

## Dose-dependent effects of isoflavone exposure during early lifetime on the rat mammary gland: Studies on estrogen sensitivity, isoflavone metabolism and DNA methylation

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**Abbreviations:** **DAI**, daidzein; **ER**, estrogen receptor; **E2**, estradiol; **GEN**, genistein; **GLY**, Glycitein; **IDD**, isoflavone-depleted diet; **IRD**, isoflavone-rich diet; **ISO**, isoflavones; **OVX**, ovariectomized control group; **PCNA**, proliferating cell nuclear antigen; **PND**, postnatal day; **PR**, progesterone receptor; **UWW**, uterine wet weight; **UEH**, uterine epithelial height, **VO**, vaginal opening

## Abstract

Scope: Isoflavone (ISO) exposure during adolescence modulates  $17\beta$ -estradiol (E2) sensitivity of the adult mammary gland. The present study investigated the dose-dependency of these effects focusing on proliferation, estrogen receptor (ER)-dependent and -independent gene expression, as well as DNA methylation and ISO metabolism.

Methods and results: Female Wistar rats were lifelong exposed to an ISO-depleted diet (IDD) or to diets enriched with a soy ISO extract (IRD) causing plasma concentrations as observed minimally (IRD<sub>low</sub>) and maximally (IRD<sub>high</sub>) in Asian women. The extract was characterized by both phytochemical analysis and E-Screen. Rats were ovariectomized at postnatal day (PND) 80 and treated with E2 from PND94 to 97.

In contrast to uterine response, body weight and visceral fat mass were affected by ISO. In the mammary gland, both E2-induced proliferation (PCNA staining) and ER activation (progesterone receptor staining) were significantly reduced by IRD<sub>high</sub> but not by IRD<sub>low</sub>, which however attenuated Gdf15 mRNA expression. DNA methylation analysis revealed significant differences in the promoter regions of Aldh11, Extl1 and WAP between IRD<sub>high</sub> and IDD.

Conclusion: Lifelong exposure to ISO results in dose-dependent differential effects on proliferation, gene expression and DNA methylation in rat mammary glands. Yet, a decrease in estrogen-responsiveness was only achieved by IRD<sub>high</sub>.

## 1. Introduction

Recent meta-analyses of epidemiological studies provide increasing evidence that intake of soy isoflavones (ISO) is associated with a reduced risk of breast cancer incidence in Asian populations [1-3]. Individual studies showed this inverse relation for premenopausal [4] and postmenopausal breast cancer risk [5]. In addition, soy consumption was associated with a lower breast cancer recurrence rate [6]. These observations are in line with animal studies describing chemopreventive properties of ISO exposure at early stages of mammary gland development for hormone dependent breast cancer [7]. In contrast, studies performed in the UK failed to link vegetarianism and resulting urinary ISO levels to the risk of hormone dependent breast cancer [8]. Levels of ISO intake in most Western societies are in average less than 1 mg per day compared to 20-50 mg/day in many Asian countries where soy is part of the traditional diet [9, 10]. Accordingly, plasma concentrations of total ISO equivalents in Western women are negligibly low compared to those of Asian women which have mean concentrations in the range of several hundred nanomolar and reach maximum concentrations up to the low micromolar levels [11, 12]. Moreover, the time point in life when the exposure to ISO starts is considered important. Lee et al. (2009) described a protective effect against breast cancer for women who consumed high amounts of soy foods consistently during adolescence and adulthood whereas no significant association with soy food consumption was found for postmenopausal breast cancer [4]. However breast cancer risk is affected by a number of additional hormonal and reproductive risk factors [13-15] which modulate estrogen activity on the breast tissue. Therefore the sensitivity of the breast tissue towards estrogens is a critical determinant with respect to breast cancer development. The major missing link for cross species comparisons is the lack of animal studies equivalent to the human dietary exposure to ISO. Dietary consumption of ISO represents a lifelong or even multigenerational, low-dose exposure with these compounds. Regarding long term studies on ISO exposure, only a two years toxicity study nested in a multigenerational reproductive study of the National Toxicology Program has been published for genistein [16]. In this study an increased incidence for breast adenomas and adenocarcinomas became apparent as a statistically significant trend in the F1 generation in the 500 ppm exposure group. Previous studies revealed that estrogen responsiveness of target organs is critically influenced by the timing of exposure to ISO. Following a two generation feeding of Wistar rats with chow enriched with an ISO-containing soy extract or isolated genistein, treatment with 17 $\beta$ -estradiol (E2) in an uterotrophic assay significantly increased the uterine weight gain in response to E2 far above stimulation in control animals on phytoestrogen-free placebo diet [17]. Conversely, the same dietary exposure to ISO inhibited E2-induced

proliferation in mammary glands of the identical set of ovariectomized animals far below proliferation levels detectable in animals on a phytoestrogen free diet. This demonstrates that ISO exposure over two generations modulates hormone responsiveness in an organ-dependent manner, which can be further modulated by timing of exposure in specific windows of adolescence [18]. However, the underlying molecular mechanisms need to be resolved.

The present study assessed the dose-dependent impact of dietary ISO exposure from conception to adulthood on estrogen sensitivity of the mammary gland in ovariectomized and E2-stimulated female Wistar rats. In order to be able to extrapolate data gained to the human situation, we determined plasma concentration and metabolism of ISO and of E2. Mechanistically, we focused on cell proliferation, as well as on the expression and promoter methylation of selected estrogen-responsive target genes. Our data indicate that in the present animal model only high ISO exposure leading to plasma concentrations of total ISO equivalents of about 2.1  $\mu\text{M}$  mirroring maximum Asian exposure reduced E2 sensitivity in the mammary gland.

## 2. Materials and Methods

### 2.1 Chemicals

ISO rich soy extract (NovaSoy650®) was purchased from ADM, Decatur, Illinois, USA. [2,3,4- $^{13}\text{C}_3$ ]Daidzein (13C3-DAI) and [2,3,4- $^{13}\text{C}_3$ ]genistein-7- $\beta$ -D-glucuronide (13C3-GEN-7-GlcA) were provided by Nigel Botting and Nawaf Al-Maharik (University of St. Andrews, UK). The ISO standard compounds were synthesized in our lab or purchased from various companies as described recently [19]. Chemicals for the quantification of estrogen levels comprising E2, E2-17-glucuronide, E2-3-glucuronide, E2-3-sulfate, E1-glucuronide, E1-sulfate, and E1 were obtained from Steraloids (Newport, USA), glucuronidase (bovine liver, Type B-1, 1644000 U/g) was obtained from Sigma Aldrich (Taufkirchen, Germany).

Kits and reagents for RNA isolation and deoxyribonuclease digestion were obtained from Sigma Aldrich (Taufkirchen, Germany). Reverse transcription kits, PCR reagents and equipment were purchased from Life Technologies (Darmstadt, Germany). Nuclease-free water was obtained from Thermo Scientific (Walldorf, Germany).

Unless indicated otherwise, methods using commercial available kits or assays were performed according to manufacturers' protocols. All other chemicals and solvents used were of analytical grade.

## 2.2 Cell culture and estrogen dependent proliferation of MCF-7 cells

MCF-7 BUS cells [20], kindly provided by Ana Soto (Tufts University, Boston, MA, USA), were grown in DMEM high glucose supplemented with 1 mM pyruvate, 2.25 g/l sodium bicarbonate, 100 U/ml penicillin, 100 µg/ml streptomycin, and 5% heat-inactivated FCS.

MCF-7 cells were seeded in Linbro®24-well plates (MP Biomedicals, Eschwege, Germany; 20,000 cells per well) in phenol red-free DMEM medium (AppliChem, Darmstadt, Germany) supplemented with 5% charcoal/dextran-treated FCS (Hyclone), 4.5 g/L D(+)-glucose, 15 mM HEPES Buffer and 2.25 g sodium bicarbonate 24 h prior to incubation with a) various concentrations of the extract, b) a mixture of ISO exhibiting the same concentration as within the extract, c) a mixture ISO aglycones and glycosides exhibiting the same concentration as within the extract, or d) with 100 pM E2 which induces maximum proliferation [21]. After 6 days, culture medium was aspirated and cell numbers and cell cycle distributions were determined: Cells were trypsinized, resuspended in culture medium and counted electronically (CASY, Schaefer System, Reutlingen, Germany). For the determination of the cell cycle distribution, cells were lysed and stained using the two-step reagents from Partec (Münster, Germany). Flow cytometry was carried out with a CyFlow®-Space (Partec) and analyzed with FlowMax®software version 2.60 (Partec).

## 2.3 Experimental animals, diets and study design

Wistar rats were obtained from Janvier Laboratories (Le Genest St Isle, France) and kept at least for 2 weeks on IDD prior to mating. All animals were kept under controlled conditions of temperature ( $20 \pm 1^\circ\text{C}$ ), relative humidity (50-80%) and illumination (12 h dark, 12h light). The animals were housed up to 6 animals per cage, had access to water *ad libitum* as well as to experimental diets used, which differed in their content of ISO. Food consumption and body weight were checked twice a week for monitoring animals' development. The diets investigated comprised a ISO-depleted diet (IDD; containing 3 ppm levels of genistein aglycone equivalents; Supplemental Table 1; Ssniff R/M-H phytoestrogen-free, Ssniff GmbH, Soest, Germany) or specific ISO-enriched diets (IRDlow and IRDhigh; Supplemental Table 2 and Supplemental Table 3; Ssniff GmbH, Soest, Germany) manufactured by adding a soy extract (NovaSoy650®, ADM, Decatur, Illinois, USA; Table 1) at the amount of 0.131g/kg (IRDlow) or 1.05g/kg diet (IRDhigh). The IRDlow diet was colored in green with a mixture of quinoline yellow, Patent Blue V and Ponceau 4R, whereas the IRDhigh diet was colored in blue with a mixture of Carmine, Patent Blue V, and Brilliant Black BN for differentiation.

For the two-generation intervention study (Figure 1), groups of breeder rats (one female and one male initially and two females and one male later on) were randomly split into three

feeding groups receiving either IDD, IRDlow, or IRDhigh. Dams received respective diets from mating until weaning. Thus, female F1 were exposed to ISO during conception, the fetal *in utero* period and through weaning and then received either IDD, IRDlow, or IRDhigh during adolescence, ovariectomy until sacrifice in adulthood (postnatal day, PND97). On PND80 females were ovariectomized and randomly split into two treatment groups. After 14 days of endogenous hormonal decline (PND94) animals were subcutaneously treated for three consecutive days with E2 (E2; 4 µg/kg bw/day, dissolved in 20% ethanol/peanut oil, Sigma Aldrich, Deisenhofen, Germany) or received vehicle control (OVX; 20% ethanol/peanut oil; 1 ml/kg bw/day), resulting in six experimental treatment groups (n=6-9 animals/group). Three hours prior to sacrifice at PND97 the animals received an additional treatment with E2 in order to monitor E2 plasma levels. All animal handling and experimental conditions were carried out according to the "Institutional Animal Care and Use Committee guidelines", regulated by the German federal law for animal welfare permission number 13.12.2010 87-51.04.2010.A336

#### **2.4 Vaginal opening**

Determination of vaginal opening was performed as previously described in Lewis et al. 2002 [22]. The animals were checked for their opening of the vagina daily.

#### **2.5 Tissue preparation**

After body weight determination, animals were decapitated and blood was collected for serum and EDTA-plasma analysis. Mammary glands, uteri and visceral fat were removed and the uterine wet weight as well as the visceral fat mass was determined. Specimen of each tissue were either snap frozen in liquid nitrogen for DNA, mRNA and protein isolation or fixed in 4% formaldehyde solution for histological examination.

#### **2.6 Histological determination of the uterine epithelial height**

The uterine epithelial height (UEH) in addition to UWW is a commonly used parameter to evaluate the estrogenic potential of a test compound. Therefore the formalin fixed and paraffin embedded uteri were cut into 7 µm sections and were mounted on slides. After rehydration the slides were stained with standard HE protocol. The height of the uterus epithelium was assessed. Approximately 100 randomly picked cells per animal were measured by using picture analysis software (AxioVison LE Rel.4.8, Zeiss, Germany).

## 2.7 Immunohistochemistry

Immunohistochemical analysis of PCNA and PR expression in mammary glands was performed as previously described [23].

## 2.8 Determination of serum leptin levels

Serum was obtained by centrifugation at +4°C and 3000 g and stored at -20°C. Serum levels of leptin were analyzed using ELISA kits for rats according to the manufacturer's protocol (mouse/rat Leptin ELISA E06, Mediagnost®, Reutlingen, Germany).

Mammary gland tissue was powdered in liquid nitrogen. Then, total RNA was isolated using GenElute™ Mammalian Total RNA Miniprep Kit followed by a DNA digest using Deoxyribonuclease I. Remaining fat from breast tissue was extracted by shaking lysates with chloroform for 30 seconds prior to binding RNA to the column. RNA content was determined via OD 260/280 using TECAN nanoquant® plate (Tecan, Crailsheim, Germany). Then, up to 1 µg total RNA was reversely transcribed using High Capacity cDNA Reverse Transcription Kit (Life Technologies, Darmstadt, Germany).

## 2.9 Analysis of transcript levels

TaqMan® Gene Expression Assays for Hprt (Hprt1-Rn01527838\_g1), Ki-67 (MKi67-Rn01451446\_m1), Gdf15 (Gdf15-Rn00570083\_m1), and Mt1a (Mt1a-Rn00821759\_g1) were performed as follows: a 20 µl reaction consisting of 1 µl cDNA, 10 µl 2x TaqMan® Gene Expression Master Mix, 8 µl nuclease-free water, and 1 µl HPRT-TaqMan® Gene Expression Assay were mixed and pipetted into wells of a 96 well reaction plate. The PCR was run on the 7900HT Fast Real-Time PCR System for real-time PCR analysis. Ct values were automatically generated by SDS Software 2.3.

The initial copy number ( $n_0$ ) for each transcript was calculated assuming  $1 \times 10^{12}$  copies at threshold Ct ( $n_{ct}$ ) and the efficiency (E) 100% (2) according to the formula:  $n_0 = n_{ct} / E^{ct}$

## 2.10 Quantitative DNA Methylation Analysis by EpiTyperMassARRAY

Candidate genes with putative epigenetic regulation of expression were selected from data on differential expression after long term ISO- and/or E2- treatment in Wistar rats ([24], GEO Acc. No. GSE38060). Promoter regions of the most significantly up- or downregulated genes were overlapped with regions showing differential enrichment of methylated DNA after ISO- and/or E2-treatment, as detected by a genome-wide methylation screen using Methyl-CpG-Immunoprecipitation (MCIp)-Sequencing (Pudenz et al., in preparation). The top 3 genes were selected for quantitative analysis using mass-spectrometry based

EpiTyperMassARRAY technology (Sequenom, San Diego, USA) as described before [25]. Using this system, methylation differences  $\geq 5\%$  are detectable by quantifying median methylation of CpG units (single CpGs or two or more CpGs analysed together). Genomic DNA was isolated with a Qiagen Blood and Tissue kit and subjected to sodium bisulfite treatment using the EZ DNA Methylation™ Kit (Zymo Research, Orange, USA), according to the manufacturers' instructions. Subsequent PCR amplification and base-specific cleavage of nucleic acids enabled separation of methylated and unmethylated CpG units according to their mass to charge ratio (m/z). Primer sequences are listed in Table 2. Genomic locations and positions of amplicons are summarized in Supplemental Figure 3.

### 2.11 Quantification of ISO in the soy extract and the animal diets

Quantification of ISO levels in the animal diets were determined by LC-DAD as described previously [18]. For determination of ISO level in the soy extract two alterations were made. Firstly, a different sample quantity (approx. 50 mg soy extract instead of approx. 250 mg diet) was processed and secondly, samples were diluted 50-times in 65% (v/v) methanol in water before analysis. For calculation of aglycone equivalents the amounts of the chemical substance ( $\mu\text{mol}$ ) for an aglycone and its corresponding glycosides were summed up and converted into mass units (mg) using the molecular weight of the aglycone.

### 2.12 Quantification of ISO in plasma

ISO and metabolites were quantified in plasma by UHPLC-MS/MS as described previously [19] with the following alterations. 100  $\mu\text{l}$  of rat plasma were thawed and 5  $\mu\text{l}$  of internal standard solution (solution containing 5  $\mu\text{M}$  [2,3,4- $^{13}\text{C}_3$ ]-daidzein and 5  $\mu\text{M}$  [2,3,4- $^{13}\text{C}_3$ ]-genistein-7- $\beta$ -D-glucuronide in DMSO) was added. The samples were diluted with 400  $\mu\text{l}$  of water and the following extraction and analysis was done as described previously. Calibration curves were done in blank plasma. Limits of quantification (LOQ) and detection (LOD) of ISO and metabolites in rat plasma are summarized in Supplemental Table 4. Chemical structures of analyzed ISO are depicted in Supplemental Figure S1.

### 2.13 Statistical Analysis

All data are represented as scatter plots with the exception of the body weight development and the MCF-7 BUS experiment. Each symbol represents data from one animal, horizontal lines are means, and vertical lines illustrate standard deviation. Statistical analysis was performed with GraphPad Prism (Version 6.03).

Normality and homoscedasticity were graphically checked with a boxplot for each group of animals. No systematic violations in normality were observed. For PCNA, GDF15 and serum leptin levels inhomogeneity of variances could not be excluded.



Statistical significance of differences was assessed by two-way ANOVA with the factors: E2 treatment, diet, and interaction between E2 treatment and diet. ANOVA was followed by Tukey's multiple comparison post hoc test with adjusted p-values. Statistical significance was established at  $*p \leq 0.05$ ,  $**p \leq 0.01$  and  $***p \leq 0.001$ . Statistical analysis of vaginal opening and the MCF-7 BUS experiment was assessed by one-way ANOVA followed by Tukey post hoc test (Origin, Version 8.6, GraphPad Prism, Version 6.03).

Associations of free and total E2 plasma level in animals with E2 treatment were described by using Pearson's correlation coefficient.

Methylation data are not normally distributed. Symbols represent median methylation differences per amplicon of one animal to the median methylation of the entire IDD control group. Vertical lines represent median methylation differences per group. Statistical analysis was done using the non-parametric Mann-Whitney test.

### 3. Results

A major goal of this paper was to assess biological consequences, specifically hormone responsiveness, of a dose-dependent lifelong exposure to ISO starting at conception and to elucidate the underlying molecular mechanisms. ISO exposure was realized by spiking an ISO free diet with a commercial ISO extract.

#### Extract characterization

To verify that estrogenicity of the soy extract can be attributed to the ISO content therein, the impact of both, extract and ISO on ER-dependent cell proliferation in cultured human breast adenocarcinoma MCF-7 cells was investigated. Maximum cell proliferation, i.e. increase in cell number and corresponding cell cycle distribution was induced by 0.5  $\mu\text{g}$  extract/ml culture medium (Supplemental Figure S2A). A mixture of GEN, DAI, and GLY mirroring the analytically determined aglycone composition of the extract at 0.5  $\mu\text{g}/\text{ml}$ , did not achieve the same stimulation of cell proliferation as the corresponding extract concentration (Fig. 2). In contrast, estrogenicity of the extract could be simulated when aglycones and glycosides were combined (Fig. 2), reflecting their occurrence in the extract (i.e. concentration of glycosides about 10 - 30 times higher than their respective aglycones).

## Dose-dependent effects of ISO on F1 females during adolescence

For the two-generation animal experiment the soy extract was added to a phytoestrogen-depleted standard diet (IDD) to obtain a low- and a high dose ISO enriched diet (IRD<sub>low</sub> and IRD<sub>high</sub>). We determined average ISO aglycone equivalents of 3 ppm for IDD (Supplemental Table 1), 68 ppm for IRD<sub>low</sub> (Supplemental Table 2), and 503 ppm for IRD<sub>high</sub> (Supplemental Table 3). Overall intake of IRD<sub>low</sub> or IRD<sub>high</sub>, based on daily food consumption of 20 g and an average body weight of 275 g, resulted in a daily ISO aglycone equivalent exposure of approximately 4.9 mg/kg BW or 36.6 mg/kg BW, respectively (around 2.5 mg/kg BW/d or 18.7 mg/kg BW/d of GEN equivalents). The IDD conversely led to an exposure level of 0.2 mg/kg BW/d of total ISO aglycone equivalents through exposure to genistein.

ISO exposure during adolescence significantly influenced the onset of puberty ( $p=0.0021$ ). In both the IRD<sub>high</sub> and the IRD<sub>low</sub> group we observed a statistically significant earlier onset of puberty of approximately 1.5 days compared to the IDD group, indicated by the time point of vaginal opening (Figure 3).

## Bioavailability of the extract

With the proven activity of the extract on vaginal opening we next asked the question, which quantities of its constituents are bioavailable *in vivo*, which are the major metabolites and whether there are differences in the quality and quantity of metabolites depending on ISO dose and E2 treatment.

We comprehensively investigated the conjugative metabolite profile of DAI and GEN in plasma (Table 3; chemical structures of the analyzed ISO and their metabolites are summarized in Supplemental Figure S1) at the end of the uterotrophic assay at PND97. In animals receiving IDD we detected at most traces of the DAI- or GEN-7-O- $\beta$ -D-glucuronide, with the exception of one rat, where a low concentration of 17.4 nM DAI-7-O- $\beta$ -D-glucuronide was found. The 7-O- $\beta$ -D-glucuronides were the predominant phase-II-metabolites in the IRD<sub>low</sub> and IRD<sub>high</sub> groups. Mean plasma concentrations in both IRD<sub>low</sub> groups (without and with E2 treatment, -E2/+E2) were 152/106 nM (-E2/+E2) for DAI-7-O- $\beta$ -D-glucuronide and 197/118 nM (-E2/+E2) for GEN-7-O- $\beta$ -D-glucuronide. For both IRD<sub>high</sub> groups mean plasma concentrations of 825/750 nM (-E2/+E2) for DAI-7-O- $\beta$ -D-glucuronide and 973/1068 nM (-E2/+E2) for GEN-7-O- $\beta$ -D-glucuronide were measured. DAI and GEN mono- and disulfates, the sulfoglucuronides as well as the 4'-O- $\beta$ -D-glucuronides were also detected but in much lower quantities (<10% related to the corresponding 7-O- $\beta$ -D-

glucuronide). Equol, which is derived from DAI by the action of the rat microbiota [26], was monitored as 7-O- $\beta$ -D-glucuronide and 4'-sulfate. Equol-7-O- $\beta$ -D-glucuronide was formed in each rat of the IRD groups with mean plasma concentrations of 41/15 nM in both IRDlow groups (-E2/+E2) and 274/271 nM in both IRDhigh groups (-E2/+E2). Equol-4'-sulfate was not detected in either of the IRDlow groups (except for one animal with 0.8 nM) but in low amounts in both IRDhigh groups with mean values of 1/2 nM (-E2/+E2). The qualitative metabolite profile was comparable among rats of all IRD groups and thus not influenced by E2 treatment or the dose of dietary IF exposure. The IRDhigh dose led to a 7.5x higher intake of ISO compared to IRDlow dose as calculated on the basis of the ISO content of the diet and the food intake. As a result total plasma concentrations of DAI and GEN (calculated as sum of all aglycone equivalents of DAI and GEN, Table 3) were significant 6.6-fold higher in the IRDhigh dose with mean plasma concentrations of 2094/2157 nM (-E2/+E2) compared to the low dose exposure (IRDlow) with mean plasma concentrations of 402/245 nM (-E2/+E2). The E2 treatment had no significant influence on total plasma concentrations of DAI and GEN ( $p = 0.875$ ). Also, no interaction effect (dose x E2 treatment) on total plasma concentrations of DAI and GEN was detected ( $p = 0.716$ ).

### **Effects of ISO exposure during adolescence on the estrogen sensitivity of body weight, body composition and the uterus**

To test effects of exposure to the IRDlow and IRDhigh diet on estrogen sensitivity, animals were ovariectomized at PND 80 and an uterotrophic assay was performed. Using two-way ANOVA, a highly significant effect for the factor diet ( $p = 0.00035$ ) and E2 ( $p = 0.0085$ ) was observed (Fig. 4A). The further statistical evaluation revealed that in the E2 intervention arm the exposure to IRDhigh significantly ( $p = 0.0001$ ) reduced bodyweight about 14% compared to IDD (Fig. 4A) whereas ISO exposure alone without E2 did not lead to a significant effect. In the IRDlow E2 group body weight was reduced about 7% compared to IDD E2 but without statistical significance ( $p = 0.158$ ). The visceral fat mass reflected the findings for body weight and was statistically significant reduced by diet (Fig. 4C,  $p = 0.0004$ ). Here we observed a significant decrease of visceral fat mass in the IRDhigh E2 group in comparison to the control group IDD E2 and the IRDlow E2 group of about 20%. Also, serum leptin levels were lowest in the IRDhigh group (Fig. 4D) with borderline significance (E2 treatment  $p = 0.0696$ , diet  $p = 0.0504$ ). No interaction of E2 treatment and diet was observed ( $p = 0.7627$ ).

Exposure to the IRDs had no effect on the uterine wet weights (UWW) (Fig. 4E,  $p = 0.546$ ). Treatment with E2 led to a significant increase of UWW ( $p < 0.0001$ ). Animals on IRDhigh seem to respond with a higher sensitivity than animals on IDD. This effect, however, did not reach the level of statistical significance. E2 treatment also increases the height of the

uterine epithelium (UEH, Fig. 4F) in all dietary groups compared to their control animals ( $p < 0.0001$ ).

### **Proliferative response of the mammary gland**

To evaluate the influence of the IRDs on the proliferative response of the mammary gland the protein expression of the proliferation marker PCNA was determined by immunohistochemistry (Fig. 5A and 5B). In all intervention groups, stimulation with E2 resulted in an increased proliferation index in the mammary gland ( $p < 0.0001$ ). PCNA protein expression was also affected by diet ( $p < 0.0001$ ) and by the combination of diet and E2 ( $p < 0.0001$ ). Interestingly, in the IRD<sub>low</sub>, but not in the IRD<sub>high</sub> group, PCNA expression was elevated also in the absence of E2. IRD<sub>high</sub>, but not the IRD<sub>low</sub> exposure prevented an increase in PCNA expression in response to E2 treatment as shown by a significant lower degree of PCNA labeled nuclei (15%) compared to control diet (IDD, 40%) and the IRD<sub>low</sub> group (50%). mRNA levels of the proliferation marker Ki-67 were also significantly increased by E2 ( $p = 0.0363$ ) and by diet ( $p = 0.0209$ ) (Fig. 5C).

### **Estrogenic responses in mammary gland tissue**

To evaluate the influence of the IRDs on estrogenic responsiveness of the mammary gland the protein expression of the progesterone receptor (PR) was determined by immunohistochemistry (Fig. 6). Moreover, mRNA expression of the estrogen responsive genes growth differentiation factor 15 (Gdf15) and metallothionein 1a (Mt1a), was evaluated by RT-qPCR in the mammary gland (Fig. 7).

As previously shown PR is a sensitive marker for E2 responsiveness in the mammary gland in ovariectomized female Wistar rats [23]. Treatment with E2 in all diet groups induced a significant increase in the percentage of PR labeled nuclei (Fig. 6,  $p < 0.0001$ ). A statistical significant effect of the diet ( $p < 0.0001$ ) and combination of diet and E2 could be observed ( $p < 0.0001$ ). In the IRD<sub>high</sub> group the percentage of PR-labeled nuclei after E2 treatment (9%) was significantly lower compared to IDD (21%) and IRD<sub>low</sub> (28%) (Fig. 6B). Furthermore, the IRD<sub>low</sub> E2 group showed a significant stronger PR expression compared to control diet IDD E2.

To investigate the effect of a diet rich in ISO on transcripts known to be sensitive to E2 without containing an estrogen responsive element in the promoter region, transcript levels of Gdf15 (summarized in [27, 28]) and Mt1a (summarized in [29]) were analyzed by Taqman PCR.

As expected, E2 highly significantly increased transcript levels of Gdf15 and Mt1a ( $p < 0.0001$  and  $p = 0.0004$ , respectively) (Fig. 7A and B). Interestingly, Gdf15 transcript levels were

significantly reduced by diet alone (Fig. 7A;  $p=0.0210$ ) or by the combination of diet and E2 ( $p=0.0213$ ). In contrast, diet neither affected Mt1a transcript levels, nor modulated the effect induced by E2. Furthermore, within the E2-exposed groups, transcript levels of Ki67 (Fig. 6C) and Gdf15 (Fig. 7) did not correlate ( $R=-0.23$  and  $-0.15$ , respectively) with plasma concentrations of E2 (Supplemental Table 5) and Mt1a correlated only weakly ( $R=-0.56$ ), demonstrating the independence of chosen endpoints towards momentary variations in plasma E2 levels due to the experimental design.

### **Isoflavones modulate estrogen-induced DNA methylation changes in rat mammary tissue**

Altered E2 responsivity of gene expression after long term dietary ISO exposure might be regulated by epigenetic mechanisms. To address this question, we used EpiTyper MassARRAY technology to quantitatively assess promoter methylation levels of three candidate genes. We selected *Aldh1L1* (10-formyltetrahydrofolate dehydrogenase), *Extl1* (exostosin-like glycosyl-transferase 1) and *Wap* (whey acidic protein) for our analyses. As reported previously, *Aldh1L1* expression was significantly downregulated in rat mammary glands after 10 day E2 stimulation, whereas *Extl1* and *Wap* were identified as the top upregulated genes [24].

In the IDD control group, the analyzed regions showed relatively high methylation levels with median methylation in the range of 61 to 75%. E2 stimulation did not influence DNA methylation in the *AldhL1* promoter (Fig. 8), whereas promoter methylation of *Extl1* and *Wap* increased by 3-12% (although these changes were not significant). IRDlow and IRDhigh exposure significantly reduced *AldhL1* promoter methylation by 5% to 8% ( $p=0.0128$  and  $p=0.0272$ ); E2 treatment had no additional influence on methylation levels. In contrast, lifelong IRDlow and IRDhigh exposure increased median methylation levels of *Extl1* and *Wap* by 3% to 14% (significant for IRDhigh with  $p=0.0066$  and  $p=0.0237$ , respectively). E2 treatment slightly increased median methylation levels in the IRDlow group (not significant), whereas IRDhigh intervention prevented E2-induced promoter methylation of both genes (significant for *Extl1*,  $p=0.0293$ ). Interestingly, methylation levels of *Extl1* and *Wap* were significantly correlated in animals of all 6 intervention groups (correlation coefficient  $R^2=0.2371$ ,  $p\leq 0.01$ ). Methylation differences for individual CpG units relative to median methylation levels of the IDD group are depicted in Supplemental Figure S4.

## 4. Discussion

In the present study dose-dependent exposure was performed by enriching an ISO free diet with an ISO extract. As a primary step in our investigations the soy extract was not only characterized analytically but also for its bioactivity in a cell growth bioassay *in vitro*. As demonstrated in Table 1 and in Fig. 2, the composition of the extract corresponded well to its estrogenic potential in cultured MCF-7 cells. Here an interesting result was that a mixture of GEN, DAI and GLY aglycones, in doses corresponding to the analytically determined aglycone composition and content, did not achieve the same stimulation of cell proliferation as the compounds within the extract matrix in the equivalent dose. This indicates that the glycosides in the extract also exert a specific estrogenic activity, at least *in vitro*. This is consistent with previous studies demonstrating that genistin and daidzin induce a weak estrogenic response in cultured MCF-7 cells [30]. The ISO determined in the extract accounted for its estrogenic potential *in vitro*, excluding the contribution of unknown compounds to the estrogenic activity of the extract. Therefore, the extract was considered a suitable source of ISO.

ISO exposure was chosen based on ISO intake of the Asian populations (summarized by [31]) on one hand and dietary intervention studies linking a defined ISO intake to resulting plasma concentrations on the other hand. In this respect, Messina et al. reported that the average daily ISO intake among eight Japanese studies ranged from 26 mg to 54 mg and that the upper level of dietary ISO intake in the Asian population can reach as much as 100 mg per day. In another study, Yamamoto et al. (2001) concluded on the basis of dietary assessment methods that the mean intake of DAI and GEN in Japan is 49.7 mg per day leading to a plasma concentration of about 600 nM [32]. Very recently results from an intervention study with postmenopausal women indicate that the intake of about 48 mg ISO per day as soy foods causes a mean ISO plasma concentration of  $1300 \pm 800$  nM in non-equol producers and  $2160 \pm 1480$  nM in equol producers. After an ISO supplement intervention with 94 mg per day (sum GEN and DAI) plasma levels reached  $2650 \pm 1490$  nM in non-equol producing women [33].

Taking into account the assumed average daily uptake of the diets by the rats and an allometric conversion of exposure to the human situation [34, 35], experimental animal chows were enriched with the extract to a final concentration of 68 ppm ISO aglycone in IRD<sub>low</sub> and 503 ppm ISO aglycone in IRD<sub>high</sub>. The mean ISO plasma concentrations (sum of GEN and DAI) at the end of the study were  $402 \pm 213$  nM and  $245 \pm 58$  nM in the two IRD<sub>low</sub> groups (without and with E2 treatment, respectively) and  $2094 \pm 866$  and  $2157 \pm 1202$  in the IRD<sub>high</sub> groups (without and with E2 treatment, respectively). Keeping in mind that the

animals were exposed to ISO lifelong (Fig. 1), the exposure scenario in the animal study resembles that of the Asian population, where the dietary exposure to ISO usually starts already in utero. Regarding the measured plasma concentrations in the rats, the IRDlow group is comparable to Asians with a low to medium consumption of soy products, whereas the IRDhigh group represents the part of the population with a high dietary soy intake. Furthermore, plasma concentrations of animals on IRDhigh diet exceeded those measured in IRDlow exposed animals about 6.6-fold. This is in line with the ISO concentrations determined in IRDhigh and IRDlow chow and demonstrates that distinct ISO concentrations in the respective diets result in a comparable ISO uptake in the animals.

Treatment and dose effects on the ISO metabolite profile were not observed (Tab. 3). As expected each individual animal was able to produce equol, which is a characteristic difference between rats and humans. Moreover, the most prominent phase-II-metabolite in the plasma of the rats was the 7-glucuronide whereas in Asian women the sulfoglucuronides predominate [36].

A major aim of this study was to investigate dose-dependent effects of the ISO exposure during early life time on estrogen sensitivity of the mammary glands. Adult animals were ovariectomized at PND80 and a three day uterotrophic assay was performed to determine the proliferative and estrogenic response. Treatment with E2 resulted in an increase of the UWW and UEH (Fig. 4E and F). Exposure to ISO by the different diets exerted no effects on UWW. This is in line with previous results using around 300 ppm of soy ISO equivalents in the diet (Molzberger et al 2013). Beside the uterotrophic response, we also carefully monitored body weight and body composition (Fig. 4A and B). E2 treatment of IRDhigh and IRDlow animals resulted in a significantly reduced body weight compared to untreated animals on the same diets. This effect was not observed in IDD fed animals. Analyzing the visceral fat mass (Fig. 4C) indicates that the observed changes in body weight may reflect a different body composition, particularly a reduced fat mass. This is in line with observations demonstrating an increase in fat metabolism by ISO [37, 38] and an increase in protein synthesis and skeletal muscle mass by GEN administered subcutaneously at a 10 mg/kg/d dose in a muscle regeneration experiment [39]. Our data indicate that even an ISO dose reflecting a lifelong nutritional exposure via soy rich diet may be sufficient to result in such effects. With respect to breast cancer risk this is of relevance because studies indicate that soy exposure may have a significant effect on the development of body fat and obesity [40] and paracrine growth factor signaling from fat tissue, and seems to be a relevant risk factor for breast cancer [41].

. A key result of the study presented here is the observation that lifelong exposure to IRD<sub>low</sub> is not sufficient to reduce the E2 induced stimulation of the expression of the proliferation marker PCNA in the mammary gland (Fig. 5A and B). This is in line with observations studying the differentiation of terminal end buds, where higher doses of dietary GEN supplementation was needed to interfere with the differentiation of terminal end buds (reviewed in [42]). In line with earlier observations [18, 23] IRD<sub>high</sub> reduced the proliferative response of the mammary gland indicating that pretreatment with ISO accounts for a lower sensitivity of mammary gland tissue towards estrogen treatment in OVX rats. In contrast, the dose of the IRD<sub>low</sub> group was not sufficient to reduce estrogen responsiveness. On the contrary IRD<sub>low</sub> in the absence of E2 induced a weak increase in the percentage of PCNA labeled nuclei and Ki-67 mRNA levels (Fig. 5C), an effect not detectable for IRD<sub>high</sub>. The physiological relevance of these findings needs to be further investigated. The Ki-67 effect was also seen at 1 mg/kg BW/d of exposure to pure GEN [43]. The mechanism behind these observations is unknown, but non-linear dose response curves are known for stimulation of proliferation by ISO in MCF-7 cells *in vitro*. Likewise, in the endocrine disruptor field numerous effects are known which follow a non-linear, very often a bell shaped dose response pattern (for review see [44]).

With respect to low dose ISO effects in the IRD<sub>low</sub> group it should also be kept in mind that IRD<sub>low</sub> reduced the time to onset of puberty similar to IRD<sub>high</sub> (Fig. 3). This response has been observed before in studies with a multigenerational setting with either GEN [16], genistin [45] or soy ISO [46, 47].

In women, lifetime estrogen exposure is a critical risk factor for breast cancer; this is why advancement of onset of menarche [48], which is mimicked by vaginal opening in animal studies, has to be interpreted as an unwanted side effect of ISO exposure. The Avon Longitudinal Study of Parents and Children [49] reported an advancement of menarche by 4 months in response to ISO, an effect which was not detectable in a study with non-human primates [50]. As an additional low dose effect, IRD<sub>low</sub> influenced body composition and body weights, too (Fig. 4A and B).

Our results show that not only proliferation, but also the expression of a variety of estrogen sensitive genes is altered. The expression patterns of the PR (Fig. 6) are very similar to those of PCNA. In agreement to previous studies [23] PR induction by E2 was significantly decreased in the IRD<sub>high</sub> group. New, and in agreement with the findings related to proliferation, is our observation that IRD<sub>low</sub> is not sufficient to result in such a protective effect. To get further insights into the molecular mechanisms involved in the altered E2 sensitivity of the breast by ISO we investigated the effect of diets rich in ISO also on transcripts known to be sensitive to E2 without containing an ERE in the promoter region. Among these genes were Gdf15 involved in various pathologies (summarized in [28]) and



inducible by E2 in cultured MCF-7 cells [27] and Mt1a which is controlled by an electrophile response element, which responds to redox status (summarized in [29]). The mRNA levels of Gdf15 were induced by E2 (Fig. 7). This response was reduced in the IRD<sub>low</sub> but not in the IRD<sub>high</sub> group. So here in contrast to the proliferative response and the response of PR, the sensitivity of a gene to an E2 stimulus was also affected by the IRD<sub>low</sub> dose, demonstrating that even a low exposure to ISO during adolescence results in a decreased E2 responsiveness, at least for selected genes. The second gene investigated was Mt1a. Here, mRNA expression was stimulated by E2, but neither IRD<sub>low</sub> nor IRD<sub>high</sub> had any effect on the responsiveness of this gene.

As reported before, numerous studies suggested a protective role of ISO exposure towards the development of breast cancer when the ingestion starts pre-pubertally. This was associated with the ability of ISO to alter the morphology of the mammary gland during pubertal breast growth [42, 51-53]. Further studies revealed that ISO are also able to induce epigenetic changes [46, 54, 55]. These lead to the assumption that the reduced estrogen sensitivity in the IRD<sub>high</sub> group compared to IDD could be due to epigenetic changes mediated by early life ISO exposure. For these reasons we investigated whether exposure to ISO resulted in DNA methylation changes. We used EpiTyper MassARRAY technology to quantitatively assess promoter methylation levels of three selected candidate genes, *Aldh1L1*, *Extl1* and *Wap*. These genes have been demonstrated to be regulated by E2 [24]. Our major finding was that lifelong ISO exposure in both IRD<sub>low</sub> and IRD<sub>high</sub> groups significantly reduced *Aldh1L1* promoter methylation. *Aldh1L1*, also known as 10-formyltetrahydrofolate dehydrogenase, plays a role in one-carbon metabolism relevant for providing S-adenosyl-methionine for methylation reactions and possesses suppressor effects [56]. Reduced methylation in the promoter region might result in enhanced expression. The effects on *Extl1* and *Wap*, both inducible by E2, were different: IRD<sub>high</sub> increased methylation levels of *Extl1* and *Wap*, but reduced methylation levels when combined with E2 as a sign of reduced estrogen sensitivity (Fig. 8). *Wap* (whey-acidic protein) is a major milk protein in rodents and regulates the proliferation of mammary epithelial cells, but is not expressed in humans [57]. *Extl1* (exostosin-like glycosyltransferase 1) was identified as one of the top E2-inducible genes in rat mammary glands [24], but its role in human breast carcinogenesis has not been investigated yet. Mechanisms underlying the differential response of *Aldh1L1*, *Extl1* and *Wap* promoter methylation to ISO treatment have to be investigated. Notably, GEN has been reported to modulate additional epigenetic mechanisms, including histone modifying genes and noncoding RNAs [58, 59]. Future studies will have to include these additional mechanisms to fully understand the impact of

soy intervention on epigenetic regulation of mammary gland estrogen sensitivity and breast cancer risk.

Taken together, our data provide evidence that lifelong nutritive exposure to low and high doses of ISO via ISO-enriched diet resulted in plasma levels relevant for human exposure which in turn induced tissue selective effects. Body weight, body fat and most importantly factors associated with mammary gland proliferation and differentiation were affected. Reduced proliferative response and reduced estrogenic sensitivity could only be observed in the IRD<sub>high</sub> group. Moreover, we could demonstrate that high ISO exposure during adolescence may result in a modulation of DNA methylation of the promoter region of selected genes. However, vaginal opening and the differential expression of specific genes could also be observed at the IRD<sub>low</sub> dose. A clear dose response pattern could not always be observed.

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## **5. References**

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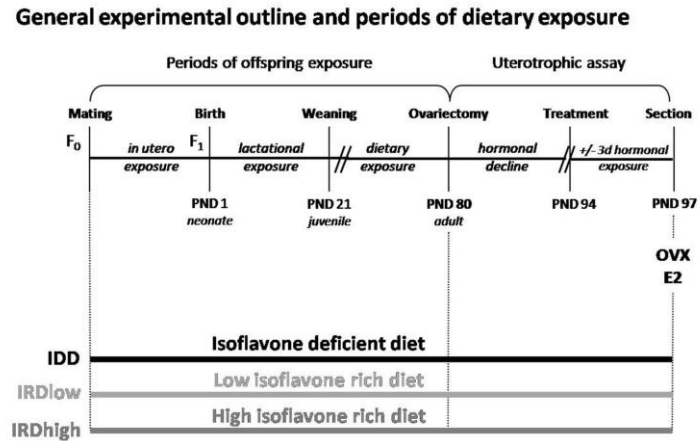
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**Figure 1.** General experimental outline and periods of dietary exposure.

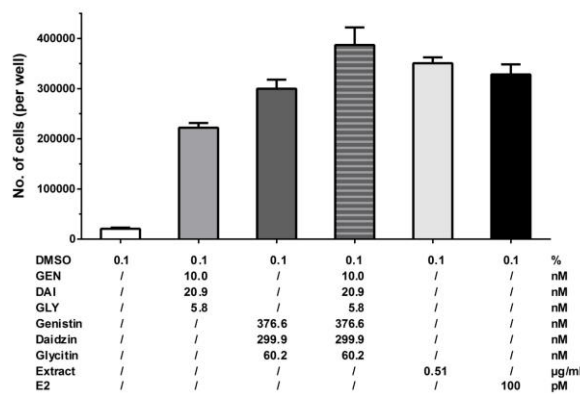
To model the lifelong dietary ingestion of ISO, animals were either exposed to an isoflavone-deficient diet (IDD) or to an isoflavone-enriched diet in two different concentrations (IRD<sub>low</sub> and IRD<sub>high</sub>). In all experimental arms animals were ovariectomized at PND80 and one half of the animals in each experimental arm was challenged with 4 µg/kg bw estradiol (E2) in comparison to those animals which received vehicle as control (OVX).

Fig. 1



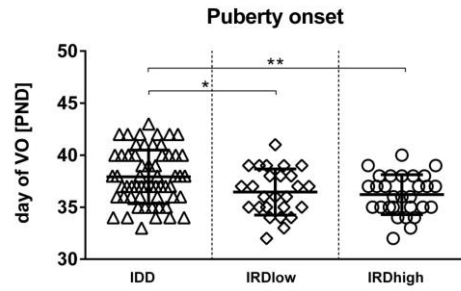
**Figure 2.** Stimulation of proliferation *in vitro* by the extract used in the animal experiment MCF-7 BUS cells were treated with solvent (DMSO, negative control), E2 (positive control), and 0.5095 µg extract/ml culture medium and the respective mixtures of genistin, daidzin, and glycitin and GEN, DAI, GLY + genistin, daidzin, and glycitin for 6 days. Thereafter, numbers of cells were determined electronically. Data represent means ± standards deviation of 3 independent experiments. Different characters a-c signify statistically different data groups (ANOVA/Tukey,  $p < 0.01$ ).

Fig. 2



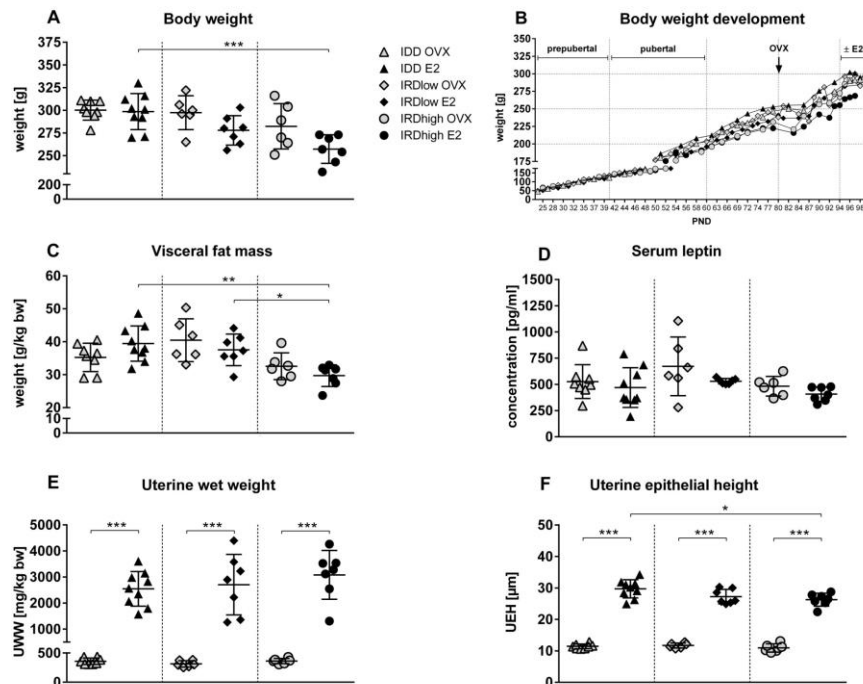
**Figure 3.** Puberty onset in the IDD, IRDlow and IRDhigh group indicated by day of vaginal opening (postnatal day, PND). ANOVA revealed an effect of the diet ( $p=0.0021$ ). Tukey post hoc test revealed significant difference with  $*p\leq 0.05$  and  $**p\leq 0.01$ .

Fig. 3



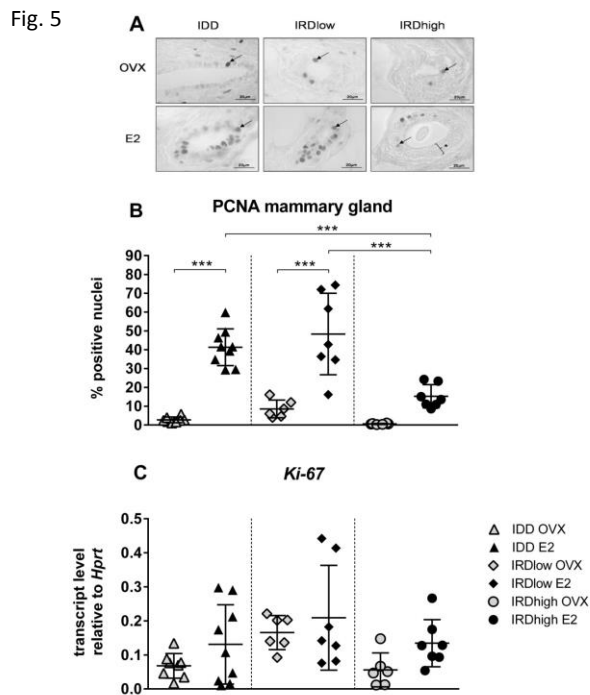


**Figure 4.** ISO effects on the estrogen sensitivity of body weight at PND97, body composition and uterus. ANOVA (factors: E2, diet and interaction of both) revealed for the body weight (A):  $p=0.0085$  (E2) and  $p=0.0003$  (diet); the visceral fat mass (C):  $p=0.0004$  (diet); the serum leptin levels (D): borderline significance with  $p=0.0696$  (E2) and  $p=0.0504$  (diet); the relative uterine wet weight (E):  $p<0.0001$  (E2); the uterine epithelial heights (F):  $p<0.0001$  (E2) and  $p=0.0306$  (diet). Tukey post hoc test revealed significant differences with  $*p\leq 0.05$ ,  $**p\leq 0.01$  and  $***p\leq 0.001$ . Body weight development (B) is shown as line chart.



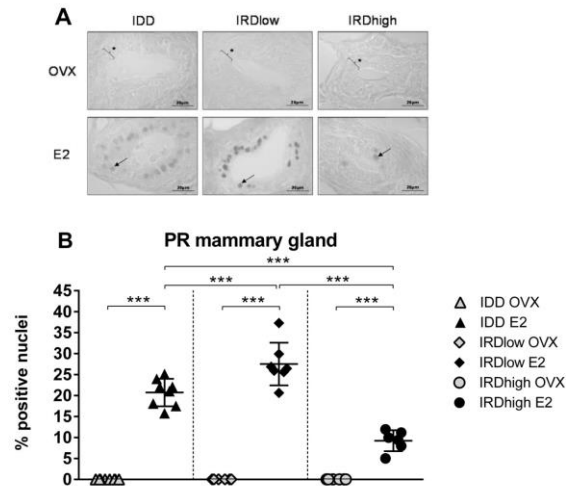
**Figure 5.** Expression of proliferation markers in the mammary gland.

(A) Representative pictures of specific anti-PCNA stained tissue sections of the mammary gland in equal magnification (630x). Positive nuclei are indicated by black arrow, glandular epithelial cells by brackets with black asterisk. (B) ANOVA of quantitative determination of PCNA staining revealed effect of E2 ( $p < 0.0001$ ) as well as an effect of the diet ( $p < 0.0001$ ) and a combination effect of E2 and diet ( $p = 0.0042$ ). Tukey post hoc test revealed significant differences with  $***p \leq 0.001$ . (C) mRNA levels of Ki-67 transcripts determined by Taqman PCR and normalized to Hprt. Statistical analysis revealed both an effect of E2 ( $p = 0.0363$ ) and of the diet ( $p = 0.0209$ ).



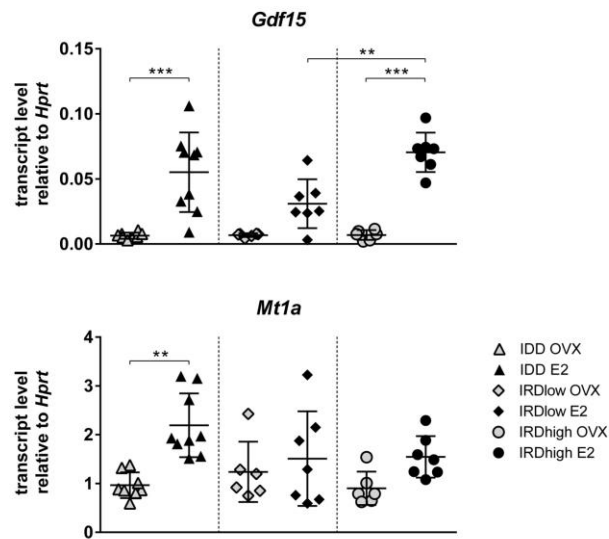
**Figure 6.** Expression of the estrogen-dependent/regulated progesterone receptor (PR). (A) Representative pictures of specific anti-PR stained sections of the mammary gland in equal magnification (630x). Positive nuclei are indicated by black arrow, glandular epithelial cells by brackets with black asterisk. (B) ANOVA of quantitative determination of PR staining revealed an effect of the diet ( $p < 0.0001$ ), the treatment with E2 ( $p < 0.0001$ ) and the interaction of both ( $p < 0.0001$ ). Tukey post hoc test revealed significant difference with  $***p \leq 0.001$ .

Fig. 6



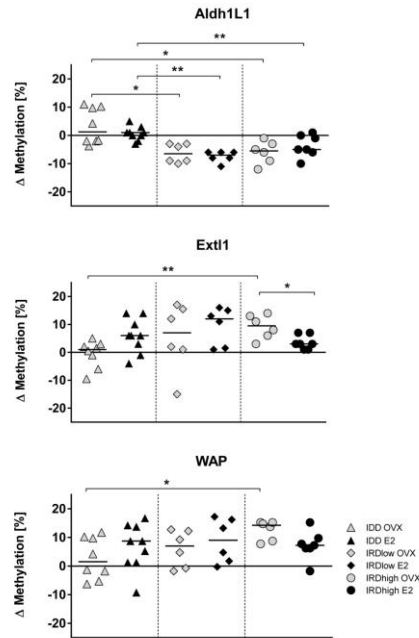
**Figure 7.** mRNA levels of *Gdf15* and *Mt1a* transcripts normalized to *Hprt*. ANOVA revealed an effect of E2 on *Gdf15* ( $p < 0.0001$ ) and *Mt1a* ( $p = 0.0004$ ) as well as an effect of the diet ( $p = 0.0210$ ) and a combination effect of E2 and diet ( $p = 0.0213$ ) on *Gdf15* transcript levels. Tukey post hoc test revealed significance differences with  $**p \leq 0.01$  and  $***p \leq 0.001$ .

Fig. 7



**Figure 8.** Quantification of DNA methylation in the promoter region of Aldh1L1, Extl1 and Wap by EpiTyper methylation analysis. Median amplicon methylation differences relative to the median methylation of the IDD OVX group (Aldh1L1, 75%; Extl1, 61%; WAP, 74%) are depicted as scatter plots with median methylation differences indicated by black horizontal lines.

Fig. 8



**Table 1:** Concentration of isoflavones in the soy extract

<b>Analytes</b>	<b>Content (mg/g soy extract)</b>	<b>RSD (%)</b>	<b>Content (%)</b>
Genistein	5.3	5.0	0.8
Genistin	319.6	4.4	47.2
6''-O-Acetyl-Genistin	19.4	4.4	2.9
6''-O-Malonyl-Genistin	n.d.		0.0
Daidzein	10.4	4.3	1.5
Daidzin	244.5	4.4	36.1
6''-O-Acetyl-Daidzin	18.3	4.3	2.7
6''-O-Malonyl-Daidzin	n.d.		0.0
Glycitein	3.2	3.6	0.5
Glycitin	52.8	4.5	7.8
6''-O-Acetyl-Glycitin	3.7	5.7	0.5
6''-O-Malonyl-Glycitin	n.d.		0.0
Sum of all analytes	677	4.4	100.0
Sum of Genistein in aglycone equivalents	216	4.4	
Sum of Daidzein in aglycone equivalents	170	4.4	
Sum of Glycitein in aglycone equivalents	39	4.3	
Overall isoflavone aglycone equivalents	425	4.4	

Mean of six determinations; RSD, relative standard deviation; n.d. not detected (6''-O-malonyl-daidzin <1.6 mg/g soy extract; 6''-O-malonyl-genistin, 6''-O-malonyl-glycitin <1.7 mg/g soy extract).

**Table 2:** Primer sequences

<b>Gene Name</b>	<b>Forward Primer</b>	<b>Reverse Primer</b>
<i>Aldh1L1</i>	AATAAAAGTTTGTGGTGTGGAGG	AAATTCCCATATATTCCCTCTATCC
<i>Ext1</i>	ATTAGGGAGGTTTGTGGATTTGTGAGG	TAATCCCCATAACACTTCCAATACC
<i>Wap</i>	TGTGAGTTTTGTTTTGGTAGTTTGG	AAAAATATCCTTTTATTCTTATCTCC

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**Table 3:** Plasma concentrations of isoflavone metabolites in rats after ingestion of isoflavone-enriched diets (IRDlow and IRDhigh). Mean  $\pm$  SD (RSD%). Values in nM.

#	Analytes	IRDlow -E2 (n=6)	IRDlow +E2 (n=7)	IRDhigh -E2 (n=6)	IRDlow +E2 (n=7)
	Aglcyone equivalents (DAI + GEN)	402 $\pm$ 213 (53)	245 $\pm$ 58 (24)	2094 $\pm$ 866 (41)	2157 $\pm$ 1202 (56)
1	GEN	4 $\pm$ 2 (56)	1 $\pm$ 1 (78)	35 $\pm$ 28 (81)	39 $\pm$ 37 (95)
2	GEN-4'-GlcA	n.d.	n.d.	28 $\pm$ 28 (101)	41 $\pm$ 47 (115)
3	GEN-7-GlcA	197 $\pm$ 112 (57)	118 $\pm$ 34 (29)	973 $\pm$ 399 (41)	1068 $\pm$ 661 (62)
4	GEN-4'-S	n.d.	n.d.	n.d. (one rat: 1.8)	1 $\pm$ 2 (149)
5	GEN-7-S	1 $\pm$ 0 (0)	1 $\pm$ 1 (125)	9 $\pm$ 10 (113)	13 $\pm$ 14 (102)
6	GEN-4',7-DiS	n.d.	n.d.	n.d. (one rat: 5.4)	n.d. (one rat: 9.8)
7	GEN-7-GlcA-4'-S	16 $\pm$ 19 (121)	4 $\pm$ 2 (60)	40 $\pm$ 38 (95)	46 $\pm$ 41 (89)
	Percentage of free GEN [%]	2.1 $\pm$ 1.0	1.1 $\pm$ 0.4	3.0 $\pm$ 1.6	2.7 $\pm$ 2.2
8	DAI	9 $\pm$ 4 (48)	5 $\pm$ 3 (62)	61 $\pm$ 32 (52)	54 $\pm$ 41 (77)
9	DAI-4'-GlcA	5 $\pm$ 4 (77)	3 $\pm$ 4 (125)	52 $\pm$ 26 (49)	54 $\pm$ 43 (80)
10	DAI-7-GlcA	152 $\pm$ 67 (44)	106 $\pm$ 18 (16)	825 $\pm$ 268 (33)	750 $\pm$ 303 (40)
11	DAI-4'-S	2 $\pm$ 1 (69)	1 $\pm$ 1 (84)	9 $\pm$ 7 (82)	10 $\pm$ 6 (60)
12	DAI-7-S	4 $\pm$ 2 (43)	2 $\pm$ 1 (58)	22 $\pm$ 18 (84)	28 $\pm$ 23 (83)
13	DAI-4',7-DiS	3 $\pm$ 2 (59)	n.d. (one rat: 3.4)	10 $\pm$ 10 (102)	19 $\pm$ 23 (124)
14	DAI-7-GlcA-4'-S	9 $\pm$ 9 (100)	3 $\pm$ 2 (52)	29 $\pm$ 26 (89)	31 $\pm$ 26 (83)
	Percentage of free DAI [%]	5.1 $\pm$ 2.1	3.7 $\pm$ 1.7	6.0 $\pm$ 2.1	5.2 $\pm$ 2.9
15	DH-GEN	n.d.	n.d.	n.d.	n.d. (one rat: 1.3)
16	6'-Hydroxyl-ODMA	n.d.	n.d.	n.d.	n.d.
17	DH-DAI	n.d.	n.d.	n.d. (one rat: 1.3)	n.d. (one rat: 1.3)
18	ODMA	n.d. (one rat: 0.5)	n.d.	1 $\pm$ 0 (75)	1 $\pm$ 0 (58)
19	Equol	n.d.	n.d.	n.d.	n.d.
20	Equol-7-GlcA	41 $\pm$ 34 (84)	15 $\pm$ 9 (65)	274 $\pm$ 131 (48)	271 $\pm$ 104 (38)
21	Equol-4'-S	n.d. (one rat: 0.8)	n.d.	1 $\pm$ 1 (65)	2 $\pm$ 4 (164)

n.d., not detected (<LOD); DAI, daidzein; GEN, genistein; GlcA, glucuronide; S, sulfate; DH, dihydro; ODMA, O-desmethylangolensin; Aglcyone equivalents (DAI + GEN), sum of DAI, DAI conjugates, GEN and GEN conjugates; For calculation of mean, SD and RSD values between LOQ and LOD were set LOQ/2 and n.d. values were set as zero.