ERGs 16 ERGs were found to be significantly regulated in the LD group, of which 7 genes were up-regulated and 9 down-regulated. In the HD group, 19 ERGs were significantly regulated, of which 8 were up-regulated and 11 down-regulated. A number of 15 ERGs were induced in the same direction in the LD and the HD group, meaning that genes that were up-regulated in the LD group were also up-regulated in the HD group, and those that were down-regulated in the LD group were also down-regulated in the HD group.

As next step GSEA was performed to elucidate which differentially regulated gene sets related to biological processes could be affected by treatment with SIF. Data revealed that a total of 639 gene sets were changed in the two treatment groups, of which 80 and 59 gene sets were up-regulated and 212 and 153 were down-regulated in LD and HD group respectively, compared to the control and based on a false discovery rate (FDR) < 0.25 (Fig. 3). Based on the KEGG database, Table 5 shows biological pathways (BPs) which are significantly up (↑) or down (↓) regulated. In total 54 KEGG based BPs were significantly changed out of which 21 and 27 gene sets were up regulated and 16 and 21 gene sets were down regulated in the LD and HD group, respectively. About half of these BPs were affected in the same direction in the LD and the HD group (i.e. either significantly up regulated or down regulated in both dose groups). The data show that all the BPs related to genetic information processing such as RNA transport and degradation and DNA replication were down regulated. Some of the BPs related to human diseases such as pathways related to colorectal and pancreatic cancer are down regulated; others such as hypertrophic and dilated cardiomyopathy were up-regulated. Also BPs related to environmental information processing, such as extracellular matrix (ECM)-receptor interactions which serves an important role in tissue and organ morphogenesis, and control of cellular processes such as endocytosis, phagocytosis, proliferation and apoptosis, were significantly up regulated. For a number of BPs a significant effect was found in one dose group only, whereas the effect in the other group was non-significant. This is for instance the case for the citrate cycle, oxidative phosphorylation, regulation of the actin cytoskeleton and others (see Table 5).

Fig. 6(a) presents the rank-rank scattered plot based on the *t*-value of the significantly changed and common PBMC genes in the two dose groups of rats. Out of 7650 filtered genes (see Fig. 3) 3575 genes were significantly changed in both LD and HD groups, and these were used to prepare the scatted plot. The size of quadrants in Fig. 6(a) is not the same, because the number of significantly down regulated genes was higher than number of significantly up regulated genes (see Fig. 3). A strong correlation in gene expression profile between both treatment groups (LD and HD) relative to the controls of the rat study is observed.

4. Discussion

In this study we evaluated the use of ovariectomized rat model to predict (post)menopausal health risk and/or benefit of SIF supplementation by comparing transcriptomics data obtained in a parallel human intervention study in which a similar SIF supplement were given to (post)menopausal women. The plasma concentration of our study shows a dose dependent increase of SIF after administration of different doses of a commercial supplement. This is also reflected by the differences in Cmax and AUC (Fig. 1 and Table 2). Only daidzein and its metabolite equol could be detected in plasma, because the commercial supplement contained a higher amount of daidzin than of the other SIF. The uptake of daidzein is very fast, peaking at 10 min (see inset of Fig. 1). In the LD group, which is the relevant dose group to be compared with the experiment in human volunteers, the levels decrease, and then rise again, with a peak at 2 h. This biphasic pattern in the plasma concentration of daidzein, suggesting enterohepatic circulation, is also observed in human intervention studies [52–54]. Franke et al. [55] mentioned that this biphasic plasma bioavailability might occur due to the location of uptake of SIF, where the first early peak represent the uptake from the small intestine and the second peak represents the uptake from the large intestine. However, in the HD group, with a 10 times higher dose than the LD group, this biphasic peak did not appear. It can be suggested that due to the higher amount of SIF in the small intestine, compared to the LD group, a longer period is needed to deconjugate the administered dose of SIF glucosides by the

Table 4
List of significantly ($p < 0.05$) up- and down regulated estrogen-responsive genes (ERG) in PBMC of ovariectomizedF344 rats treated with a low (LD, 2 mg/kg b.w.) or high (HD, 20 mg/kg b.w.) dose of SIF from a commercial supplement, compared to the control. Up and down regulation are indicated with up and down arrows. A list of ERG in the rat can be found in the Dragon ERG database (http://datam.i2r.a-star.edu.sg/ergdbV2/).

Gene	Description	LD	HD
ANGPT1	Angiopoietin 1	↑	↑
ANXA4	Annexin A4	ns	↑
BCL2	B-cell CLL/lymphoma 2	↓	↓
CCND2	Cyclin D2	ns	↓
CDKN1B	Cyclin-dependent kinase inhibitor 1B	↓	↓
CEACAM1	Carcinoembryonic antigen-related cell adhesion molecule 1 (biliary glycoprotein)	↑	↑
HK1	Hexokinase 1	↑	↑
HPCAL1	Hippocalcin-like 1	↓	↓
IL6R	Interleukin 6 receptor	↓	ns
ITGA6	Integrin, alpha 6	↑	↑
MARK2	MAP/microtubule affinity-regulating kinase 2	ns	↓
NCOA1	Nuclear receptor coactivator 1	↓	↓
NDRG2	NDRG family member 2	↓	↓
PAM	Peptidylglycine alpha-amidatingmonooxygenase	↑	↑
PHF5A	PHD finger protein 5A	↓	↓
PSMA7	Proteasome (prosome, macropain) subunit, alpha type 7	↓	↓
RB1	Retinoblastoma 1	↑	↑
SCP2	Sterol carrier protein 2	ns	↓
SFRP4	Secreted frizzled-related protein 4	↑	↑
SLC25A4	Solute carrier family 25 (mitochondrial carrier; adenine nucleotide translocator), member 4	↓	↓

ns = not significant.

intestinal enzymes, leading to a longer absorption time in the small intestine, thereby preventing the occurrence of a biphasic absorption behavior. Sepehr et al. [1] also did not observe any biphasic behavior in the plasma profile of SIF in Sprague Dawley rats receiving an oral gavage of 20 mg/kg b.w. of a commercial soy supplement.

In feces no conjugated SIFs were detected. This is in line with other authors [38,56] reporting that SIFs are completely deconjugated by gut microbiota. In both the LD and HD group the equol concentration was significantly higher compared to daidzein, indicating an efficient conversion of daidzein into equol by rat colonic microbiota [57,58]. The later appearance of equol in plasma compared to daidzein is also consistent with its production in the large intestine [1,59]. All rats are equol producers and several colonic bacteria are involved in the production of equol in rats [59]. However, only 25–30% of the adult Western population is able to produce equol due to lack of the specific colonic microbiota. This percentage is significantly lower than in Eastern Asian populations where more than 50%

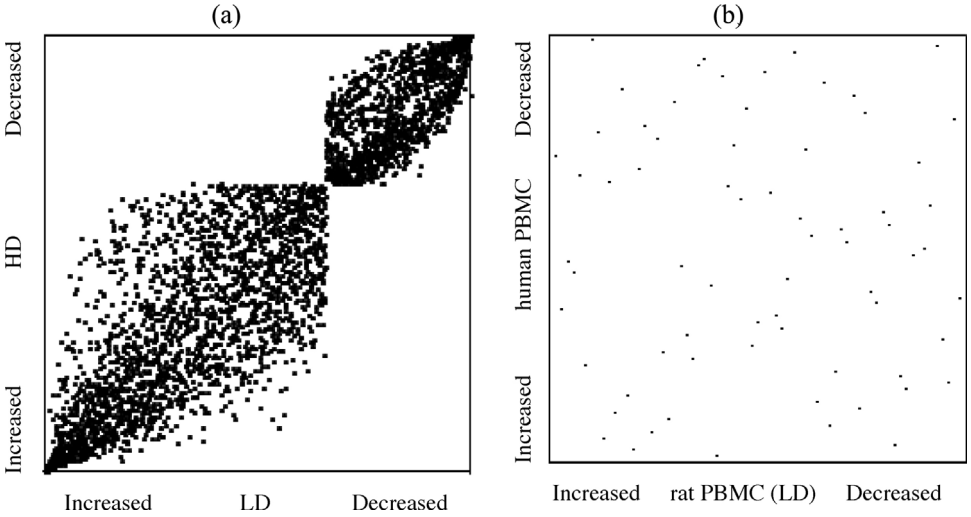

Fig. 6. Rank–rank scattered plot for significantly changes in gene expression among the LD and the HD group relative to the controls (a), and for the LD group compared to (post)menopause women (b). A similar picture was found for HD rats vs. (post)menopause women (data not shown). Out of 7650 filtered genes (see Fig. 3) 3575 genes were significantly changed ($p < 0.05$) in both LD and HD groups; while out of 7650 filtered genes, only 69 genes were significantly changed ($p < 0.05$) in both rat and homologous human PBMC. These 3575 and 69 genes were used to build the scattered plot. Each dot represents the *t*-value of a single gene. Corresponding human homologous genes were retrieved from the study with (post)menopausal women taking the same supplement, in a similar dose as the LD rats [23]. 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. The density of the dots visualize similar trends in regulation among the two different groups.

Table 5
Gene set enrichment analysis (GSEA) of KEGG based significantly regulated (FDR < 0.25) biological pathways (BPs) in the PBMC of ovariectomized F344 rats treated with a commercial supplement compared with control. LD and HD stands for low and high dose groups, while up and down regulated enrichments are indicated by up and down arrows. The names of the different pathways are literally quoted from KEGG together with the number of genes involved in the specific pathways.

BPs related to	BP	No. of Genes	cLD_c	cHD_c
Mebabolism	Citrate cycle (TCA cycle)	24	↓	ns
	Oxidative phosphorylation	82	↓	ns
	Glutathione metabolism	30	↑	↑
	N-glycan biosynthesis	36	↓	↓
	Porphyrin and chlorophyll metabolism	17	↑	↑
Genetic information processing	Basal transcription factors	22	↓	ns
	Spliceosome	94	↓	↓
	Aminoacyl-tRNA biosynthesis	25	↓	ns
	Ribosome biogenesis in eukaryotes	56	↓	↓
	Ribosome	35	↓	↓
	RNA transport	105	↓	↓
	mRNA surveillance pathway	52	↓	↓
	RNA degradation	49	↓	↓
	Proteasome	40	↓	ns
	Protein export	18	↓	↓
	Protein processing in endoplasmic reticulum	129	↓	ns
	DNA replication	28	↓	↓
	Base excision repair	18	↓	↓
	Mismatch repair	19	ns	↓
Environmental information processing	ABC transporters	15	↑	ns
	Neuroactive ligand-receptor interaction	33	↑	ns
	ECM-receptor interaction	19	↑	↑
Cellular processes	Lysosome	92	ns	↑
	Endocytosis	126	↑	↑
	Phagosome	90	↑	↑
	Regulation of actin cytoskeleton	104	↑	ns
	Focal adhesion	81	↑	↑
	Gap junction	37	↑	↑
Organismal System	Complement and coagulation cascades	16	↑	↑
	Toll-like receptor signaling pathway	54	↓	ns
	Hematopoietic cell lineage	45	↑	↑
	Natural killer cell mediated cytotoxicity	63	ns	↓
	T cell receptor signaling pathway	77	↓	↓
	Intestinal immune network for IGA production	20	ns	↓
	PPAR signaling pathway	26	↑	↑
	Cardiac muscle contraction	29	ns	↑
	Vascular smooth muscle contraction	50	↑	ns
	Pancreatic secretion	37	↑	ns
	Aldosterone-regulated sodium reabsorption	18	↓	ns
	Long-term depression	31	↑	ns
	Circadian rhythm – mammal	15	↓	↓
Human disease	Pathways in cancer	142	↓	↓
	Chagas disease (americantrypanosomiasis)	59	↓	↓
	Malaria	23	↑	↑
	Colorectal cancer	46	↓	↓
	Pancreatic cancer	41	ns	↓
	Primary immunodeficiency	26	ns	↓
	Parkinson's disease	81	↓	ns
	Huntington's disease	109	↓	ns
	Prion diseases	18	↓	ns
	Hypertrophic cardiomyopathy (hcm)	23	↑	↑
	Arrhythmogenic right ventricular cardiomyopathy (arvc)	20	↑	ns
	Dilated cardiomyopathy	25	↑	↑
	Staphylococcus aureus infection	18	↑	↑

of all adults can produce equol [25,48,59]. Sepehr et al. [1] reported that dosing Sprague-Dawley rats (both male and female) with a mixture of all three SIF glucosides or a commercial SIF supplement increases the production of equol compared to dosing with aglycone alone. In vitro studies with human colonic bacteria suggested that a high carbohydrate environment stimulates the colonic fermentation and increases the rate of conversion of daidzein into equol [16,17].

From the transcriptomic analysis it appears that out of the 19,311 gene probes on the array chip about 20–25% genes were significantly changed in the different dose groups compared to the control (Fig. 3). To the best of our knowledge there is no literature available on the effect of soy supplementation on gene expression in rat PBMC, therefore, it was not possible to directly compare our PBMC data with published data. However in the parallel study of van der Velpen [23] PBMC of (post)menopausal

equol-producing human volunteers, exposed to the same SIF supplement, only 2% of the total number of genes was significantly changed. This suggests that rats might be more susceptible to effects of SIF than humans. It is unclear, however, to what extent the inbred nature of the rats could have contributed to this difference. In both studies the transcriptomic data were produced and analyzed in the same laboratory, thus preventing intra laboratory variation between the two studies. It is generally known that rats have a higher metabolic potency compared to humans which is also true for SIF [17]. Therefore a higher enzymatic activity in rats might probably results in a higher number of affected genes [60]. In the rat study also a strong dose dependent correlation was shown by the rank-rank scattered plot (Fig. 6a) and the log (base 2) analysis (Fig. 5). We also found that about 20% of the total rat ERGs was significantly changed: 16 and 19 ERGs were significantly regulated in LD and HD group, respectively out of 87 ERGs. Some of the affected ERGs are involved in important biological endpoints that might be related to beneficial health effects like lower risks for breast cancer as observed in epidemiological studies [13,61]. For example, the RB1 (retinoblastoma1) gene, which is up regulated in the present study after SIF treatment, is a tumor suppressor gene, and a negative regulator of the cell cycle [62]. Hanahan and Weinberg [64] stated that down regulation of the retinoblastoma protein pathway by the RB1 gene is a "hallmark of cancer". Down regulation of RB1 is positively correlated with tumor formation and in our present experiment this RB1 gene was up regulated, indicating a possible beneficial effect of SIF supplementation on tumor formation.

To investigate whether the observed effects in rats are predictive for effects in humans, the gene expression data from the present rat study were compared with the data of van der Velpen et al. [23] who performed a parallel human intervention study. They found that of the 1069 human ERGs mentioned in the Estrogen Responsive Gene Database [51] only 17 human ERGs were significantly changed, of which 7 were up regulated and 10 were down regulated. We have investigated whether these 17 human ERGs are also significantly regulated in the present rat study. Table 6 shows the significantly expressed ERGs in PBMC of human volunteers reported by van der Velpen et al. [23] and their expression in PBMC of rats of the current study. It is shown that 10 out of these 17 genes were common in both rat and human PBMC. Seven of these 10 genes were significantly changed, but only 3 were changed in the same direction in rats and humans: CACYBP and NME2 were up-regulated and STXBP1 was down-regulated. It is interesting to note that more than half of the number of human ERGs is also found in rat PBMC and that most of them are significantly affected by SIF treatment. However, most of the common genes are regulated in opposite directions in rats and humans. This means that if they are up regulated in human PBMC they are down regulated in rat PBMC or if they are down regulated in human PBMC they are up regulated in rat PBMC. Therefore rat PBMC do not seem to be a suitable model to predict effects of SIF treatment on ERGs in human PBMC. It should, however, be noted that these human genes identified as ERGs by the Estrogen Responsive Gene Database [51], and also found in rat PBMC, are not identified as rat ERGs by the Dragon Database. Further information is needed to conclude whether these genes are also estrogen responsive in rats.

When the significantly changed and common PBMC genes in the LD group of the rat study and the human intervention study by van der Velpen et al. [23] were compared, it was found that only 69 common genes were significantly changed in both rat and human PBMC. However, in the rank-rank scattered plot (Fig. 6b) no overall correlation for

Table 6
List of the significantly expressed human estrogen responsive genes (ERGs) in PBMC of human volunteers reported by van der Velpen et al. [23] and their expression in PBMC genes in the present rat study. LD and HD stands for low and high dose groups while up and down regulated gene expressions are indicated by up and down arrow marks.

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

a – absent; ns – not significant.

the changes in PBMC of rats and humans was observed. Also for the HD group of the rat study no correlation with the results and the human intervention study was found (data not shown). This lack of correlation is in line with the observation that of the common affected ERGs in rat and human PBMC. Only three were changed in the same direction. Another important example of a gene that is affected in a different direction in rat and human is BCL2 which is up regulated in human indicating induction of apoptosis [33] but down regulated in rat.

In the present study we have observed beneficial effect of SIF on ovariectomized rat but also significant species differences in the changes in gene expression in PBMC of rats and humans following comparable exposure to a commercial SIF supplement. Setchell et al. [63] also found significant species differences of the circulating concentrations of aglycones among rodent and human. Based on differences in the proportion of unconjugated SIF in plasma of humans in rodents, in particularly for certain strains of mice, they also questioned the value of these rodent models for the assessment of effects of SIF in humans. However, in the article of Setchell et al. [63] different isoflavone sources and administration protocols were used to treat animals and human volunteers. Animals were treated with much higher dose levels compared to humans. In addition the adult human data span wide range of ages (i.e. from 21 to 65 years). Therefore, the higher aglycone concentrations of IF in the plasma of rats compared to that of humans may also be due to the higher dose levels used in the rat studies and enzymatic variation among different age group of human individuals. In our present study we have overcome these difficulties by using the same source and dose level to compare plasma bioavailability and PBMC gene expression of ovariectomized rat with equol producing menopause women. To further increase the comparability of rat data with human data, only equol producing menopausal women were included in the parallel human study because all rats are equol producers, while only 25–30% of the Western populations are equol producers [59].

However, the comparison of the results of the current rat study with those of the parallel study with human volunteers [23] indicates that the rat might not be a suitable model to predict effects of SIF in humans. It should, however, be recognized that, although the relative exposure period in rats and the human volunteers (i.e. 0.27% for rat and 0.22% for humans, see Section 2.4) in relation to their respective life span is comparable, longer term repetitive administration of rats to SIF may be needed to draw a final conclusion on the suitability of the rat model, because longer exposure might lead to a difference in gene expression [62].

Conflicts of interest

The authors have declared no conflict of interest.

Transparency document

The Transparency document associated with this article can be found in the online version.

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